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The role of dietary oxidized cholesterol and oxidized fatty acids in the development of atherosclerosis

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The etiology of atherosclerosis is complex and multifactorial but there is extensive evidence indicating that oxidized lipoproteins may play a key role. At present, the site and mechanism by which lipoproteins are oxidized are not resolved, and it is not clear if oxidized lipoproteins form locally in the artery wall and/or are sequestered in atherosclerotic lesions following the uptake of circulating oxidized lipoproteins. We have been focusing our studies on demonstrating that such potentially atherogenic oxidized lipoproteins in the circulation are at least partially derived from oxidized lipids in the diet. Thus, the purpose of our work has been to determine in humans whether oxidized dietary oxidized fats such as oxidized fatty acids and oxidized cholesterol are absorbed and contribute to the pool of oxidized lipids in circulating lipoproteins. When a meal containing oxidized linoleic acid was fed to normal subjects, oxidized fatty acids were found only in the postprandial chylomicron/chylomicron remnants (CM/RM) which were cleared from circulation within 8 h. No oxidized fatty acids were detected in low density lipoprotein (LDL) or high density lipoprotein (HDL) fractions at any time. However, when alpha-epoxy cholesterol was fed to human subjects, alpha-epoxy cholesterol in serum was found in CM/RM and also in endogenous very low density lipoprotein, LDL, and HDL and remained in the circulation for 72 h. *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. We have suggested that cholesteryl ester transfer protein is mediating the transfer. Thus, alpha-epoxy cholesterol in the diet is incorporated into CM/RM fraction and then transferred to LDL and HDL contributing to lipoprotein oxidation. We hypothesize that diet-derived oxidized fatty acids in chylomicron remnants and oxidized cholesterol in remnants and LDL accelerate atherosclerosis by increasing oxidized lipid levels in circulating LDL and chylomicron remnants. This hypothesis is supported by our feeding experiments in animals. When rabbits were fed oxidized fatty acids or oxidized cholesterol, the fatty streak lesions in the aorta were increased by 100%. Moreover, dietary oxidized cholesterol significantly increased aortic lesions in apo-E and LDL receptor-deficient mice. A typical Western diet is rich in oxidized fats and therefore could contribute to the increased arterial atherosclerosis in our population.

Keywords: Atherosclerosis / Oxidized cholesterol / Oxidized diet / Oxidized fatty acids

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1 Introduction

There is compelling evidence that oxidized lipoproteins are atherogenic and play a key role in the pathogenesis of coronary heart disease [1, 2]. Oxidized lipoproteins have been identified in atherosclerotic lesions in both animals and humans [3]. However, the origin of oxidized lipoproteins *in vivo* is not clear since the site and mechanism whereby lipoproteins are oxidized have not been resolved. It has been suggested that the oxidation of lipoproteins may occur locally in the artery wall or that circulating oxidized lipoproteins are sequestered in atherosclerotic lesions [4]. In our laboratory we have concentrated our efforts to demonstrate that oxidized lipids in the diet contribute to oxidized lipoproteins in the circulation. Our hypothesis is that dietary oxidized lipids are absorbed by the intestine and then incor-

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Abbreviations: CM/RM, chylomicrons/chylomicron remnants; **HDL**, high density lipoproteins; **LDL**, low density lipoproteins; **VLDL**, very low density lipoproteins

porated into serum lipoproteins and such diet-derived oxidized lipoproteins in the circulation are potentially atherogenic.

It is well established that the typical diet in Western countries contains high concentrations of lipid oxidation products [5–7]. When exposed to heat, air, light and oxidizing agents, cholesterol and polyunsaturated fatty acids undergo spontaneous oxidation forming oxidation products. Food processing, especially heat treatment and drying, induces lipid oxidation in foods including dairy products, eggs, meat, and fish. Oxidized cholesterol is also present in bakery products since the major ingredients, eggs and butter, contain large amounts of oxidized cholesterol.

Dietary fat enters the circulation as chylomicrons, is carried in the circulation as chylomicrons/chylomicron remnants (CM/RM), and then is taken up by the liver [8]. Previously, we have established that in rodents, oxidized fatty acids and oxidized cholesterol in the diet are absorbed, incorporated into serum CM/RM, and are transported to the liver [9, 10]. In this study, we have investigated whether in humans dietary oxidized lipids in the diet contribute to oxidized lipoproteins in the circulation.

2 Incorporation of oxidized fats in human lipoproteins

In our initial experiments in humans, we examined whether oxidized fatty acids are incorporated into postprandial CM/RM following a meal that contained oxidized fatty acids derived from heated corn oil [11]. As shown in Fig. 1, the

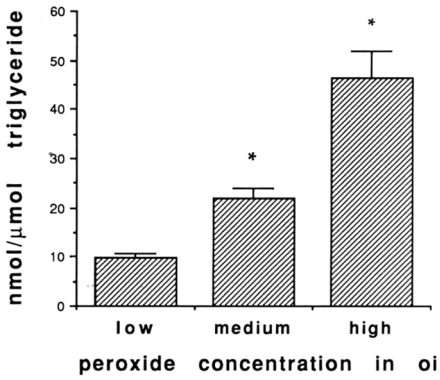

Figure 1. Lipid oxidation in the CM/RM fraction measured as conjugated dienes. Six subjects were fed corn oil containing increasing lipid peroxides. Control, medium oxidized oil, and highly oxidized oil contained 6.5–10, 30–50, and 180–120 nmol conjugated dienes/mg oil, respectively. Serum samples were obtained at 4 h and the CM/RM fraction was isolated. $^{*}p < 0.005$ when control group fed control oil was compared to the group fed either medium oxidized oil or highly oxidized oil. Data are presented as mean ± SEM.

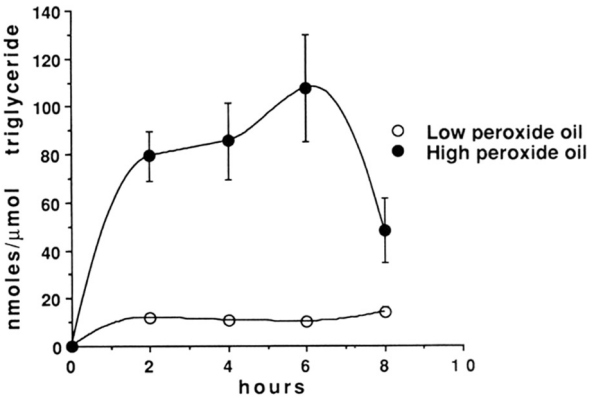

Figure 2. Profiles of postprandial total conjugated dienes in the CM/RM fraction (nmol conjugated dienes/μmol triglyceride) in four men who consumed control (6.5 nmol conjugated dienes/mg oil) and oxidized oil (180 nmol conjugated dienes/mg oil) diets. Conjugated dienes and triglycerides were determined at indicated time points.

quantity of oxidized fatty acids found in CM/RM was proportional to the amount of oxidized fatty acids in the oil in the test meal (measured as conjugated dienes). These results directly demonstrate that oxidized fatty acids in the diet are absorbed by the small intestine and then packaged into chylomicrons which are transported into the bloodstream in humans. Figure 2 shows the time course of CM/RM clearance after the consumption of a control and a meal containing oxidized oil. Over the 8 h time period it takes to clear CM/RM, there were considerably higher levels of oxidized fatty acids in individuals that were fed the oxidized meal than those that were fed the control meal. In all subjects studied, feeding oxidized fatty acids resulted in an increase in oxidation only in the CM/RM fraction and not in any other lipoprotein fraction such as very low density lipoprotein (VLDL), high density lipoprotein (HDL) or low density lipoprotein (LDL).

Since it is known that this dietary effect is exaggerated in animal models of diabetes [12], we have examined whether humans with diabetes also have increased levels of oxidized fatty acids in CM/RM following the ingestion of oxidized fatty acids. We found that the ingestion of oil containing increasing quantities of lipid peroxides leads to a corresponding increase in levels of lipid peroxides in the CM/RM fraction in both control and diabetic subjects (Fig. 3). Materials and methods are described in detail in [13]. At 2.5 h after the consumption of the oxidized oil test meal, in diabetic subjects in poor glycemic control, there was a large increase in serum oxidized fatty acids when compared to controls. Diabetic patients in good glycemic control had similar levels of oxidized fatty acids in their CM/RM as control subjects. Additionally, as shown in Fig. 4, in diabetic patients in poor glycemic control the levels of oxidized lipids in CM/RM remained elevated for an extended

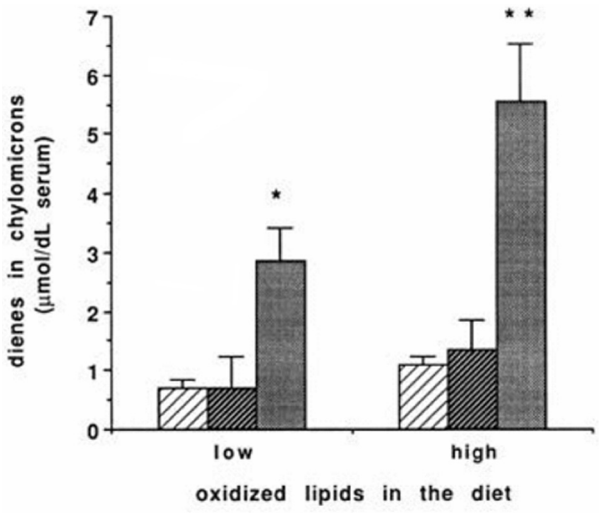

Figure 3. Lipid oxidation in postprandial serum CM/RM fraction. Control subjects (left bar) or diabetic patients in good (middle bar) and poor glycemic control (right bar) were administered diets containing either low or high amounts of lipid peroxides; 2.5 h after the consumption of the meal, CM/RM were isolated and conjugated dienes were measured to determine the oxidation. *For the low oxidized lipid diet, $p < 0.01$ when control subjects are compared with diabetic patients in poor glycemic control and $p < 0.05$ when diabetic patients in good diabetic control are compared to diabetic patients in poor glycemic control. **For the high oxidized lipid diet, $p < 0.01$ when control subjects are compared to diabetic patients in poor glycemic control and $p < 0.05$ when diabetic patients in good glycemic control are compared with diabetic patients in poor control. Difference between control subjects and diabetic patients in good glycemic control was not significant. Data are presented as mean ± SEM.

postprandial period. The area under the curve of conjugated dienes in the CM/RM fraction was increased approximately three-fold in the poorly controlled diabetic patients. Thus, in poorly controlled diabetic patients oxidized fatty acids in the diet result in an increase in oxidized fatty acids in the circulation for an extended postprandial time period.

Thus, we have demonstrated that in diabetic subjects with poor glycemic control dietary oxidized fatty acids induce an exaggerated and sustained increase in the levels of oxidized fatty acids in CM/RM when compared to controls or diabetic patients with good glycemic control. CM/RM generated postprandially can potentially deposit cholesterol in the vessel wall [14] and the prolonged presence of CM/RM in the circulation may increase the atherogenic risk of diabetic patients with poor glycemic control. In patients with diabetes, the atherogenic risk may be exaggerated when chylomicron remnants are oxidized and these increased postprandial levels of potentially atherogenic oxidized lipids may contribute to the accelerated atherosclerosis associated with diabetes.

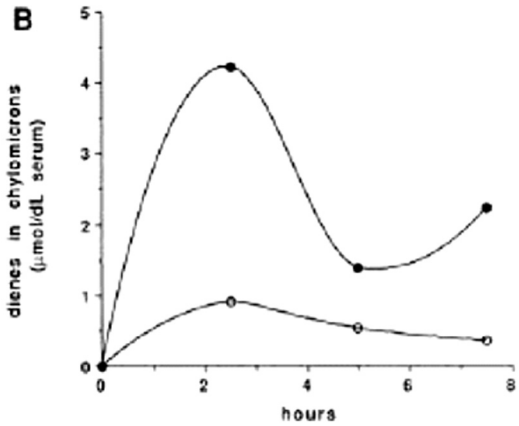

Figure 4. Time course of CM/RM triglyceride and diene clearance. Six diabetic patients in poor glycemic control and six control subjects were administered oxidized corn oil (0.100 – 0.150 μmol conjugated dienes/μmol triglyceride) and the triglyceride levels and the quantity of conjugated dienes were measured at indicated times. Two subject groups, when compared by analysis of variance with repeated measurements, differed significantly ($p = 0.001$).

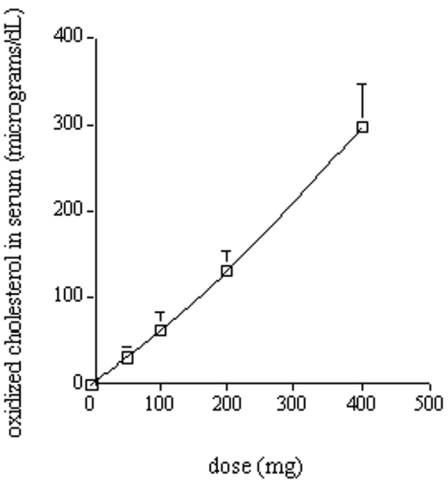

Figure 5. Line graph showing the effect of different alpha-epoxy cholesterol quantities in the test meal on alpha-epoxy cholesterol levels in postprandial serum. Serum levels of alpha-epoxy cholesterol strongly correlate ($r = 0.997$; $p < 0.001$; $n = 3$) with the amount of alpha-epoxy cholesterol in the test meal (50 – 400 mg).

Next we determined whether in humans oxidized cholesterol in the diet is absorbed by the small intestine and contributes to the pool of oxidized lipids in humans and whether dietary oxidized cholesterol may be incorporated in serum lipoproteins including CM/RM, VLDL, HDL, and LDL [15]. In our initial studies, we determined the effect of different quantities of oxidized cholesterol (alpha-epoxy cholesterol) in the test meal on alpha-epoxy cholesterol levels in postprandial serum (Fig. 5). All oxidized cholesterol determinations were performed using GLC. We found

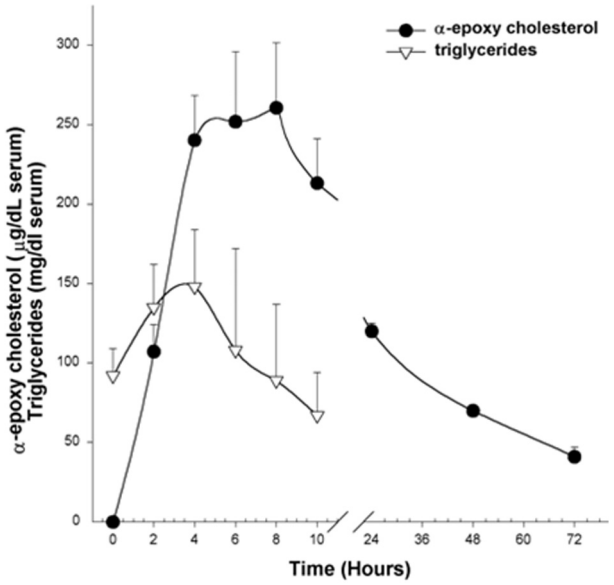

Figure 6. Time course of alpha-epoxy cholesterol and triglycerides in serum. Six control subjects were administered a test meal containing alpha-epoxy cholesterol (400 mg), and the quantity of alpha-epoxy cholesterol and triglycerides, in serum, was measured at indicated times. Levels of alpha-epoxy cholesterol are expressed as μg/dL serum and triglycerides as mg/dL serum. For the 24, 48, and 72 h time points, $n = 3$. Data are expressed as mean ± SE.

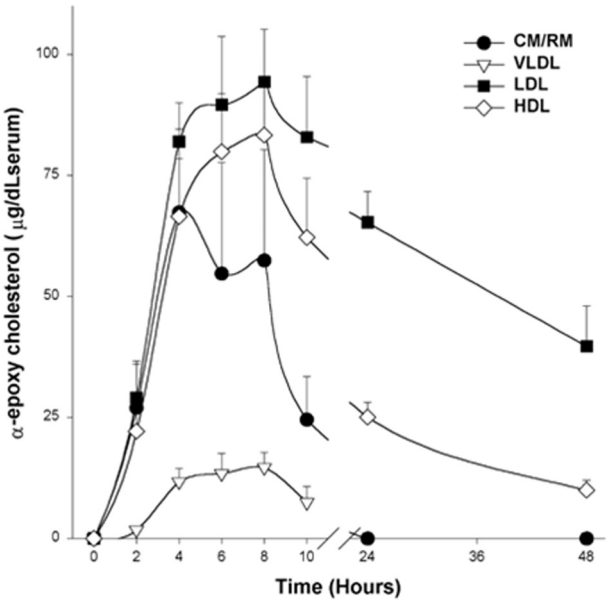

Figure 7. Time course of alpha-epoxy cholesterol distribution among the serum lipoproteins. Six control subjects were administered a meal containing alpha-epoxy cholesterol (400 mg) and serum samples were obtained at indicated times. Lipoproteins from serum were isolated by affinity chromatography followed by sequential ultracentrifugation. Amount of alpha-epoxy cholesterol in CM/RM, VLDL/IDL, LDL, and HDL was measured using GLC. For the 24, 48, and 72 h time points, $n = 3$. Data are expressed as mean ± SE.

that the serum levels of alpha-epoxy cholesterol strongly correlate with the amount of alpha-epoxy cholesterol in the test meal (50 – 400 mg). 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.

We next examined the time course of the increase in serum alpha-epoxy cholesterol levels after feeding the test meal. As shown in Fig. 6, after feeding a test meal containing 400 mg alpha-epoxy cholesterol, the levels of serum triglycerides peaked at 2 – 3 h and returned to baseline 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. Because the postprandial increase in serum triglycerides is mainly associated with CM/RM, this difference in the time course between oxidized cholesterol and triglycerides suggests that alpha-epoxy cholesterol in the serum is not solely associated with CM/RM but may be present in other serum lipoprotein particles. 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, with LDL displaying the highest levels. Figure 7 shows the time course of appearance of alpha-epoxy cholesterol 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 then were 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 remnants and at 72 h the circulating alpha-epoxy cholesterol was found only in endogenous serum lipoproteins, especially LDL.

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 [10]. Sec-

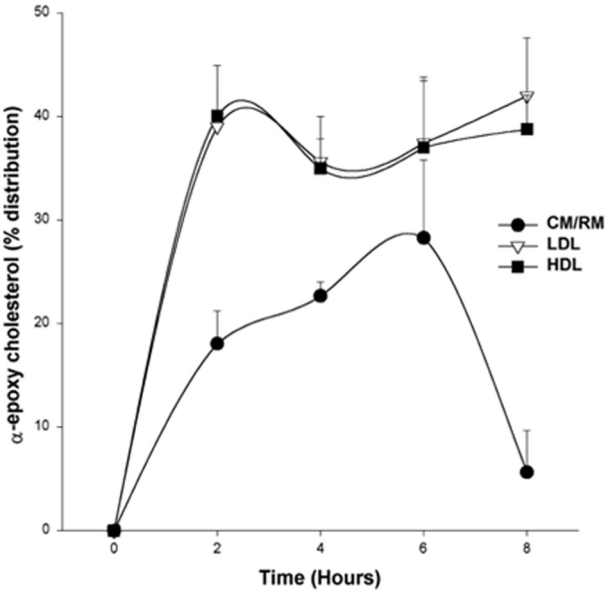

Figure 8. Line graph showing distribution of alpha-epoxy cholesterol among donor CM/RM and acceptor LDL and HDL. At designated time intervals after the consumption of the test meal, alpha-epoxy cholesterol containing CM/RM was isolated from serum and then incubated with alpha-epoxy free serum for 12 h at 37°C. Amount of alpha-epoxy cholesterol transferred to LDL and HDL and remaining in CM/RM was measured using GLC, $n = 3$.

ond, during the metabolism of particles, oxidized cholesterol could be directly transferred to LDL and HDL in the circulation presumably through the action of cholesteryl ester transfer protein (CETP) [16]. 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. When alpha-epoxy cholesterol containing CM/RM were incubated with fasting human serum, alpha-epoxy cholesterol was transferred to endogenous lipoproteins from CM/RM isolated as early as 2 h after the consumption of the test meal. As shown in Fig. 8, oxidized cholesterol is indeed transferred from CM/RM particles to endogenous LDL and HDL and is distributed nearly equally between these lipoprotein fractions. These results are in agreement with our observations *in vivo* (Fig. 6) 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. 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 the rat serum which does not contain CETP.

3 The effect of oxidized dietary fat on fatty streak formation in animals

Oxidized lipids have been repeatedly implicated in the development of atherosclerosis [1, 2]; however, there is no direct evidence that oxidized cholesterol is atherogenic in humans. The most direct evidence for the atherogenicity of oxidized lipids comes from feeding experiments in animals. We have shown that adding oxidized fatty acids [17] and oxidized cholesterol [18] to the diet increases fatty streak formation in rabbit aortas. Feeding a diet enriched in oxidized fatty acids to cholesterol-fed rabbits resulted in an incorporation of oxidized fatty acids into serum β-VLDL (Fig. 9) and a significant increase in fatty streak lesions in

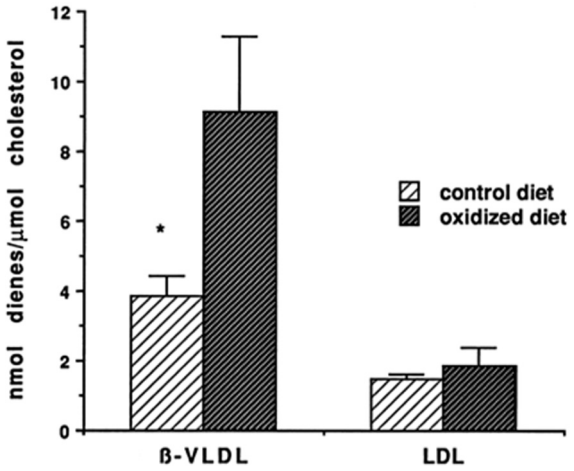

Figure 9. Bar graph shows lipid oxidation in the β-VLDL ($d = 1.006 – 1.019$) and LDL ($d = 1.019 – 1.063$) fractions measured as conjugated dienes. Rabbits were fed either the control or oxidized diet containing oxidized fatty acids. $^*p < 0.05$ for dienes compared in β-VLDL fractions. $n = 7$ for each group. Data are presented as mean ± SEM.

the aorta (Fig. 10). Similar to oxidized fatty acid diets, feeding diets containing oxidized cholesterol also resulted in an increase in fatty streak lesions in the aortas of rabbits (Fig. 11) [18]. Rabbits fed the oxidized diet also had a greater than 100% increase in the deposition of total cholesterol in the pulmonary artery that was due primarily to an increase in cholesteryl ester (Fig. 12). These results demonstrated for the first time that diets containing either oxidized polyunsaturated fatty acids or oxidized cholesterol cause an acceleration of fatty streak lesion formation in the aorta indicating that oxidized lipids in the diet are atherogenic.

In the rabbit, the majority of cholesterol in the circulation is carried in β-VLDL [17]. This animal model has been criti-

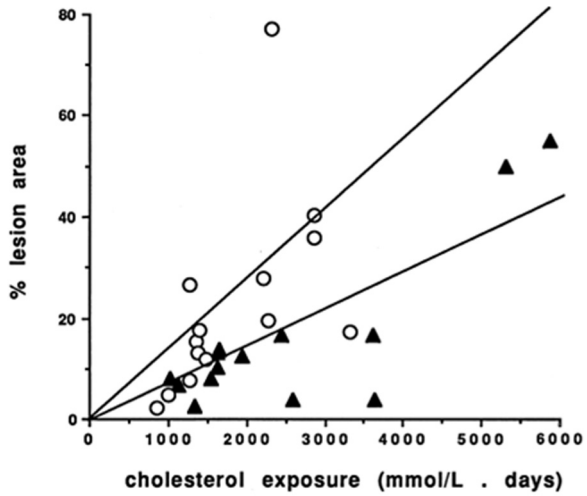

Figure 10. Scatterplot showing the dependence of aortic lesion areas on cholesterol exposure for rabbits fed either control or oxidized lipid diet. Cholesterol exposure was calculated as the AUC of serum cholesterol level (expressed as mmol cholesterol/L × days). $n = 14$ for each diet group.

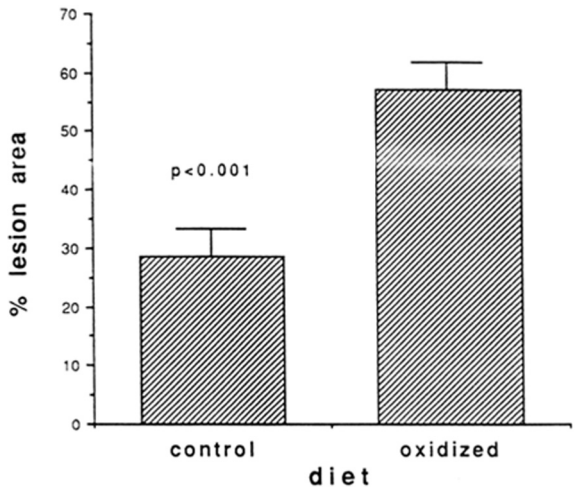

Figure 11. Bar graphs show the percent aortic area covered by fatty streaks in rabbits. Rabbits were fed either control or oxidized cholesterol diet for 12 wk. There were 14 rabbits in each diet group. Data are presented as mean ± SEM.

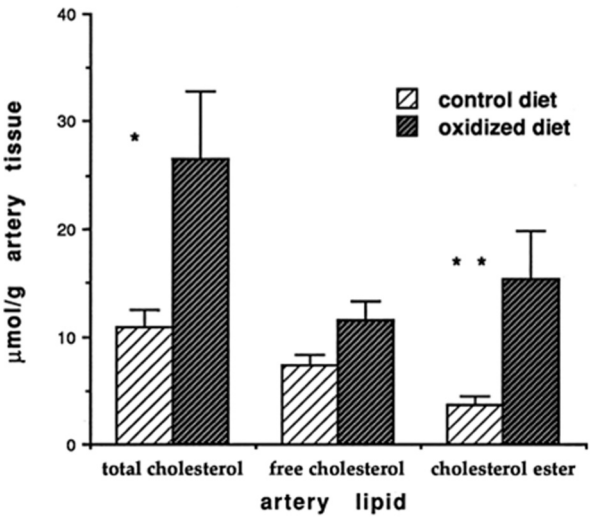

Figure 12. Bar graphs showing cholesterol and cholesterol ester deposition in rabbit pulmonary arteries after rabbits were fed either control or oxidized lipid diet for 12 – 14 wk. $^{*}p < 0.02$, $^{**}p < 0.05$. $n = 8$ for each group. Data are presented as mean ± SEM.

cized in that it fails to mimic human lipoprotein metabolism and that the aortic lesions that develop with cholesterol feeding are not identical to those seen in humans. Murine models, such as LDL-receptor (LDLR) deficient and apo-E deficient mice, have been generated that develop extensive atherosclerosis and have been widely used as models in which to study the atherogenic process. These animals mimic human lipoprotein disorders that are associated with an increased risk of coronary heart disease. In LDLR deficient mice, similar to humans with familial hypercholesterolemia, the majority of cholesterol in the circulation accumulates in serum LDL particles particularly after ingestion of a high cholesterol diet [19]. In apo-E deficient mice, similar to humans with familial dysbetalipoproteinemia, the accelerated atherosclerosis is due to increased serum chylomicron and VLDL remnants. In these models, aortic atherosclerosis resembles human lesions [20].

When we measured serum oxidized cholesterol levels in these mice fed either control or oxidized cholesterol diet [21], increased levels of oxidized cholesterol after ingestion of oxidized cholesterol containing diets were detected in both LDLR and apo-E deficient mice indicating that in these mice, dietary oxidized cholesterol is absorbed and enters the circulation contributing to the overall oxidative stress. In LDLR deficient mice, no cholesterol oxidation products could be detected by our method in the serum of mice fed the control diet that contained no detectable oxidized cholesterol (Fig. 13). In contrast, in LDLR deficient mice fed a diet containing oxidized cholesterol, measurable levels of 7-ketocholesterol and alpha-epoxy cholesterol were detected. Oxidized cholesterol detected in the serum of LDLR deficient mice fed an oxidized cholesterol diet is likely to be derived from dietary sources.

In apo-E deficient mice, oxidized cholesterol was detected in the serum of both diet groups (Fig. 14). 7-Ketocholesterol, 7β-hydroxycholesterol, β-epoxycholesterol, and 7α-hydroxycholesterol were present in the serum of mice fed the control diet that contained no oxidized cholesterol. Mice that were fed the oxidized cholesterol diet had a four-fold increase in serum concentrations of 7-ketocholesterol and a 100% increase in 7β-hydroxycholesterol – the two

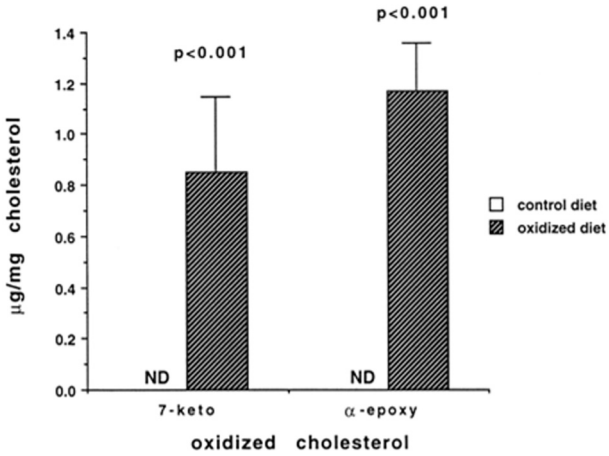

Figure 13. Oxidized cholesterol levels in the serum of LDLR deficient male mice. Mice were fed for 7 months either a control diet containing 1.0% cholesterol ($n = 6$) or an oxidized cholesterol diet containing 1.0% cholesterol where 5% of the added cholesterol was oxidized ($n = 6$). 7-Keto (7-ketocholesterol); alpha-epoxy (alpha-epoxy cholesterol); ND (not detected). Data are presented as mean ± SEM.

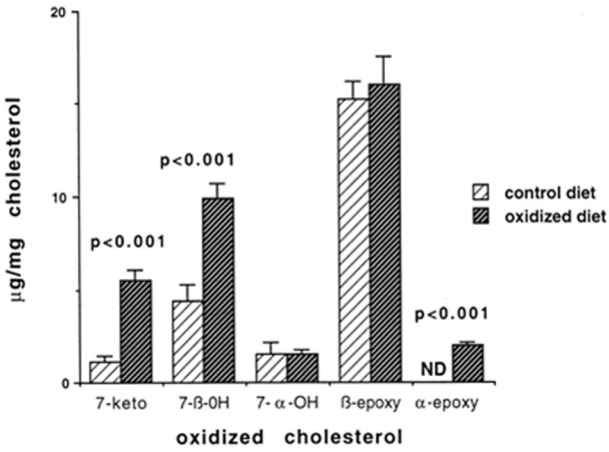

Figure 14. Oxidized cholesterol levels in the serum of apo-E deficient female mice. Mice were fed for 4 months either a control diet containing 0.15% cholesterol ($n = 6$) or an oxidized cholesterol diet containing 0.15% cholesterol where 5% of the added cholesterol was oxidized ($n = 6$). 7-Keto (7-ketocholesterol); 7-β-OH (7-β-hydroxycholesterol); 7-alpha-OH (7α-hydroxycholesterol); β-epoxy (β-epoxycholesterol); alpha-epoxy (alpha-epoxy cholesterol); ND (not detected). Data are presented as mean ± SEM.

main oxidized cholesterol components identified in the diet. Moreover, mice fed the oxidized diet contained alpha-epoxy cholesterol in their serum which was undetectable in mice fed the control diet. No increase was observed in 7α-hydroxycholesterol and β-epoxycholesterol levels in the serum of apo-E deficient mice after feeding the oxidized cholesterol diet. Thus, in apo-E deficient mice, oxidized cholesterol was present in the serum even when fed the control diet that did not contain any detectable cholesterol oxidation products. The explanation for the presence of cholesterol oxidation products in apo-E deficient mice fed the diet containing no detectable oxidized cholesterol and not in LDLR deficient mice is not clear. However, one can speculate that oxidized cholesterol is observed in the serum in animal models where the clearance of diet-derived CM/RM is impaired as occurs in cholesterol-fed rabbits [18] and apo-E deficient mice [20]. This impairment in remnant clearance might allow for the accumulation of oxidized cholesterol that is present in the diet at very low levels although *in vivo* generation cannot be ruled out.

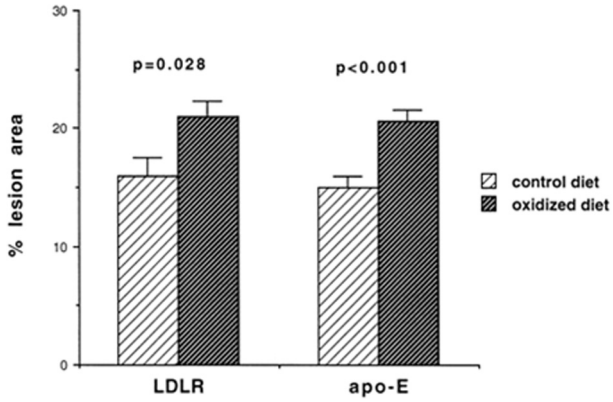

Figure 15. Bar graphs showing areas of fatty streak lesions in aortas of LDLR and apo-E deficient mice fed control and oxidized diets. In LDLR deficient mice, there were 11 animals in the control and the oxidized group. In apo-E deficient mice, there were 13 animals in the control group and 17 animals in the oxidized animal group. Data are presented as mean ± SEM.

At the end of the experiment, aortas from LDLR and apo-E deficient mice were removed and the percent fatty streak lesions determined (Fig. 15) [21]. In LDLR deficient mice, despite the lower serum cholesterol concentration, feeding an oxidized cholesterol diet resulted in a 32% increase in fatty streak lesions. Similarly, in apo-E deficient mice, feeding an oxidized cholesterol diet resulted in a 38% increase in fatty streak lesions. These results demonstrate that oxidized cholesterol in the diet increases fatty streak lesions in both LDLR and apo-E deficient mice. These results confirm and extend our observations in cholesterol-fed rabbits, where we demonstrated that the addition of oxidized cholesterol to the diet increased atherosclerosis by 100% [18].

4 Conclusion

We have shown that oxidized dietary lipids such as oxidized fatty acids and oxidized cholesterol in the diet are absorbed by the intestine and are incorporated into serum lipoproteins in both animals and humans and thereby contribute to

the formation of oxidized lipoproteins in the circulation. In animals, oxidized lipoproteins contribute to fatty streak formation in the aorta and atherosclerosis. Our results demonstrate that oxidized fat in the diet increases fatty streak lesions in aortas of rabbits and both LDLR and apo-E deficient mice. Moreover, the pathogenesis of atherosclerosis is different in these animal models. Apo-E deficient mice represent a model for impaired CM/RM clearance [20] whereas LDLR deficient mice represent a model for impaired LDL clearance [19]. In the cholesterol-fed rabbit the pathogenesis of atherosclerosis is due to increased formation of β-VLDL [17]. Thus, we have shown that oxidized cholesterol in the diet promotes fatty streak lesion formation in several different animal models of atherosclerosis.

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