Oxidized Cholesterol in the Diet Accelerates the Development of Aortic Atherosclerosis in Cholesterol-Fed Rabbits

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Abstract—Oxidized lipoproteins may play a role in atherosclerosis. Recently, we have demonstrated that the levels of oxidized fatty acids in the circulation correlate directly with the quantity of oxidized fatty acids in the diet and that dietary oxidized fatty acids accelerate atherosclerosis in rabbits. The present study tests the hypothesis that oxidized cholesterol in the diet accelerates the development of atherosclerosis. Rabbits were fed a diet containing 0.33% nonoxidized cholesterol (control diet) or the same diet containing 0.33% cholesterol of which 5% was oxidized (oxidized diet). Serum cholesterol levels increased to a similar extent in both groups, with the majority of cholesterol in the β-VLDL fraction. Moreover, in the serum β-VLDL fraction and liver, there was a significant increase in the oxidized cholesterol levels. Most importantly, feeding a diet enriched in oxidized cholesterol resulted in a 100% increase in fatty streak lesions in the aorta. Western diets contain high concentrations of oxidized cholesterol products, and our results suggest that these foods may be a risk factor for atherosclerosis. (Arterioscler Thromb Vasc Biol. 1998;18:977-983.)

Key Words: oxidized cholesterol β-VLDL atherosclerosis oxidized diet

The etiology of cardiovascular disease is complex and multifactorial, but there is substantial evidence that oxidized lipoproteins play an important role in atherosclerosis. This hypothesis is supported by a large number of in vitro studies and experimental animal studies using antioxidants and by epidemiological investigations. However, the site and mechanism by which lipoproteins are oxidized is not resolved, and it is not clear whether the oxidation of LDL and other lipoproteins occurs in the artery wall, as suggested by several investigators, and/or whether oxidized lipids are sequestered in atherosclerotic lesions following the uptake of circulating oxidized serum lipoproteins.

Recent studies in our laboratory have demonstrated that oxidized fatty acids in the diet play a significant role in lipoprotein oxidation. We have shown that in rodents and humans, oxidized fatty acids in the diet are absorbed by the small intestine and incorporated into chylomicrons. In rodents, oxidized dietary fatty acids are also incorporated into the endogenous serum VLDL+LDL fraction. The levels of oxidized chylomicrons and VLDL+LDL directly correlated with the quantity of oxidized lipids in the diet. Furthermore, we have shown that oxidized lipids in the diet are delivered to the liver via chylomicrons, incorporated into VLDL, and resecreted into the circulation, thereby providing a mechanism by which dietary oxidized lipids can affect the oxidative state of endogenous lipoproteins.

Most importantly, we recently have demonstrated that oxidized lipids in the diet are atherogenic. We have shown that feeding a diet enriched in oxidized fatty acids to cholesterol-fed rabbits resulted in a significant increase in fatty streak lesions in the aorta. These results demonstrate for the first time that diets containing oxidized fatty acids accelerate atherosclerotic lesions. Owing to the popularity of fried foods and the widespread fast-food industry, oxidized polyunsaturated fatty acids are common in the Western diet and could constitute a risk factor for cardiovascular disease.

Similar to fatty acids, cholesterol also undergoes free radical–mediated oxidation via hydroperoxide formation, resulting in the production of numerous oxygenated derivatives (oxidized cholesterols or oxysterols). Moreover, it is well established that due to processing, heating, or prolonged storage, the Western diet contains large quantities of oxidized cholesterol. Thus, in the present study, we have tested the hypothesis that oxidized cholesterol in the diet accelerates atherosclerosis.

Methods

Animal Model

To facilitate comparison with our previous study, which demonstrated that oxidized fatty acids in the diet accelerate atherosclerosis, in this study we used a similar experimental protocol. Twenty-six 10- to 11-week-old male New Zealand White rabbits (2 kg initial weight) were divided into two equal groups and were individually housed in stainless steel cages at the San Francisco Veterans Affairs Animal Housing. The animal care facility is accredited by the American Association for Accreditation of Laboratory Care, and all

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procedures were reviewed and approved by the Institutional Animal Care Subcommittee.

Animal Diets and Experimental Protocol
One group (control diet group) was fed a diet (150 g rabbit chow per day) to which 0.33% cholesterol was added. The cholesterol used in diet preparations was stored at −70°C under N₂. The second group (oxidized diet group) was fed a similar diet (0.33% cholesterol), except approximately 5% of the total added cholesterol was oxidized, i.e., these rabbits received 25 mg oxidized cholesterol per day. We prepared oxidized cholesterol for our test diet by heating at 100°C for 8 hours as described by Kim and Nawar.12 The composition of cholesterol oxidation products is highly dependent on the oxidizing conditions,12 and under these conditions, approximately 95% of the cholesterol was not altered and 5% was oxidized, yielding numerous oxidation products. Typically, the oxidized cholesterol had the following cholesterol oxidation products that were identified by us: 7% cholest-5-ene-3β,7α-diol (7α-hydroxycholesterol), 20% cholest-5-ene-3β,7β-diol (7β-hydroxycholesterol), 16% 5β-epoxy-5β-cholestan-3β-ol (5β-epoxycholesterol), 12% 5α-epoxy-5α-cholestan-3β-ol (5α-epoxycholesterol), 42% 5β-hydroxycholesterol, 5α-hydroxycholesterol-5-ene-7-one (7-cholestenol), and 3% cholest-5-ene-3β,25-diol (25β-hydroxycholesterol). The area for these six oxysterols accounted for 52% of the total. Both diets were prepared weekly, and the amounts of the above-described diets were adjusted such that all animals ate their daily food allowance and had a similar food intake. Cholesterol was added to these diets in an ether solution, and the solvent was evaporated under an N₂ stream. The control diet was fed a similar diet (0.33% cholesterol), containing remnants of dietary [chylomicron] and hepatic (VLDL) lipoproteins. In agreement with the results of others17 the oxidized diet group 29.99 ± 2.36 mmol/L EDTA and 5 mmol/L BHT in the diet did not significantly alter the serum cholesterol in the diet did not significantly alter the serum cholesterol levels in rabbits were markedly increased at 2 weeks, reaching maximum levels at 4 weeks. At the end of the experiment (12 weeks), the average serum cholesterol level for control and oxidized diet groups was 28.12 ± 2.95 mmol/L and for the oxidized diet group 29.99 ± 3.37 mmol/L (not significant [NS]). Figure 1 shows the cholesterol distribution among lipoprotein particles. In agreement with the results of others17 and our previous study,1 the oxidized cholesterol concentration was plotted versus time (12 weeks) as described1 and is given in mmol · L⁻¹ · d⁻¹. Serum triglyceride levels were measured at the beginning and end of the experiment.

Serum Lipid Analysis
At 12 weeks, rabbits were killed in pairs (one control rabbit for each rabbit fed the oxidized diet) after an overnight fast and their blood was collected. Lipoproteins were isolated by sequential ultracentrifugation18 in the density ranges 1.006 to 1.019 g/mL (β-VLDL containing remnants of dietary [chylomicron]) and hepatic (VLDL) origin, including IDL), 1.019 to 1.063 g/mL (LDL), and 1.063 to 1.225 g/mL (HDL). All serum and lipoprotein samples included 10 µmol/L EDTA and 5 µmol/L BHT. Cholesterol was measured in serum and lipoprotein fractions to determine the cholesterol distribution among lipoproteins. Oxidized cholesterol levels in serum lipoprotein fractions were determined in a Hewlett Packard model HP 5890 gas-liquid chromatograph as described by Hughes et al13 using a DB-1 bonded-phase capillary column (30 m × 0.25 mm inner diameter, J & W Scientific). An HP 3600 integrator series II was used for data analysis. Lipoproteins were extracted as described by Folch et al.,15 and the lipid-containing fraction was analyzed for cholesterol and oxidized cholesterol (7β-hydroxycholesterol, 7α-hydroxycholesterol, 25α-hydroxycholesterol, 7-cholesterol, α-epoxycholesterol, and β-epoxycholesterol). Standards for these oxidized cholesterol determinations were obtained from Steraloids, Inc. To minimize the destruction of any oxidized cholesterol products, the saponification in this procedure was performed at room temperature for 16 to 18 hours as described by Addis et al.16

Oxidized Cholesterol Determination in the Liver
Oxidized cholesterol in the hepatic lipid fraction was determined by GLC as described above. Livers (1 g) were homogenized in 1 mL saline containing 10 µmol/L EDTA, 5 mmol/L BHT and the lipid was extracted from the homogenates by the procedure of Folch et al.15

Morphological Examination of Atherosclerotic Lesions
As in our previous studies,7 at the end of the experiment, rabbits were anesthetized with ketamine (35 mg/kg) plus xylazine (10 mg/kg). The chests were opened and the rabbits were bled by cardiac puncture and then killed by a pentobarbital overdose (200 mg/kg weight). After laparotomy, the aortas and livers were removed. The aorta was dissected from the aortic valve to the iliac bifurcation and the adventitia was removed as much as possible to prevent errors resulting from Sudan staining of the vessel. The aorta was opened longitudinally and pinned flat on a styrofoam surface. After overnight fixation in 10% formalin (Buffered Formalde-Fresh, Fisher Scientific Co), the aorta was rinsed in 70% ethanol for 10 minutes and then stained with 0.5% Sudan IV in 35% ethanol and 50% acetone for 20 minutes. Destaining was carried out for 20 minutes in 80% ethanol. Lipid deposition in aorta was determined by morphological assessment of the percentage of the aorta covered by lesions visualized by fat staining of the region between the aortic root and bifurcation. The fatty streak lesions in the enlarged photographs were traced on a digital tablet (Kurta IS/ADB, Inmac Inc), and the areas of the lesions were measured using MacDraft software on a Macintosh Computer.

Analytical Methods
Serum cholesterol in serum and lipoproteins (kit No. 352 to 20 by Sigma Chemical Co) and triglycerides (kit No. 339 to 20 by Sigma Chemical Co) were determined by enzymatic assays as specified by the manufacturer.

Statistical Analysis
Unless stated otherwise, all results are expressed as mean ± SEM. Student’s t test was used to test the significance between the means, which was set at P < 0.05. All computations were done using the Statistica 4.1 application for Macintosh (StatSoft Inc).

Results
Serum Cholesterol and Triglyceride Levels
After the initiation of either the nonoxidized cholesterol (control) or oxidized cholesterol diet, serum cholesterol concentrations in rabbits were markedly increased at 2 weeks, reaching maximum levels at 4 weeks. At the end of the experiment (12 weeks), the average serum cholesterol level for the control diet group was 28.12 ± 2.95 mmol/L and for the oxidized diet group 29.99 ± 3.37 mmol/L (not significant [NS]). Figure 1 shows the cholesterol distribution among lipoprotein particles. In agreement with the results of others17 and our previous study,1 the oxidized cholesterol concentration was plotted versus time (12 weeks) as described1 and is given in mmol · L⁻¹ · d⁻¹. Serum triglyceride levels were measured at the beginning and end of the experiment.
serum triglyceride concentration in the control and oxidized diet groups were 0.78±0.57 and 0.88±0.58 mmol/L, respectively (NS). At the start of the experiment, the average triglyceride concentration in all rabbits was 0.61±0.11 mmol/L.

Oxidized Lipoproteins in the Serum

When freshly isolated serum β-VLDL was examined, oxidized cholesterol was detected in both groups of rabbits (Figure 2). The major cholesterol oxidation products that were present in the diet were also detected in the serum β-VLDL fraction. However, the levels of several oxidized cholesterols in the β-VLDL fraction were higher in rabbits fed the oxidized diet. 7α-Hydroxycholesterol increased by approximately 200%, 7β-hydroxycholesterol by 50%, and α-epoxycholesterol by 80% in β-VLDL after the oxidized cholesterol diet. However, it should be noted that 7-ketocholesterol, which was the main oxidized cholesterol component in the diet, showed only a very small increase in the serum β-VLDL fraction after the oxidized cholesterol diet. This effect may be due to a different transport mechanism of this cholesterol oxidation product to the liver. It has been suggested by Addis et al18 that serum albumin and not lipoproteins might be the major carrier of ketocholesterol to the liver. Thus, oxidized cholesterol in the diet at least partially contributes to the oxidized cholesterol levels in β-VLDL. However, the presence of basal levels of oxidized cholesterol in β-VLDL in rabbits fed the control diet containing no oxidized cholesterol indicates that the endogenous production of oxidized cholesterol also contributes to the oxidized cholesterol in rabbit serum.

There was no difference in the oxidized cholesterol levels in serum LDL fraction after feeding oxidized cholesterol to rabbits (Figure 3). 7α-Hydroxycholesterol that was present in the oxidized diet and β-VLDL fraction was not detected in LDL. This finding supports previous findings that cholesterol oxidation products are not distributed equally among all plasma lipoproteins.19,20 Due to low serum levels, the HDL fraction was not examined for oxidized cholesterol.

Oxidized Cholesterol in the Liver

The oxidized cholesterol content of liver at the conclusion of the experiment is presented in Figure 4. As described for serum...
\( \beta \)-VLDL fraction, significantly more oxidized cholesterol was found in livers after consumption of the diet containing oxidized cholesterol. The oxidized cholesterol that shows the greatest change was 7\( \beta \)-hydroxycholesterol, which was increased by 100% (0.16±0.07 versus 0.33±0.02 \( \mu \)g/mg liver tissue, \( P<0.02 \)). A similar 100% increase was also found in \( \beta \)-epoxycholesterol (0.11±0.04 versus 0.22±0.02 \( \mu \)g/mg liver tissue, \( P<0.05 \)). 7-Ketocholesterol increased from 0.14±0.06 to 0.29±0.03 \( \mu \)g/mg liver tissue (\( P=0.06 \)). Thus, these results suggest that oxidized cholesterol is absorbed, incorporated into lipoproteins, and transported to the liver. 7-Ketocholesterol does not appear to be transported by \( \beta \)-VLDL but by some other plasma carrier, possibly BSA, as suggested by Addis et al.\(^{18} \) The presence of oxidized cholesterol in livers of rabbits fed the control diet, which did not contain detectable oxidized cholesterol, indicates that endogenous production of oxidized cholesterol contributes to hepatic oxidized cholesterol levels. The hepatic nonoxidized cholesterol content in control and oxidized diet groups was similar (21.19±1.61 versus 21.86±2.26 \( \mu \)g/mg liver tissue, NS).

**Effect of Oxidized Dietary Cholesterol on Fatty Streak Formation**

At the end of the experiment, the aortas were removed and fatty streak lesions in the aortas were measured.\(^7 \) The comparison of the percent lesion areas in aortas in both groups of rabbits is shown in Figure 5. The control and oxidized diet group had lesion areas of 28.54±4.89% and 57.11±4.84%, respectively (\( P<0.001 \)). Thus, very small quantities of oxidized cholesterol in the rabbit diet (25 mg/d) increased fatty streak lesions by 100%. This demonstrates that cholesterol in the diet is considerably more atherogenic when present in the oxidized form.

Since it has been established by other investigators\(^{21,22} \) and by us\(^7 \) that a wide biological variability occurs among rabbits with respect to individual responsiveness to dietary cholesterol and that the severity of arterial lesions correlates with the serum cholesterol concentration, the data was also calculated as a function of serum cholesterol exposure (expressed as mmol cholesterol \( \cdot \) L\(^{-1} \) \( \cdot \) d). When lesions in rabbit aortas are expressed as ratios of aortic lesion divided by the cholesterol exposure for each rabbit, our data also show a 100% increase in aortic lesions in the oxidized cholesterol diet group (0.0082±0.0013 versus 0.0163±0.0017; \( P<0.001 \)). Thus, our results, even when adjusted for serum cholesterol levels, demonstrate that the atherogenicity of dietary cholesterol is significantly increased by oxidation.

**Discussion**

There is a large body of evidence showing that oxidized lipoproteins are involved in atherosclerosis;\(^{1,2} \) however, the origin of oxidized lipoproteins in vivo is uncertain. Recent studies in our laboratory have demonstrated that oxidized fatty acids in the diet contribute to serum lipoprotein oxidation. We have shown in rodents\(^3,5 \) and humans\(^6 \) that oxidized fatty acids in the diet are absorbed by the small intestine and incorporated into the serum chylomicron fraction. In rodents,
oxidized dietary fatty acids are also incorporated into the endogenous VLDL fractions. Moreover, we have demonstrated that oxidized fatty acids in the diet accelerate atherosclerosis in cholesterol-fed rabbits. In this study, we demonstrate that oxidized cholesterol in the serum of rabbits is both synthesized endogenously and derived from food. The evidence for endogenously produced oxidized cholesterols is the observation that after feeding rabbits a control diet that contains no detectable levels of oxidized cholesterol, both rabbit serum β-VLDL and LDL contained oxidized cholesterols as identified by GLC. As shown in Figures 2 and 3, there is a basal level of oxidized cholesterols in the serum of rabbits fed a control diet. The exact source of endogenous oxidized cholesterols is not clear, but it could be produced by enzymatic and/or nonenzymatic oxidation. Enzymatic oxidation mainly occurs in liver and stereidogenic tissues, and several cholesterol oxides are produced in the liver in the course of enzymatic oxidation of cholesterol for the production of bile acids. Furthermore, the elevated oxidized cholesterol could also reflect cholesterol peroxidation through free radical-mediated nonenzymatic processes. The radical species responsible for cholesterol oxidation are derived from activated oxygen, which could occur in a variety of tissues, or within the artery cell wall, as suggested by several investigators. Such endogenously produced cholesterol oxidation products in rabbit serum have been described previously by other investigators. In vivo formation of oxidized serum cholesterol has been also shown by Breuer and Bjorkhem using an O2 inhalation technique.

After feeding rabbits diets containing oxidized cholesterol, we found a significant increase in cholesterol oxidation products in serum β-VLDL (Figure 2). It should be noted that the cholesterol oxidation product distribution in the diet was not reflected in the increase in serum lipoprotein fractions, since the highest elevation was observed in 7α- and 7β-hydroxycholesterols, relatively minor components in the oxidized diet. These discrepancies are probably due to differences in the extent of absorption of each individual oxysterol, the variability of oxidized cholesterol transfer from lymph chylomicrons to other lipoproteins, and the distribution of each individual oxysterol among serum lipoprotein fractions or other serum oxidized cholesterol carriers such as BSA, and possible differences in oxidized cholesterol clearance.

Similar to the serum β-VLDL fraction, oxidized cholesterol was also observed in the livers of animals fed a cholesterol diet containing no detectable oxidation products (Figure 4); however, there was an increase in 7β-hydroxycholesterol, β-epoxycholesterol, and 7-ketocholesterol in the liver after feeding the cholesterol diet containing oxidation products. Because previously the liver has been suggested to be the main site of oxidized cholesterol accumulation subsequent to feeding, it is likely that this increase is secondary to the absorption of oxidized cholesterols from the diet. Thus, these findings suggest that similar to oxidized fatty acids, oxidized cholesterols, when present in the diet, are absorbed by the small intestine, incorporated into serum lipoproteins, and delivered to the liver. However, 7-ketocholesterol, the major cholesterol oxidation product in the oxidized diet, was not increased in the serum β-VLDL or LDL fraction but was greatly increased in the liver. This finding supports a previous observation by Addis et al, suggesting that serum albumin may be one of the carriers of oxidized cholesterol to the liver. This could account for the absence of a large increase of 7-ketocholesterol in the serum lipoproteins after feeding the oxidized diet.

The absorption of dietary oxidized cholesterol has been demonstrated previously. Peng et al have shown that within 24 hours after feeding trace amounts of radiolabeled oxidized cholesterol to normolipemic rabbits, the oxidized cholesterol label appeared in serum VLDL and LDL fractions. There is also evidence that dietary oxidized cholesterol is absorbed in rats. When administered by gavage, radiolabeled cholesterol was recovered in serum VLDL and lymph chylomicrons. Similar absorption of oxidized cholesterol has also been described in humans. Diet-derived oxidized cholesterol has been identified in both chylomicrons and LDL. Thus, several different studies by a number of investigators have shown that dietary oxidized cholesterol is absorbed, packaged into lipoproteins, and, as suggested previously, delivered to the liver.

The major finding of the present study was that oxidized cholesterol is more atherogenic than nonoxidized cholesterol. Our results (Figure 5) demonstrate that feeding a diet enriched in oxidized cholesterol (25 mg/d) results in a 100% increase in fatty streak lesions in the rabbit aorta. A similar large difference in fatty streak lesions was observed even when percent fatty streak lesions in the aorta were adjusted for serum cholesterol exposure.

Several previous studies have examined the atherogenesis of oxidized dietary cholesterol in animal models. Jacobson et al observed a fivefold increase in coronary atherosclerosis in White Carneau pigeons after feeding oxidized cholesterol in amounts that are comparable to the average US dietary intake. On the other hand, Higley et al reported that oxidized cholesterol has a protective effect on cholesterol-induced atherosclerosis in rabbits. However, in this study, oxidized cholesterol concentrations in the diet were high (120 to 240 mg/d), and preliminary studies in our laboratory and observations of Osada et al indicate that such high concentrations of oxidized cholesterol impair the absorption of cholesterol from the diet. Thus, it is likely in the experiments of Higley et al that high quantities of oxidized cholesterol in the diet reduced the absorption of total cholesterol, which resulted in decreased serum cholesterol levels and consequently decreased fatty streak lesions in the aorta. Our study is the first study in mammals to demonstrate that oxidized cholesterol in the diet accelerates atherosclerosis.

Atherosclerosis is a complex process that is still not completely understood. There are several potential mechanisms by which oxidized cholesterol in circulating lipoproteins could accelerate atherosclerosis. First, oxidized cholesterol is cytotoxic to many cells, including endothelial cells, and numerous studies have shown that 7α-hydroxycholesterol, 7β-hydroxycholesterol, and 7-ketocholesterol are all very cytotoxic to arterial wall cells in vitro. In our study, we have demonstrated that rabbits ingesting a diet containing oxidized cholesterol have elevated 7α-hydrocholesterol, 7β-
hydroxycholesterol, and 7-ketocholesterol in their serum β-VLDL fraction. Endothelial injury has been proposed to be a major factor in initiating the atherogenic process that leads to fatty streak formation.44 Other investigators have reported that oxidized cholesterol induces endothelial cell injury in rabbits in vivo. Imai et al45 and Taylor et al46 orally administered oxidized cholesterol and reported vascular injury as manifested by dead or dying smooth muscle cells. Similar findings of endothelial damage have also been reported in rats.47–49 Thus, oxidized cholesterol causing endothelial injury is one potential mechanism by which oxidized cholesterol in the diet could accelerate fatty streak formation.

A second potential mechanism by which oxidized cholesterol in lipoproteins could accelerate atherosclerosis is by inducing foam cell formation. Studies by other investigators have shown that oxidized β-VLDL is degraded by macrophages at an accelerated rate compared with native β-VLDL.50,51 Additionally, oxidized β-VLDL leads to increased lipid accumulation in smooth muscle cells.52,53 Thus, after consuming a meal containing oxidized cholesterol, the vascular tissues are exposed to lipoproteins containing oxidized cholesterol, which by a variety of mechanisms could initiate or accelerate aortic fatty streak formation and atherosclerosis.

It is well established that the typical diet in Western countries contains high concentrations of cholesterol oxidized products. Food processing, especially heat treatment and drying, induces cholesterol oxidation. Oxidized cholesterol esters are detected in various food products, including dairy products, eggs, meat, and fish.8–11 Many bakery products also contain oxidized cholesterol due to the presence of butter and eggs. Oxidized cholesterol esters found in these food sources clearly provide an exogenous source of oxidized cholesterol.

In summary, the present study demonstrates that oxidized cholesterol in the diet accelerates fatty streak lesion formation in rabbit aortas. Dietary oxidized cholesterol may be a risk factor for atherogenesis, and therapeutic interventions that reduce oxidized dietary cholesterol intake may have a role in the prevention and treatment of atherosclerosis.

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References


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