

# Oxidized cholesterol in the diet is a source of oxidized lipoproteins in human serum

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**Abstract** The aim of this study was to determine in humans whether oxidized cholesterol in the diet is absorbed and contributes to the pool of oxidized lipids in circulating lipoproteins. When a meal containing 400 mg cholestan-5 $\alpha$ ,6 $\alpha$ -epoxy-3 $\beta$ -ol ( $\alpha$ -epoxy cholesterol) was fed to six controls and three subjects with Type III hyperlipoproteinemia,  $\alpha$ -epoxy cholesterol in serum was found in chylomicron/chylomicron remnants (CM/RM) and endogenous (VLDL, LDL, and HDL) lipoproteins. In controls,  $\alpha$ -epoxy cholesterol in CM/RM was decreased by 10 h, whereas in endogenous lipoproteins it remained in the circulation for 72 h. In subjects with Type III hyperlipoproteinemia,  $\alpha$ -epoxy cholesterol was mainly in CM/RM. In vitro incubation of the CM/RM fraction containing  $\alpha$ -epoxy cholesterol with human LDL and HDL that did not contain  $\alpha$ -epoxy cholesterol resulted in a rapid transfer of oxidized cholesterol from CM/RM to both LDL and HDL. In contrast, no transfer was observed when human serum was substituted with rat serum, suggesting that cholesteryl ester transfer protein is mediating the transfer. Thus,  $\alpha$ -epoxy cholesterol in the diet is incorporated into the CM/RM fraction and then transferred to LDL and HDL, contributing to lipoprotein oxidation. Moreover, LDL containing  $\alpha$ -epoxy cholesterol displayed increased susceptibility to further copper oxidation in vitro. It is possible that oxidized cholesterol in the diet accelerates atherosclerosis by increasing oxidized cholesterol levels in circulating LDL and chylomicron remnants.—Staprans, I., X-M. Pan, J. H. Rapp, and K. R. Feingold. Oxidized cholesterol in the diet is a source of oxidized lipoproteins in human serum. *J. Lipid Res.* 2003. 44: 705–715.

**Supplementary key words** Type III hyperlipoproteinemia • chylomicron/chylomicron remnants • cholestan-5 $\alpha$ ,6 $\alpha$ -epoxy-3 $\beta$ -ol

A large body of evidence supports the hypothesis that oxidized lipoproteins, particularly oxidized LDL, play a pathogenic role in atherosclerosis (1, 2). Oxidized LDL has numerous atherogenic properties with a variety of cell types in culture, including induction of inflammatory

genes, stimulation of monocyte chemotactic factor production, the potentiation of monocyte-endothelial cell adhesion, accelerated deposition of lipids in macrophages, cytotoxicity with endothelial cells, and modulation of growth factors. Additionally, oxidized LDL-like particles and lipid peroxidation products are present in atherosclerotic lesions (3, 4). Moreover, it has been shown that anti-oxidants in the diet can slow the progression of atherosclerosis in animal models (5–7).

Thus, there is strong evidence that oxidized lipoproteins play a key role in atherogenesis, but the site and mechanisms by which lipoproteins are oxidized is far from resolved. It is not clear whether oxidized lipoproteins form locally in the artery wall, as suggested by several investigators (1, 2), or are sequestered in atherosclerotic lesions following the uptake of circulating oxidized lipoproteins. We have been focusing our studies on the role of diet and have demonstrated that potentially atherogenic oxidized lipoproteins in the circulation are at least partially derived from oxidized lipids in food and contribute to atherosclerosis in animal models.

The American diet contains large quantities of oxidized fatty acids (8, 9) and oxidized cholesterol (10–13) due to the fact that a large portion of the fat and cholesterol in the diet is often prepared in a fried, heated, or processed form. Studies in our laboratory have demonstrated that oxidized fatty acids in the diet result in oxidized lipids in serum lipoproteins in rodents and rabbits. In rats, the levels of oxidized fatty acids in the serum are proportional to the quantity of oxidized fatty acids in the diet, with increased dietary oxidized fatty acids leading to increased exogenous and endogenous serum oxidized lipoprotein levels (14). The quantity of oxidized fatty acids in chylomicrons isolated from the mesenteric lymph drainage of the small intestine and postprandial serum also correlates directly with the amount of oxidized fatty acids adminis-

Abbreviations: CM/RM, chylomicrons/chylomicron remnants; CETP, cholesteryl ester transfer protein.

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tered to the animal (15, 16). Thus, oxidized fatty acids in the diet are absorbed by the small intestine, transported in chylomicrons to the liver, and utilized to form VLDL that are secreted into the circulation (16). Most importantly, we have demonstrated that oxidized fatty acids in the diet are proatherogenic and accelerate fatty streak formation in the aorta of cholesterol-fed rabbits (17).

It has also been shown in several laboratories that, similarly to fatty acids, oxidized cholesterol in the diet is incorporated into serum lipoproteins of rats (18, 19) and rabbits (20–22). Recently, we have demonstrated that oxidized cholesterol in the diet also accelerates fatty streak lesion formation in aortas of cholesterol-fed rabbits (22). In LDL receptor-deficient and apolipoprotein E (apoE)-deficient mice (murine models in which atherosclerosis more closely resemble human lesions), again the feeding of oxidized cholesterol resulted in a significant increase in aortic fatty streak lesions (23). Thus, our observations in animals clearly demonstrate that diets containing oxidized lipids, either oxidized fatty acids or cholesterol, increase the development of atherosclerosis.

In studies in humans, we have shown that the quantity of oxidized fatty acids in the diet also correlates with the levels of oxidized lipids in postprandial serum chylomicrons/chylomicron remnants (CM/RM) (24, 25). Oxidized fatty acids in the diet are absorbed by the small intestine, incorporated into chylomicrons and chylomicron remnants, and appear in the bloodstream where they contribute to the total body pool of oxidized lipid. Moreover, these postprandial lipoproteins containing diet-derived oxidized fatty acids were more susceptible to oxidation *in vitro*.

The purpose of the present study was 1) to determine whether in humans oxidized cholesterol in the diet is absorbed by the small intestine and contributes to the pool of oxidized lipids, 2) to determine the distribution of dietary oxidized cholesterol among all serum lipoproteins, including CM/RM, VLDL, HDL, and LDL, and 3) to determine whether  $\alpha$ -epoxy cholesterol containing LDL is more susceptible to further oxidation.

## MATERIALS AND METHODS

### Study subjects

This study was performed on six control subjects (four males and two females) and three male subjects with familial Type III hyperlipoproteinemia (apolipoprotein phenotype E<sub>2</sub>/E<sub>2</sub> and elevated lipids) (26). All control subjects were selected from volunteers employed at the Veterans Affairs Medical Center, San Francisco. Blood was drawn from each subject after a 12 h fast (0 time) for measurement of fasting serum triglycerides, cholesterol, and  $\alpha$ -epoxy cholesterol levels. All subjects were nonsmokers, were moderately active, and consumed a typical American diet. None of the subjects was on vitamin or antioxidant therapy. Control subjects had normal serum triglyceride (<2.3 mmol/l) and cholesterol (<5.2 mmol/dl) levels. None of the subjects was obese (BMI < 30), had diabetes, congestive heart failure, or gastrointestinal disorders. Control subjects were not taking any lipid-lowering medication. Subjects with familial Type III hyperli-

poproteinemia were recruited from the Lipid Clinic at University of California, San Francisco, and all their medications were discontinued for at least 3 weeks prior to the experiment. This study was approved by the Committee on Human Research at University of California, San Francisco, and written consent was obtained from all experimental subjects.

### Experimental diets and study protocol

Cholestan-5 $\alpha$ ,6 $\alpha$ -epoxy-3 $\beta$ -ol ( $\alpha$ -epoxy cholesterol) was used as a source of oxidized cholesterol in a test meal. It was purchased from Steraloids Inc. (Newport, RI), and was selected because it is one of the major oxidized cholesterol components in heated or stored foods (10–13). It has also been detected in human serum (27–29) and atherosclerotic lesions (30, 31). It is efficiently absorbed as indicated by studies with experimental animals (19). Moreover, it has been demonstrated that  $\alpha$ -epoxy cholesterol is not formed enzymatically during cholesterol transport *in vivo* (32).

After a 12 h fast, six control subjects and three subjects with familial Type III hyperlipoproteinemia were given a dose of 400 mg  $\alpha$ -epoxy cholesterol dissolved in olive oil (0.5 ml/kg body weight) and added to 100 g of carbohydrate (mashed potatoes). For control purposes, in another experiment the same subjects were given a similar dose of nonoxidized cholesterol as a test meal and serum and lipoprotein fractions were tested for  $\alpha$ -epoxy cholesterol generated during the isolation procedure. The subjects tolerated the test meal well, and none had gastrointestinal symptoms. At 2 h, 4 h, 6 h, 8 h, and 10 h after the consumption of the test meal, 50 ml blood samples were obtained for the determination of serum triglycerides, cholesterol, and  $\alpha$ -epoxy cholesterol levels. In three control subjects, serum samples were also obtained at 24 h, 48 h, and 72 h. All serum samples were stored in ice and contained 10  $\mu$ M EDTA and 5  $\mu$ M BHT throughout the sample processing. The  $\alpha$ -epoxy cholesterol levels were measured in serum and the amount of  $\alpha$ -epoxy cholesterol was expressed as micrograms of oxidized cholesterol per decaliters of serum. The subjects were not permitted to consume any food for the 10 h test period. Water was allowed *ad libitum*.

To determine the dose response, three control subjects, in addition to ingesting the 400 mg dose, were given decreasing amounts of  $\alpha$ -epoxy cholesterol (200 mg, 100 mg, and 50 mg), and serum was collected at 6 h for the determination of the oxidized cholesterol levels. In each individual, there was at least a 2 week time period between the consumption of any of the test meals.

### Lipoprotein isolation and characterization

Initially, serum samples collected at 2 h time intervals after the administration of the test meal were separated into fractions using apoB-100 immunoaffinity columns (the column does not bind apoB-48) as described by Schneeman et al. (33): 1) apoB-100 containing lipoproteins (VLDL, IDL, LDL) and 2) CM/RM and HDL lipoprotein fractions. Thus, a highly enriched diet-derived apoB-48 fraction could be separated from most apoB-100-containing serum lipoproteins (34). Serum (4 ml) was applied on columns and the unbound fraction containing apoB-48 CM/RM and HDL were collected to be further separated by density centrifugation (35) into CM/RM fraction ( $d < 1.019$ ), and HDL fraction ( $d = 1.063$ – $1.225$ ). The bound fraction was eluted from the column, collected as described (33, 34), then further fractionated into VLDL ( $d < 1.006$ ), IDL ( $d = 1.006$ – $1.019$ ), and LDL ( $d = 1.019$ – $0.063$ ) by ultracentrifugation (35). All lipoproteins from all time points examined were characterized by determining triglyceride, cholesterol, and  $\alpha$ -epoxy cholesterol content. The recovery of  $\alpha$ -epoxy cholesterol was 85–95% when the sum of  $\alpha$ -epoxy cholesterol in each lipoprotein was compared with  $\alpha$ -epoxy cholesterol in serum.

## Determination of $\alpha$ -epoxy cholesterol transfer from diet-derived CM/RM fraction to endogenous LDL and HDL

The transfer of  $\alpha$ -epoxy cholesterol from CM/RM fraction to serum-endogenous lipoproteins was determined by incubating diet-derived  $\alpha$ -epoxy cholesterol containing CM/RM fraction with fasting serum that was free of  $\alpha$ -epoxy cholesterol. Three normal subjects were fed an  $\alpha$ -epoxy cholesterol-containing meal as described above to obtain CM/RM fraction at 2 h, 4 h, 6 h, and 8 h after the consumption of the test meal. Postprandial serum samples from each time point were collected and applied to a apoB-100 affinity column to obtain the unbound fraction for CM/RM isolation as described above. Four milliliters of fasting serum ( $d > 1.006$ ) were incubated at 37°C for 12 h (36) with the CM/RM fraction that was derived from 4 ml serum. After a 12 h incubation, the CM/RM, LDL, and HDL fractions were isolated from the incubation mixture by sequential centrifugation, and the amounts of  $\alpha$ -epoxy cholesterol in these fractions were determined by gas-liquid chromatography (GLC) as described below. In these experiments,  $\alpha$ -epoxy cholesterol in the CM/RM fraction was  $\sim 2\%$  of the total cholesterol mass. The transfer was expressed as a percentage of  $\alpha$ -epoxy cholesterol distribution among LDL, HDL, and the remaining CM/RM. The original CM/RM fraction was designated as 100%. To eliminate the possibility that oxidized cholesterol in endogenous lipoproteins was generated during the experimental procedure, in a separate experiment, incubation was also carried out in the presence of CM/RM fraction that did not contain oxidized cholesterol.

To determine the rate of transfer during the incubation over a 12 h time period,  $\alpha$ -epoxy cholesterol containing purified CM/RM fraction (isolated from serum 6 h after the consumption of the test meal) was incubated at 37°C with fasting serum and the transfer was monitored for 12 h. Samples were removed and examined for  $\alpha$ -epoxy cholesterol in CM/RM fraction and in endogenous LDL and HDL. For control purposes, the CM/RM fraction was incubated with a similar amount of rat serum that does not contain cholesteryl ester transfer protein (CETP).

### Susceptibility of LDL to *in vitro* oxidation

Susceptibility of LDL to oxidation was performed using the procedure of Esterbauer et al. (37). Freshly isolated, antioxidant-free LDL from four control subjects was incubated (200  $\mu$ g cholesterol/ml incubation mixture) with  $\text{CuSO}_4$  (final concentration 1.5  $\mu$ mol/l) at 37°C, and conjugated dienes were measured at 234 nm every 15 min for 5 h. Susceptibility to oxidation was expressed as the "lag time," and was determined from the intercept of lines drawn through the linear portion of the lag and propagation phases for each sample as described by us previously (24). The lag time was compared for LDL isolated from serum samples obtained at 0 time and 8 h after the consumption of the test meal containing 400 mg  $\alpha$ -epoxy cholesterol.

### Analytical methods

Total cholesterol and triglyceride concentration in serum and lipoproteins was measured using Sigma (St. Louis, MO) kits #352-20 and #339-20, respectively.

$\alpha$ -Epoxy cholesterol in serum and serum-lipoprotein fractions and free and esterified cholesterol in LDL were determined by GLC using the procedure described by Hughes et al. (38) and previously by us (22, 23). Lipid samples were derivatized with Sylo BFT and injected into gas chromatograph (Hewlett-Packard 5890, Palo Alto, CA) fitted with DB-1 column (J&W Scientific, Folsom, CA). Free and esterified  $\alpha$ -epoxy cholesterol and cholesterol in lipoproteins was determined by measuring the difference between the amount before (free cholesterol) and after saponification (total cholesterol). Fatty acid composition in LDL

was determined as described by us previously for lymph chylomicrons (24). Triglycerides were isolated by TCL, hydrolyzed, and fatty acids were transmethylated with boron trifluoride methanol. Vitamin E in LDL was measured as described previously (24). Standard  $\alpha$ -epoxy cholesterol was obtained from Steraloids Inc. (Newport, RI), and a mixture of fatty acid standards was obtained from Matreya, Inc (Pleasant Gap, PA).

### Statistics

Data are presented as mean  $\pm$  SEM. The mean differences between groups were assessed with Student's *t*-test. The mean lag time for LDL susceptibility to oxidation was determined using paired Student's *t*-test. Significance was expressed as  $P < 0.05$ .

## RESULTS

### Subjects

Table 1 summarizes the age, BMI, and lipid values of the control and Type III hyperlipoproteinemic subjects. As expected, in the hyperlipoproteinemic subjects, serum lipid values are elevated because of a decrease in the clearance of serum chylomicron and VLDL remnants (IDL).

### The effect of dietary $\alpha$ -epoxy cholesterol on the levels of $\alpha$ -epoxy cholesterol in the serum

In our initial studies, we determined the effect of different  $\alpha$ -epoxy cholesterol quantities in the test meal on  $\alpha$ -epoxy cholesterol levels in postprandial serum. We found that the serum levels of  $\alpha$ -epoxy cholesterol strongly correlated ( $r = 0.997$ ;  $P < 0.001$ ;  $n = 3$ ) with the amount of  $\alpha$ -epoxy cholesterol in the test meal (50–400 mg). To ensure detection and to obtain accurate measurements, in these studies the quantity of  $\alpha$ -epoxy cholesterol administered in the test meal was relatively large (400 mg) because the sensitivity of GLC for detecting small quantities of  $\alpha$ -epoxy cholesterol is limited. At baseline before the feeding of the test meal, we did not detect any  $\alpha$ -epoxy cholesterol in the serum. However, it has been previously reported (27–29) that human fasting serum contains levels of  $\alpha$ -epoxy cholesterol as low as 0.01  $\mu$ g/dl, as determined by mass spectrometer. Such trace amounts cannot be detected using our methodology where the lower limit

TABLE 1. Characteristics of study subjects

	Control Subjects n = 6	Subjects with Hyperlipoproteinemia n = 3
Age (years)	52.9 $\pm$ 3.0	60.0 $\pm$ 2.5
BMI	25.8 $\pm$ 0.7	27.3 $\pm$ 0.7
Triglyceride <sup>a</sup>	0.90 $\pm$ 0.19	4.0 $\pm$ 0.24 ( $P < 0.001$ )
Cholesterol <sup>a</sup>	5.31 $\pm$ 0.29	8.20 $\pm$ 1.18 ( $P < 0.02$ )
HDL cholesterol <sup>a</sup>	1.54 $\pm$ 0.17	0.99 $\pm$ 0.10
LDL cholesterol <sup>a</sup>	2.67 $\pm$ 0.22	1.82 $\pm$ 0.35
$\alpha$ -Epoxy cholesterol/ cholesterol ( $\mu$ g/mg) <sup>b</sup>	22.20 $\pm$ 4.90	1.32 $\pm$ 0.21 ( $P < 0.05$ )

$\alpha$ -Epoxy cholesterol, cholestan-5 $\alpha$ ,6 $\alpha$ -epoxy-3 $\beta$ -ol. All values are given as means  $\pm$  SEM.

<sup>a</sup> mmol/dl.

<sup>b</sup>  $\alpha$ -Epoxy cholesterol-cholesterol ratio in postprandial dietary chylomicrons/chylomicron remnants (CM/RM) fraction collected 6 h after the consumption of the test meal.

of detection is 40  $\mu\text{g}/\text{dl}$  serum, and less than 1.0% of the ingested dose is found in 100 ml of postprandial serum. Of importance is the observation that no  $\alpha$ -epoxy cholesterol was detected in serum samples when subjects were fed nonoxidized cholesterol, indicating that  $\alpha$ -epoxy cholesterol was not generated during the isolation procedures.

### Dietary $\alpha$ -epoxy cholesterol incorporation into serum lipoproteins in control subjects

We next examined the time course of the increase in serum  $\alpha$ -epoxy cholesterol levels after feeding the test meal. As shown in **Fig. 1**, after feeding a test meal containing 400 mg  $\alpha$ -epoxy cholesterol and olive oil, the levels of serum triglycerides peaked at 2–3 h and returned to base line at 7 h. In contrast, the levels of  $\alpha$ -epoxy cholesterol in the serum rapidly increased, reaching a peak value at 4 h, and remained elevated for more than 72 h. This postprandial increase in serum triglycerides is in agreement with previous studies in our laboratory (24, 25) and by other investigators (33). Because the postprandial increase in serum triglycerides is mainly associated with chylomicrons, this difference in the time course between oxidized cholesterol and triglycerides suggests that  $\alpha$ -epoxy cholesterol in the serum is not solely associated with chylomicrons but may be present in other serum lipoprotein particles. No change in the serum cholesterol levels was detected after the administration of the test meal. The majority of  $\alpha$ -epoxy cholesterol in serum lipoproteins was present in an ester form, since only traces of free  $\alpha$ -epoxy cholesterol were detected when samples were not subjected to saponification.

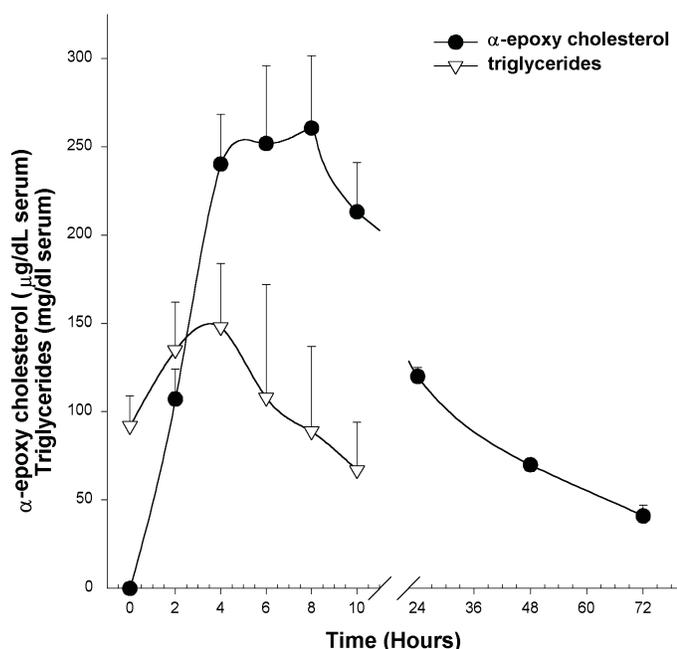
After the administration of the test meal, over a 10 h period  $\alpha$ -epoxy cholesterol was present in all lipoprotein fractions, LDL displaying the highest levels. **Figure 2** shows the time course of the appearance of  $\alpha$ -epoxy cho-

lesterol in serum CM/RM, VLDL, LDL, and HDL after feeding a meal containing 400 mg  $\alpha$ -epoxy cholesterol. In the CM/RM fraction,  $\alpha$ -epoxy cholesterol levels reached a peak at 2–4 h and were then sustained at a relatively constant level until 8 h, decreasing at 10 h. In VLDL, very little  $\alpha$ -epoxy cholesterol was detected at all time points examined. In both LDL and HDL,  $\alpha$ -epoxy cholesterol was detected as early as 2 h after the test meal, reached a peak at 8 h, and then remained in the circulation for several days. Thus, the incorporation of dietary  $\alpha$ -epoxy cholesterol into endogenous lipoproteins occurred rapidly and remained in the circulation for extended periods of time exceeding 72 h. In fact, at 24 h, only trace amounts of  $\alpha$ -epoxy cholesterol were present in CM/RM, and at 72 h the circulating  $\alpha$ -epoxy cholesterol was found in endogenous serum lipoproteins, especially LDL. Moreover, after the isolation of serum HDL, the infranate contained no detectable  $\alpha$ -epoxy-cholesterol, indicating that all  $\alpha$ -epoxy cholesterol is associated solely with serum lipoproteins and not bound to serum albumin.

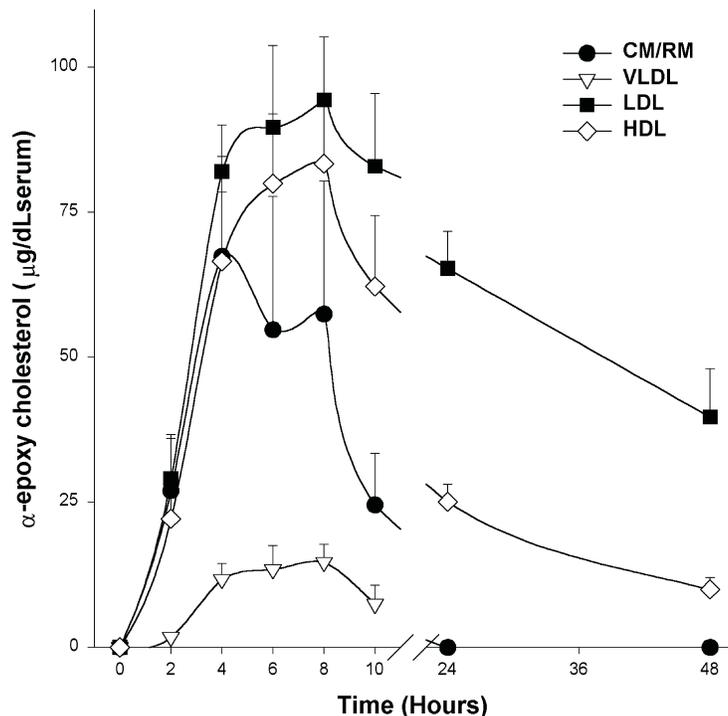
### Dietary $\alpha$ -epoxy cholesterol incorporation into serum lipoproteins in subjects with Type III hyperlipoproteinemia

We next examined the lipoprotein distribution of  $\alpha$ -epoxy cholesterol in subjects with Type III hyperlipoproteinemia in whom the clearance of CM/RM cholesterol is impaired. As shown in **Fig. 3**, similar to controls, the levels of serum chylomicron triglycerides peaked at 4 h and returned to the baseline value by 8 h. In contrast, however, the levels of  $\alpha$ -epoxy cholesterol increased with time and remained high for the 10 h study period. It should be noted that, as expected, the sustained baseline values were elevated in these subjects.

**Figure 4** shows that in subjects with Type III hyperlipoproteinemia, the majority of  $\alpha$ -epoxy cholesterol was



**Fig. 1.** Time course of cholestan-5 $\alpha$ ,6 $\alpha$ -epoxy-3 $\beta$ -ol ( $\alpha$ -epoxy cholesterol) and triglycerides in serum. Six control subjects were administered a test meal containing  $\alpha$ -epoxy cholesterol (400 mg), and the quantity of  $\alpha$ -epoxy cholesterol and triglycerides in serum was measured at indicated times. The levels of  $\alpha$ -epoxy cholesterol are expressed as  $\mu\text{g}/\text{dl}$  serum and triglycerides as  $\text{mg}/\text{dl}$  serum. For the 24 h, 48 h, and 72 h time points,  $n = 3$ . Data are expressed as mean  $\pm$  SE.

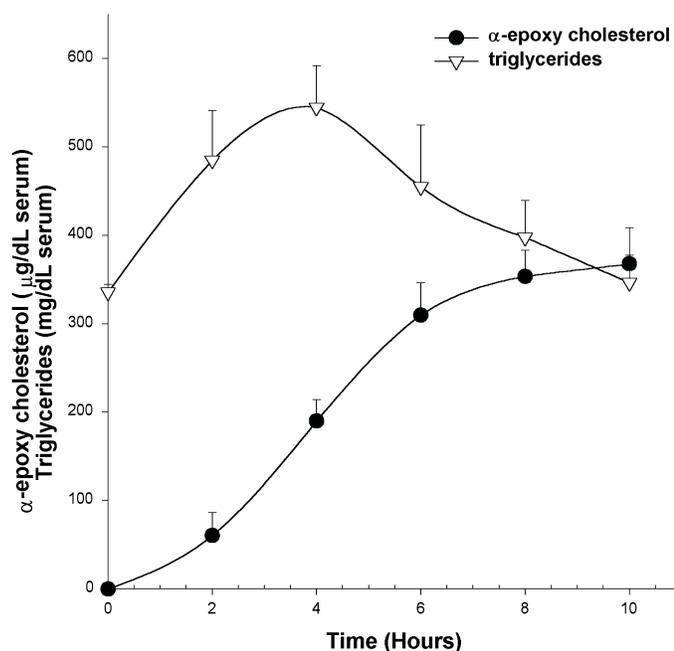


**Fig. 2.** Time course of  $\alpha$ -epoxy cholesterol distribution among the serum lipoproteins. Six control subjects were administered a meal containing  $\alpha$ -epoxy cholesterol (400 mg), and serum samples were obtained at indicated times. Lipoproteins from serum were isolated by affinity chromatography followed by sequential ultracentrifugation, as described in Materials and Methods. The amount of  $\alpha$ -epoxy cholesterol in chylomicrons/chylomicron remnants (CM/RM), VLDL/IDL, LDL, and HDL was measured using gas-liquid chromatography (GLC). For the 24 h, 48 h, and 72 h time points,  $n = 3$ . Data are expressed as mean  $\pm$  SE.

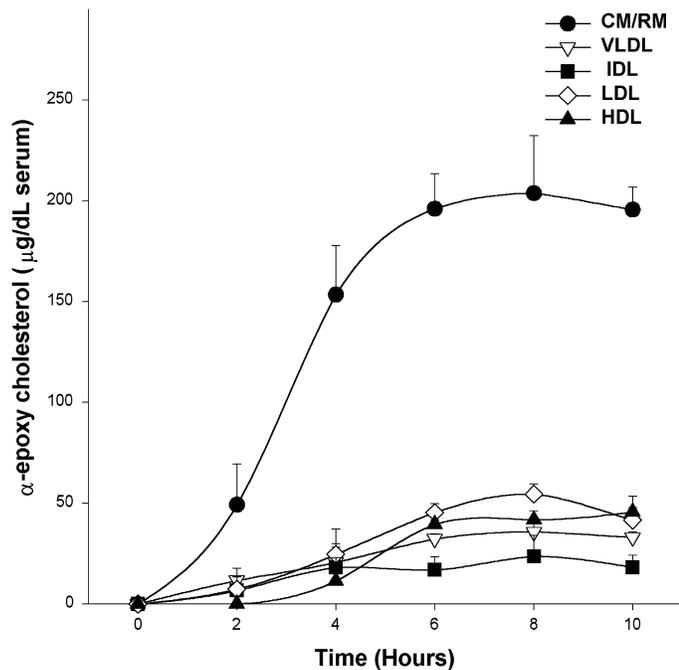
present in the circulating CM/RM fraction. In contrast to controls, the incorporation of  $\alpha$ -epoxy cholesterol into LDL and HDL was markedly reduced. However, because of the delayed CM/RM clearance and the large cholesterol pool in CM/RM in subjects with Type III hyperlipoproteinemia, the fraction of  $\alpha$ -epoxy cholesterol in CM/RM is significantly lower. In CM/RM obtained 6 h after the consumption of the test meal, the ratio of  $\alpha$ -epoxy cholesterol to total CM/RM cholesterol in subjects with Type III hyperlipoproteinemia was significantly lower than in CM/RM of controls ( $22.20 \pm 4.90$  vs.  $1.32 \pm 0.21$   $\mu\text{g}/\text{mg}$  cholesterol;  $P < 0.05$ ) (Table 1).

#### **$\alpha$ -Epoxy cholesteryl ester transfer from diet derived CM/RM fractions to endogenous LDL and HDL**

To determine whether  $\alpha$ -epoxy cholesterol transfers from exogenous CM/RM fraction to endogenous lipoproteins, we incubated diet derived CM/RM fraction with fasting oxidized cholesterol-free human serum ( $d > 1.006$ ) at  $37^\circ\text{C}$  for 12 h. The amount of  $\alpha$ -epoxy cholesterol in the donor CM/RM fraction and in the recipient endogenous lipoproteins LDL and HDL was monitored simultaneously by GLC as described in Materials and Methods. CM/RM samples were obtained at various time intervals (2 h, 4 h, 6 h, 8 h) after the consumption of the test



**Fig. 3.** Time course of  $\alpha$ -epoxy cholesterol, triglyceride, and cholesterol in serum. Three subjects with Type III hyperlipoproteinemia were administered a test meal containing  $\alpha$ -epoxy cholesterol (400 mg), and the quantity of  $\alpha$ -epoxy cholesterol and triglycerides in serum was measured at indicated times. The levels of  $\alpha$ -epoxy cholesterol are expressed as  $\mu\text{g}/\text{dl}$  serum and triglycerides, and cholesterol as  $\text{mg}/\text{dl}$  serum. Data are expressed as mean  $\pm$  SE.



**Fig. 4.** Time course of  $\alpha$ -epoxy cholesterol distribution among the serum lipoproteins. Three subjects with Type III hyperlipoproteinemia were administered a meal containing  $\alpha$ -epoxy cholesterol (400 mg), and serum samples were obtained at indicated times. Lipoproteins were isolated by affinity chromatography followed by sequential ultracentrifugation as described in Materials and Methods. The amount of  $\alpha$ -epoxy cholesterol in CM/RM, VLDL/IDL, LDL, and HDL was measured using GLC.

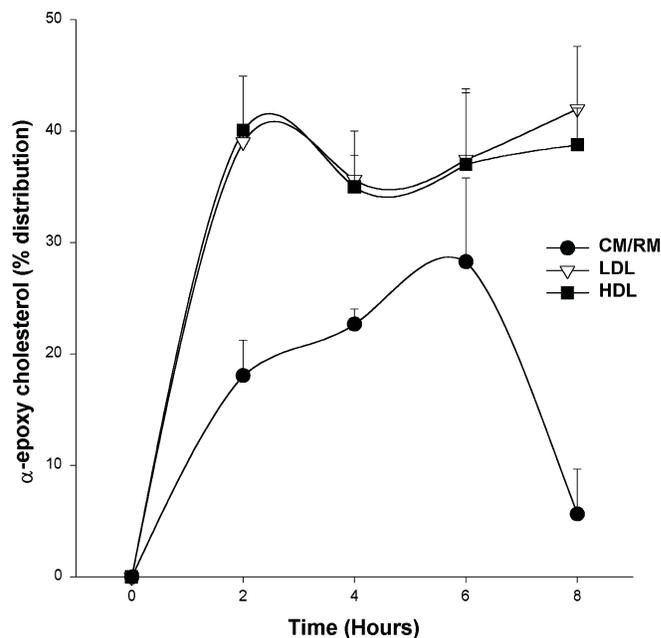
meal, as triglyceride levels in CM/CM were depleted by lipoprotein lipase with the increasing time in the circulation. As shown in **Fig. 5**,  $\alpha$ -epoxy cholesterol was transferred to endogenous lipoproteins from CM/RM isolated as early as 2 h after the consumption of the test meal, and the transfer was similar to both LDL and HDL fractions. With CM/RM isolated 8 h after the test meal, most of the  $\alpha$ -epoxy cholesterol was present in endogenous lipoproteins. No transfer was observed when lipoprotein-free serum was substituted for lipoprotein-containing serum. These results are in agreement with our observations *in vivo* (**Fig. 2**), where  $\alpha$ -epoxy cholesterol is present in endogenous lipoproteins as early as 2 h after the ingestion of the oxidized cholesterol. Thus, our data clearly show that oxidized cholesterol, when ingested, is incorporated into CM/RM fraction and is transferred within the plasma compartment from exogenous to endogenous lipoproteins, and this transfer accounts at least partially for the presence of oxidized cholesterol in LDL and HDL in the circulation.

In the above transfer experiments, the 12 h incubation time was based on published methods (36, 39). In an experiment with CM/RM isolated 6 h after the consumption of the test meal, more than 60% of  $\alpha$ -epoxy cholesterol had transferred from the CM/RM fraction during the initial 2 h period with little change thereafter. Moreover,  $\alpha$ -epoxy cholesterol was equally distributed between LDL and HDL. No transfer was observed when human serum was substituted with rat serum, suggesting that CETP, which is present in human serum but not in rat serum, may be important for this transfer.

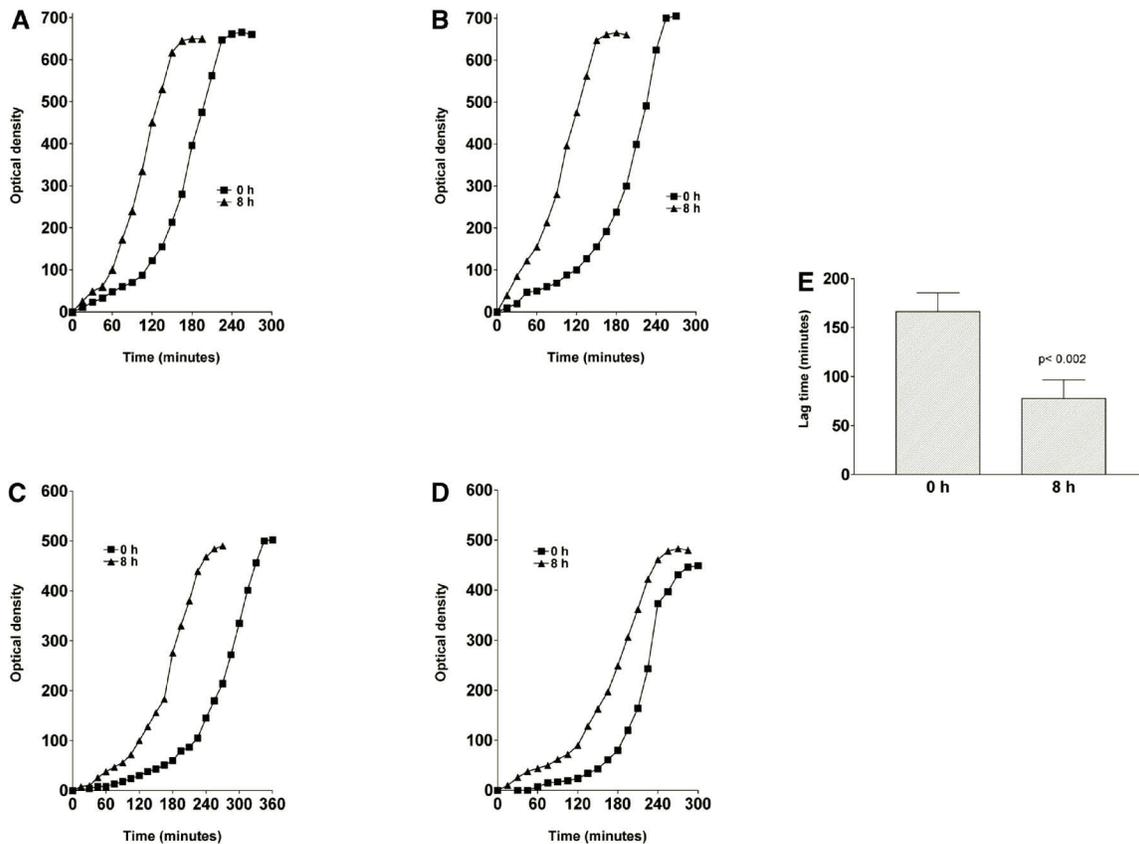
#### Susceptibility of LDL to oxidation

To determine whether the presence of  $\alpha$ -epoxy cholesterol in LDL results in functional changes, we next deter-

mined the ability of copper to oxidize LDL that contains  $\alpha$ -epoxy cholesterol versus control LDL from the same subject. The results are shown in **Fig. 6** and are presented for four subjects as A, B, C, and D. LDL susceptibility and maximum dienes varied among different subjects; however, in all subjects the lag time for the copper oxidation



**Fig. 5.** A line graph showing distribution of  $\alpha$ -epoxy cholesterol among donor CM/RM and acceptor LDL and HDL. At designated time intervals after the consumption of the test meal,  $\alpha$ -epoxy cholesterol containing CM/RM were isolated from serum and then incubated with  $\alpha$ -epoxy free serum for 12 h at 37°C. The amount of  $\alpha$ -epoxy cholesterol transferred to LDL and HDL and remaining in CM/RM was measured using GLC. N = 3.



**Fig. 6.** A graph showing in vitro oxidation of LDL isolated from four subjects (A, B, C, D) at 0 time and 8 h after the consumption of the oxidized cholesterol containing test meal. Absorbance at 234 nm was determined every 15 min as described in Materials and Methods. The mean lag time  $\pm$  SE (E) was calculated from the individual lag times derived from each subject from the intercept of lines drawn through the linear portion of the lag and propagation phases.

was reduced in LDL samples that contained oxidized cholesterol. The mean lag time ( $77.50 \pm 19.20$  vs.  $166.25 \pm 19.19$  min;  $P < 0.002$ ;  $n = 4$ ) for the oxidation of LDL isolated at 0 time and 8 h after the consumption of the test meal is shown in Fig. 6E. It should be noted that the differences in oxidation shown in Fig. 6 were observed on fresh LDL samples. Samples that were stored at  $4^{\circ}\text{C}$  for several days had a decrease in the lag time; however, the difference between the 0 h LDL and LDL samples containing oxidized cholesterol persisted. Thus, the ingestion of oxidized cholesterol in the diet leads to not only an increase in the levels of oxidized cholesterol in LDL but also increases the susceptibility of LDL to further oxidation.

### Characterization of LDL

In order to explain the difference in the susceptibility of LDL samples to copper oxidation isolated, we next examined the composition of LDL preparations obtained from four control subjects before the consumption and 8 h after the consumption of the test meal. The results are summarized in Table 2. No significant differences were obtained in  $\alpha$ -tocopherol levels, fatty acid composition, or free to esterified cholesterol ratio. Thus, the only difference detected in LDL samples obtained after the consumption of the test meal was the incorporation of  $\alpha$ -epoxy cholesterol.

### DISCUSSION

It has been previously demonstrated in rabbits (20–22) and in rodents (18, 19, 23) that dietary oxidized cholesterol is absorbed and enters the circulation via CM/RM particles. As expected, in the present study we found that the absorption of dietary oxidized cholesterol in humans is similar to that observed in other species. Our results demonstrate that in humans oxidized cholesterol in the

TABLE 2. The composition of LDL ( $n = 4$ )

	0 h	8 h
Cholesterol	100 mg	100 mg
% Free cholesterol	$23.21 \pm 0.15$	$22.89 \pm 0.65$
% Cholesterol ester	$76.76 \pm 0.15$	$77.61 \pm 1.0$
$\alpha$ -Epoxy cholesterol	0	$90.67 \pm 6.09^b$
Triglyceride <sup>a</sup>	$15.90 \pm 2.21$	$12.95 \pm 1.38$
% Palmitate (16:0)	$21.39 \pm 3.13$	$19.80 \pm 2.08$
% Stearate (18:0)	$17.45 \pm 2.68$	$19.8 \pm 2.08$
% Oleate (18:1)	$48.71 \pm 3.07$	$51.65 \pm 1.44$
% Linoleate (18:2)	$4.04 \pm 0.58$	$3.80 \pm 0.38$
% Linolenate (18:3)	$6.43 \pm 1.00$	$4.95 \pm 0.58$
% Arachidonate (20:4)	$1.99 \pm 0.14$	$2.39 \pm 0.35$
$\alpha$ -Tocopherol <sup>a</sup>	$3.85 \pm 0.31$	$4.21 \pm 0.12$

Values are given as means  $\pm$  SEM.

<sup>a</sup> Expressed as  $\mu\text{g}/100$  mg cholesterol.

<sup>b</sup>  $P < 0.02$ .

diet is absorbed by the small intestine and then incorporated into CM/RM particle; however, it is not only the exogenous lipoproteins (CM/RM) produced by the intestine that are enriched in oxidized cholesterol but endogenous lipoproteins (LDL, HDL) also contain cholesterol that is oxidized. In control subjects, the enrichment of CM/RM particles with oxidized cholesterol is seen soon after the ingestion of the oxidized cholesterol containing meal, is markedly decreased by 10 h, and is not detected at 24 h (Fig. 2). Similarly, oxidized cholesterol is also present in LDL and HDL soon after the meal, but in contrast to CM/RM particles, which are rapidly cleared from the circulation, the levels of oxidized cholesterol in these endogenous lipoprotein particles persist for an extended time period. Thus, the ingestion of a single meal containing oxidized cholesterol results in elevated oxidized cholesterol in serum lipoproteins for several days. In subjects with Type III hyperlipoproteinemia, where the clearance of CM/RM is delayed, less  $\alpha$ -epoxy cholesterol is incorporated into LDL and HDL, and the majority of oxidized cholesterol remains in the circulating chylomicron remnant fraction. Thus, after a meal enriched in oxidized cholesterol, atherogenic particles such as LDL in controls and chylomicron remnants in subjects with Type III hyperlipoproteinemia are enriched in diet derived oxidized cholesterol. The mechanism by which oxidized cholesterol is absorbed by the small intestine is unknown; whether it is similar to nonoxidized cholesterol and plant sterols is unclear, and the role of ABCA1, ABCG5, or ABCG8 has not been defined.

There are two potential pathways by which dietary oxidized cholesterol can be transferred from CM/RM particles to endogenous serum lipoproteins. First, CM/RM particles containing oxidized cholesterol could be taken up by the liver and the oxidized cholesterol then incorporated into newly secreted endogenous lipoprotein particles (16). Second, during the metabolism of particles, oxidized cholesterol could be directly transferred to LDL and HDL in the circulation, presumably through the action of CETP. To address this question, we carried out *in vitro* studies to determine whether diet-derived CM/RM can serve as  $\alpha$ -epoxy cholesterol donors for endogenous lipoproteins while in the circulation. As shown in Fig. 5, oxidized cholesterol is indeed transferred from CM/RM particles to endogenous LDL and HDL, and is distributed nearly equally between these lipoprotein fractions.

In subjects with Type III hyperlipoproteinemia, less  $\alpha$ -epoxy cholesterol was detected in the endogenous lipoprotein fractions when compared with controls (Fig. 4). Because the transfer is dependent on the relative  $\alpha$ -epoxy cholesterol concentration of the donor CM/RM when compared with total cholesterol pool (36, 40), the reduced transfer to endogenous lipoproteins can be explained by the reduced fraction of  $\alpha$ -epoxy cholesterol in the total cholesterol pool in the postprandial CM/RM of subjects with Type III hyperlipoproteinemia. As seen in Table 1, the ratio of oxidized cholesterol to total cholesterol CM/RM particles in control subjects was markedly higher than in subjects with Type III hyperlipoprotein-

emia. An additional contributing factor for the reduced  $\alpha$ -epoxy cholesterol levels in the endogenous serum lipoproteins of subjects with Type III hyperlipoproteinemia could be the delayed uptake of  $\alpha$ -epoxy cholesterol containing CM/RM by the liver. Because  $\alpha$ -epoxy cholesterol in the liver can be utilized for endogenous lipoprotein production (16), it is possible that less  $\alpha$ -epoxy cholesterol is available to be incorporated in these lipoproteins of patients with Type III hyperlipoproteinemia. However, the rapid appearance of oxidized cholesterol in endogenous lipoproteins and the low levels in VLDL point to the possibility of oxidized cholesterol transfer from exogenous to endogenous lipoproteins.

The mechanism for this transfer of  $\alpha$ -epoxy cholesterol to LDL and HDL is not known at the present time; however, it is known that in human plasma, CETP mediates the exchange of cholesteryl esters and triglycerides between HDL and apoB-containing lipoproteins (41). It has been suggested that *in vivo* LDL and HDL are donors for cholesteryl ester, and triglyceride-rich lipoproteins are the acceptors (41). Moreover, it has been shown by Serdyuk and Morton (42) that CETP also facilitates a homotransfer of cholesteryl ester between serum lipoproteins that appear to occur at a much faster rate than the exchange between cholesteryl ester and triglyceride. The rapid incorporation of oxidized cholesterol ester into LDL and HDL observed in this study suggests the possibility that cholesteryl ester from CM/RM could be exchanged with cholesteryl ester in endogenous lipoproteins (42).

Moreover, Lin and Morel (39) have shown that oxidized cholesterol in HDL can transfer to isolated LDL *in vitro*. Our studies demonstrate that  $\alpha$ -epoxy cholesterol transfers from CM/RM to LDL and HDL, and therefore diet-derived triglyceride-rich lipoproteins can serve as donors of oxidized cholesterol for endogenous lipoproteins both *in vitro* and *in vivo*. The possibility that this process is facilitated by CETP is supported by our observations that most of  $\alpha$ -epoxy cholesterol in CM/RM is present in an esterified form, and there was no transfer when the human serum was substituted with rat serum.

The absorption of dietary oxidized fatty acids has been examined in our laboratory and other laboratories, and it has been shown that both rodents (14–16, 43) and rabbits (17, 44) absorb oxidized fatty acids from the diet. Moreover, in rodents we were able to demonstrate the hepatic uptake of CM/RM particles that contained oxidized fatty acids, and the secretion of VLDL particles by the liver enriched in these oxidized fatty acids (16).

In humans, we found that after a single meal containing oxidized fatty acids, oxidized fatty acids are incorporated into CM/RM particles that are cleared from the circulation by 8 h. Thus, it is clear that both oxidized fatty acids, and oxidized cholesterol are absorbed by the small intestine, incorporated into CM/RM particles, and delivered to the liver, where they can be utilized for the formation of endogenous serum lipoproteins. The major difference is that a single meal containing oxidized cholesterol results in an appearance of oxidized cholesterol in endogenous lipoproteins, whereas a single meal containing oxi-

dized fatty acids does not. No oxidized fatty acids were detected in VLDL, LDL, or HDL. The explanation for this difference is not clear at the present time; however, it is possible that oxidized fatty acids are utilized by a diverse metabolic pathway such as uptake by the adipose tissue, muscle, and other tissues, or oxidized fatty acid containing endogenous lipoprotein particles are taken up more rapidly from the circulation by scavenger receptors.

A limitation of the present study was that a large amount of oxidized cholesterol was fed in the test meal. However, for assessing  $\alpha$ -epoxy cholesterol in each lipoprotein fraction, given the low sensitivity of our method, we used large amounts of oxidized cholesterol in our test meal. Moreover, our measurements were performed after a single meal and not after a daily-sustained ingestion of a diet containing low amounts of oxidized cholesterol. It should be noted that we found that there was a strong correlation between the quantity of oxidized cholesterol in the test meal and the quantity of oxidized cholesterol in the serum across a wide range of dietary oxidized cholesterol (50 mg to 400 mg), suggesting that our observations will be relevant even when lower amounts of oxidized cholesterol are ingested.

Oxidized cholesterol has repeatedly been implicated in the development of atherosclerosis (44); however, the mechanism by which cholesterol epoxides might promote oxidative stress is not clear. There is no direct evidence that oxidized cholesterol is atherogenic in humans. It has been shown that oxidized cholesterol displays numerous proatherogenic properties in vitro (45, 46). In animals, numerous studies have demonstrated that oxidized cholesterol in the diet may contribute to the atherogenic process. Vine et al. (21) showed that dietary oxidized cholesterol resulted in a marked increase in cholesterol deposition in the aorta. Rong et al. (47) demonstrated in rabbits that oxidized cholesterol causes endothelial dysfunction and increases macromolecular permeability and cholesterol deposition in the aorta. Jacobsen et al. (48) and Peng et al. (49) demonstrated an increase in atherosclerotic lesions in the aortas of White Carneau pigeons and squirrel monkeys, respectively. We have demonstrated that adding oxidized cholesterol to the diet increases fatty-streak formation in rabbit aortas (22). Additionally, oxidized cholesterol in the diet increased the development of atherosclerosis in apoE and LDL-receptor deficient mice (23). Given these results in animal models, it is likely that oxidized cholesterol in the diet would also accelerate atherosclerosis in humans. In fact, oxidized cholesterol is found in human foam cells and advanced atherosclerotic lesions (30, 31). In individuals with atherosclerosis, plasma levels of oxidized cholesterol appear to be higher than in healthy controls (50), and coronary atherosclerosis is reflected by autoantibodies against oxidized LDL and oxidized cholesterol in the serum (51). In the typical American diet, 5% of dietary cholesterol is oxidized because of the ingestion of fried, heated, and processed foods in which cholesterol is oxidized during the food preparation (8, 9, 10–13).

The present study demonstrates that this dietary oxi-

dized cholesterol is absorbed and incorporated into both exogenous and endogenous serum lipoprotein particles. In addition, the increase in oxidized cholesterol in LDL is associated with an increased susceptibility to further oxidation, as shown by decreased lag time when oxidized by copper (Fig. 6), indicating that these particles have an increased propensity to undergo further oxidation that may occur in the vessel wall. At present, the mechanism by which dietary oxidized cholesterol in LDL particles increases the susceptibility to copper oxidation is unknown. Particle size and density (52, 53), fatty acid composition (54, 55), free cholesterol concentration (52), and antioxidant content (37, 53) have all been implicated in the susceptibility to in vitro oxidation; however, there seems to be a lack of agreement among these various investigators regarding which of these factors plays the major role. We found no differences in either the fatty acid composition or antioxidant content in LDL samples isolated after the consumption of the test meal containing oxidized cholesterol. It is of course possible that alterations such as conformational changes (52, 53) resulting in a different exposure of fatty acid side chains or antioxidants to copper oxidation could be occurring.

Oxidation of lipoproteins is currently considered a key event in the pathogenesis of atherosclerosis. We have demonstrated that oxidized cholesterol in the diet clearly contributes to the levels of oxidized lipoproteins in the circulation, including atherogenic chylomicron remnants and LDL. Thus, it is possible that this increase in circulating oxidized lipoproteins following the ingestion of oxidized cholesterol could interact with endothelial cells and/or enter the vessel walls, then accelerate atherosclerosis by a number of mechanisms, including inducing endothelial cell dysfunction, monocyte chemotaxis, and foam cell formation. ■■

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