Cholesterol Oxidation Products Induce Vascular Foam Cell Lesion Formation in Hypercholesterolemic New Zealand White Rabbits

James X. Rong, Lijiang Shen, Yi H. Chang, Arnis Richters, Howard N. Hodis, Alex Sevanian

Abstract—Circulating cholesterol oxidation products (ChOx) have long been implicated in the etiology of early atherosclerosis; however, direct in vivo evidence elucidating their role in atherogenesis is only recently becoming available. This study investigated ChOx effects on vascular lesion formation in New Zealand White rabbits under controlled hypercholesterolemic conditions. By closely monitoring plasma cholesterol levels and adjusting dietary cholesterol intake during a 78-day period, total plasma cholesterol exposures (cumulative plasma cholesterol levels over time) were controlled between 27 000 and 34 000 mg/dL×day (final plasma cholesterol concentration, 467±77 mg/mL), representing a threshold range for sudanophilic lesion formation in the aorta. Twenty injections of a ChOx mixture (70 mg per injection) were made bearing an oxysterol composition similar to that found in circulating oxidatively modified low density lipoprotein. At sacrifice, the ChOx-injected rabbits (n=5) had (1) significantly higher plasma ChOx levels, (2) significantly increased cholesterol content in the aortas, mainly as esterified cholesterol, and (3) significantly greater sudanophilic lesion size and frequency in the aortas compared with vehicle-injected control rabbits (n=5). The aortic cholesterol content and extent of sudanophilic lesion area were correlated significantly with total plasma ChOx exposure (P<0.003 and P<0.0001, respectively) but not with total cholesterol exposure. The results indicate that for moderate experimental hypercholesterolemia, a situation more relevant to physiological hypercholesterolemia in humans, circulating ChOx may play an important role in inducing formation of early atherosclerotic lesions. Because ChOx are often present in cholesterol-containing diets, foam cell lesion formation induced by ChOx rather than cholesterol cannot be overlooked. (Arterioscler Thromb Vasc Biol. 1999;19:2179-2188.)

Key Words: oxysterols ■ cholesterol feeding ■ foam cell lesions ■ hypercholesterolemia

There is growing evidence that cholesterol oxidation products (ChOx) contribute to the development of atherosclerosis.1 Studies specifically addressing this issue have shown that ChOx possess several characteristics that may promote atherosclerosis. These include cytotoxic/apoptotic potential to vascular cells such as fibroblasts,2 endothelial cells,3–5 and smooth muscle cells6–8; impairment of vascular endothelial barrier function9,10; inhibition of endothelial NO release11 and arterial relaxation12; inhibition of cholesterol synthesis or utilization13,14; perturbation of intracellular cholesterol trafficking15–17; and activation of acyl CoA:cholesterol acyltransferase (ACAT) activity.18,19 ChOx are abundant in oxidatively modified LDL (ox-LDL),20 and high levels of circulating ChOx are found in rabbits fed a cholesterol-containing diet.21,22 However, direct evidence linking circulating ChOx to early vascular lesion formation is only recently becoming available.23,24

Recently, we described the pharmacokinetic properties of ChOx in New Zealand White (NZW) rabbits25 by using a ChOx mixture with a composition similar to that found in hypercholesterolemic rabbit plasma21 and in circulating ox-LDL from hypercholesterolemic primates and humans.20,26 Our findings showed that consecutive intravenous injections caused a progressive increase in circulating ChOx levels in normcholesterolemic rabbits, increased vascular permeability, and increased cholesterol accumulation in the aorta. However, atherosclerotic/foam cell lesions were not observed, because plasma cholesterol levels remained in the normal range.

Diet-induced hypercholesterolemia in rabbits has been a widely used model system for studying the development of human atherosclerosis.27 To achieve rapid lesion development, exceedingly high plasma cholesterol levels (>1500 mg/dL)28,29 have been produced by feeding high levels of cholesterol (≥0.5% of diet) or by other dietary methods. As a consequence, the lesions that are usually produced are topographically and morphologically dissimilar to those seen in humans.30 This dissimilarity is due in part to the fact that

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Humans usually do not ingest such large quantities of cholesterol; do not, in general, have plasma cholesterol levels exceeding 800 mg/dL; and process and tolerate cholesterol intake better than do rabbits. However, recent studies employing low levels of dietary cholesterol (<0.5% by diet weight) produced moderate hypercholesterolemia (plasma cholesterol levels ranging from 200 to 800 mg/dL) with gradual lesion formation that more closely resembles early atherosclerosis in humans. Moreover, a number of studies have attempted to define the relationship between atherosclerotic lesion formation and diet-induced hypercholesterolemia in NZW rabbits fed low levels of cholesterol. A total plasma cholesterol exposure (cumulative plasma cholesterol levels over time) can be used to establish a threshold below which aortic lesions are minimal but above which the extent of aortic lesion formation is correlated positively with total plasma cholesterol exposure. On the basis of these studies, we examined the role of ChOx in the development of early atherosclerosis in NZW rabbits whose hypercholesterolemic status was controlled to approximate the threshold required for foam cell lesion formation. Our findings reveal a pivotal role of circulating ChOx in the development of early atherosclerosis under moderately hypercholesterolemic conditions that more closely approximate hypercholesterolemia in humans.

### Methods

#### Rabbit Feeding

Ten male NZW rabbits (~2.5 kg) were acquired from a local breeder (Irish Farms, Norco, Calif) and maintained by the USC vivaria in accordance with the National Institutes of Health guidelines. After a 7-day quarantine period, all rabbits were fed a cholesterol-containing diet prepared by dissolving Sigma-grade (99%+) cholesterol (Sigma Chemical Co) in distilled ether and spraying the solution onto the standard rabbit chow (Teklad No. 8630). The food was allowed to air-dry at room temperature (22°C) and was then stored in airtight containers at 4°C for no more than 2 weeks before use. ChOx content in the cholesterol-supplemented chow was below the limits of detection; however, detection was also limited by the sample size used for analysis. The final cholesterol content added to the chow was 0.1%, 0.2%, and 0.3% by weight, representing 3 levels of cholesterol-containing diets used in this study. Any food remaining in the feeders was replaced with fresh chow every 2 days.

Rabbit plasma cholesterol levels were elevated to and controlled at 200–800 mg/dL throughout the remainder of the study. The rabbits were randomized according to their original plasma cholesterol levels and responsive-ness to cholesterol feeding and were divided into 2 groups consisting of 5 rabbits each.

#### ChOx Injection

The ChOx mixture was stored in ethanol (23.3 mg/mL), and its composition is shown in Table 1, being identical to that described previously. The purity of the individual ChOx was checked before use and found to be >99% during the course of these studies. Injections were initiated 3 weeks after cholesterol feeding (plasma cholesterol level, 450.6 ± 80.5 mg/dL). Before each injection, the ChOx solution (3 mL) was transferred to a sterile 15-mL polypropylene centrifuge tube containing lecithin (140 mg, Avanti Polar Lipids), and the ethanol was evaporated under N2. Saline (0.9% NaCl, 4.5 mL) was added to the residue, the tube was capped under Ar, and the contents were sonicated in a cup-horn sonicator (Heat Systems, Inc) at 4°C until a stable emulsion was obtained (usually requiring 5 minutes). The emulsion was stable for at least 3 hours at room temperature. The emulsion was immediately transferred to a syringe and injected into the rabbit by using a 23-gauge butterfly catheter via the lateral ear vein at a flow rate of 3 mL/min. All procedures were performed under sterile conditions. As shown in Figure 1 (dotted arrows), animals were injected every other day, involving 3 series of 6 or 7 injections with a rest period of 7 days between each series of injection. Each animal received a total of 20 injections, amounting to 1400 mg of ChOx over 78 days. Controls rabbits were injected with the lecithin vehicle alone. No animals died throughout the procedures. The average weight of the rabbits at the end of the experiments was 4.1±0.13 kg, with no differences between the ChOx-injected and control groups.

#### Blood Sampling

Blood was drawn from the central ear artery of each nonfasted animal at baseline, before, and once a week after initiating the cholesterol-feeding protocol (Figure 1, solid arrows). Blood was collected before ChOx or lecithin injection on days when sampling and injection regimens coincided. Plasma was separated from blood (collected with EDTA) by centrifugation (3000 rpm, 4°C, 20 minutes). A 100-μL aliquot was taken for cholesterol determination by use of an automated enzymatic procedure (Cobas Mira automatic analyzer, Roche Diagnostic Inc) that utilizes a Centers for Disease Control and Prevention (Atlanta, Ga) standardized protocol, and the remainder was stored under N2 (−70°C) until further analysis. The cholesterol levels were confirmed by gas chromatography (GC).

#### Chylomicron Isolation

Chylomicrons were separated from plasma by the method of Karpe and Hamsten as modified by Mero et al. In brief, solid NaCl (705 mg) was added to plasma (5.0 mL) to increase its density to 1.10 kg/L. A total volume of 4.0 mL of the plasma was transferred to a polyallomer ultracentrifuge tube (Ultra-Clear, 14×89 mm, Beckman Instruments). A density gradient consisting of 3.0 mL each of 1.065, 1.020, and 1.006 kg/L NaCl solutions was then carefully layered on top of the plasma. Ultracentrifugation was performed in a Beckman L8-55 ultracentrifuge equipped with an SW-40 Ti swinging-bucket rotor.
was expressed as the sum of individual ChOx. ChOx content in biological samples was performed by the internal standard method to identify and quantify cholesterol and individual ChOx. Total ChOx fitted with a DB-1 capillary column (J&W Scientific Inc) equipped vials, and analyzed with a Shimadzu GC-14 gas chromatograph. Cholesterol and ChOx were collected, hydrolyzed by cold alkaline "Diol" solid-phase extraction columns (VWR Scientific). The cho- lesterol and ChOx were extracted by a modified Bligh-Dyer procedure and evaporated to dryness under N2. TC was determined as described previously. Alternatively, CEs and FC were separated using the method of Kaluzny et al with some modifications. In brief, total lipids were redissolved in chloroform (0.5 mL), applied to an aminopropyl column (VWR Scientific) preconditioned by washing with hexane (4 mL), and allowed to dry under vacuum aspiration. The column was then eluted with chloroform/isopropanol (2:1 vol/vol, 4 mL total volume). The eluate (neutral lipids) was collected, reconstituted in hexane (0.2 mL), and applied to a new aminopropyl column prepared as above. Hexane (4 mL) was applied and the eluate (mainly unoxidized CEs) collected. Another new aminopropyl column was then attached in a piggyback fashion to the existing column, and hexane (6 mL) containing 1% diethyl ether and 10% methylene chloride was eluted through both columns. The eluate (triglycerides and oxidized CEs) was collected and pooled with the previous CE eluate. The columns were then eluted with methanol (8 mL) and the eluate (FC) collected. The CE and FC fractions were then evaporated to dryness and subjected to cold alkaline saponification for GC analysis (described above). TC is expressed as the sum of CEs and FC.

**Determination of Total Cholesterol (TC), Cholesteryl Esters (CEs), and Free Cholesterol (FC)**

As indicated in Figure 2, the entire aortic arch, upper half of the thoracic aorta, and upper abdominal aorta (between the celiac and right renal branch) from the right half of the rabbit aorta were weighed and minced in a tissue homogenizer (Tekmar Instruments). Total lipids were extracted by a modified Bligh-Dyer procedure and evaporated to dryness under N2. TC was determined as described previously. Alternatively, CEs and FC were separated using the method of Kaluzny et al with some modifications. In brief, total lipids were redissolved in chloroform (0.5 mL), applied to an aminopropyl column (VWR Scientific) preconditioned by washing with hexane (4 mL), and allowed to dry under vacuum aspiration. The column was then eluted with chloroform/isopropanol (2:1 vol/vol, 4 mL total volume). The eluate (neutral lipids) was collected, reconstituted in hexane (0.2 mL), and applied to a new aminopropyl column prepared as above. Hexane (4 mL) was applied and the eluate (mainly unoxidized CEs) collected. Another new aminopropyl column was then attached in a piggyback fashion to the existing column, and hexane (6 mL) containing 1% diethyl ether and 10% methylene chloride was eluted through both columns. The eluate (triglycerides and oxidized CEs) was collected and pooled with the previous CE eluate. The columns were then eluted with methanol (8 mL) and the eluate (FC) collected. The CE and FC fractions were then evaporated to dryness and subjected to cold alkaline saponification for GC analysis (described above). TC is expressed as the sum of CEs and FC.

**Statistics**

Statistical analysis was performed using Origin 5.0 (Microcal Software, Inc). Quantitative results were reported as mean ± SEM. Statistical evaluations for differences between paired observations were made with a two-sample t test. The total plasma cholesterol/ChOx exposure over the course of the study was determined by applying a trapezoidal approximation to the cumulative plasma cholesterol/ChOx level versus time curve. The relationship between the percent sudanophilic lesion area and the total plasma cholesterol/ChOx exposure was determined by linear correlation analysis.

**Results**

**Control of Plasma Cholesterol Level and Total Plasma Cholesterol Exposure**

As shown in Figure 3A, there was no difference between the cholesterol levels for ChOx-injected rabbits versus controls. Total plasma cholesterol exposure is defined as the area under the cumulative plasma cholesterol levels versus time curve. Figure 3B shows that there was no difference in total plasma cholesterol exposure between the 2 groups (P = 0.69).
Plasma Total ChOx Levels and Total Plasma ChOx Exposure

As shown in Figure 4A, total ChOx levels increased in both groups due to cholesterol feeding. However, ChOx levels in the controls reached a plateau (21.9 ± 1.4 mg/dL) as plasma cholesterol concentrations leveled off at 500 mg/dL and were maintained at this level by adjusting dietary cholesterol intake with 0% to 0.3% cholesterol-containing chow. In contrast, ChOx levels in ChOx-injected rabbits increased continuously, reaching a maximum of 49.7 ± 7.4 mg/dL. As shown in Figure 4B, the total plasma ChOx exposure was 1.8-fold greater in the ChOx-injected rabbits than in the controls (P < 0.0002).

Contribution of Chylomicrons to Plasma Cholesterol/ChOx Exposure

As shown in Figure 5, at the end of the injection regimen and just before sacrifice, the chylomicron fraction contained slightly greater proportions of total plasma cholesterol in the controls than in the ChOx-injected rabbits. The proportion of ChOx in the chylomicron fraction was 2-fold greater when expressed as a percentage of the plasma ChOx in the controls (78.3 ± 10.9%) than in the ChOx-injected rabbits (40.2 ± 6.3%, P < 0.04 versus control), suggesting different origins for the plasma ChOx between the controls and the ChOx-injected rabbits. This finding also indicates that the diet served as a source of ChOx for both groups, but that a substantial portion of the circulating ChOx in the ChOx-injected group was, understandably, of nondietary origin.

ChOx Content in the Liver

The liver ChOx content at the conclusion of the experiments is presented in Figure 6. Livers from the ChOx-injected...
rabbits contained significantly greater ($P<0.02$) ChOx levels than did livers from pure cholesterol–injected rabbits, indicating possible involvement of the liver in eliminating or accumulating injected ChOx.

**Sudanophilic Lesions in Rabbit Aortas**

At sacrifice, sudanophilic lesions were present proximal to the brachiocephalic, common carotid, and left subclavian branches in the aortic arch, as well as the celiac, cranial mesenteric, and renal branches in the abdominal aorta in both the ChOx-injected rabbits and controls. Little or no lesions were found in the thoracic and lower abdominal aortas. Microscopically, H&E staining (Figure 7) indicated that the prominent lesions consisted of foam cells, degenerative smooth muscle, and clear spaces, which were mostly lipid-filled, as confirmed by oil red O staining (data not shown). To determine the extent of the lesions morphometrically, lesion surface percent was used because aortic thickening was only minimally present and volumetric quantification could be subject to a number of errors. Owing to the site-prone distribution of the lesions, the aorta was divided into aortic arch, thoracic aorta, and abdominal aortic segments, and lesion areas were quantified as described in Methods. Comparisons of percent lesion area in aortic segments from both groups of rabbits are shown in Figure 8. In the aortic arch, the ChOx-injected rabbits had a 2-fold greater lesion area coverage than did controls ($P<0.02$). This result accounted for the significantly greater percent lesion area for the whole aorta from the ChOx-injected rabbits ($P<0.002$ versus the controls), since no significant differences were observed in the thoracic ($P<0.15$) and abdominal ($P<0.51$) aortic segments.

The extent of sudanophilic lesions in rabbit aortas as a function of total plasma cholesterol/ChOx exposure is presented in Figure 9. There was a poor correlation ($R=0.28$, $P>0.44$) between lesion area and total plasma cholesterol exposure (Figure 9A). In contrast, a strong correlation ($R=0.93$, $P<0.0001$) was found between lesion area and total plasma ChOx exposure (Figure 9B).

**TC, CE, and FC Contents in Aortic Segments**

The comparison of TC, CE, and FC contents in aortic segments from both groups of animals is shown in Table 2 (CE and FC were not determined in the aortic arch owing to the limited availability of tissue.) In ChOx-injected rabbits, the aortic arch contained the greatest levels of TC, followed by the upper abdominal and thoracic aortic segments. Interestingly, in the lecithin-injected controls, cholesterol levels appeared to be greatest in the upper abdominal aorta among the analyzed segments. This is consistent with the finding that, in the controls, slightly higher percentages of sudanophilic lesion areas were present in the upper abdominal aorta than in the aortic arch (data not shown). ChOx-injected rabbit aortas contained significantly greater levels of TC ($P<0.01$, aortic arch; $P<0.05$, thoracic and upper abdominal aorta) than did control aortas. The CE content in upper abdominal and thoracic aortic segments in the ChOx-injected rabbits was greater ($P<0.07$ in abdominal aorta, $P<0.05$ in thoracic aorta) than in controls, whereas no differences in FC were observed. This indicated that the increased cholesterol content in the ChOx-injected rabbit aorta was mainly associated with esterified cholesterol.
The TC content in the rabbit aortic arch as a function of total plasma cholesterol/ChOx exposure is presented in Figure 10. There was a poor correlation ($R = 0.30, P < 0.40$) between tissue TC content and total plasma cholesterol exposure (Figure 10A). In contrast, a strong correlation ($R = 0.83, P < 0.003$) was observed between the aortic tissue TC content and ChOx exposure (Figure 10B).

**Discussion**

This study was based on previous findings that consecutive intravenous injections of a ChOx mixture with a composition similar to that found in circulating ox-LDL led to a progressive increase in circulating ChOx levels in normocholesterolemic NZW rabbits and also to an increase in vascular permeability. The vascular injury was accompanied by increased cholesterol accumulation in the aorta. In the present

<table>
<thead>
<tr>
<th>TC in aortic arch</th>
<th>7530 ± 540*</th>
<th>4471 ± 593</th>
</tr>
</thead>
<tbody>
<tr>
<td>TC in thoracic aorta</td>
<td>4348 ± 323†</td>
<td>3395 ± 228</td>
</tr>
<tr>
<td>CE in thoracic aorta</td>
<td>2746 ± 157† (66.0 ± 3.8)%</td>
<td>1795 ± 273 (53.2 ± 3.2)%§</td>
</tr>
<tr>
<td>FC in thoracic aorta</td>
<td>1603 ± 275</td>
<td>1691 ± 180</td>
</tr>
<tr>
<td>TC in upper abdominal aorta</td>
<td>6629 ± 381†</td>
<td>5143 ± 422</td>
</tr>
<tr>
<td>CE in abdominal aorta</td>
<td>4272 ± 282‡ (64.0 ± 4.0)%§</td>
<td>3223 ± 397 (53.0 ± 3.0)%§</td>
</tr>
<tr>
<td>FC in abdominal aorta</td>
<td>2357 ± 270</td>
<td>1920 ± 81</td>
</tr>
</tbody>
</table>

Values are in micrograms per gram wet weight. n denotes the number of animals.

*P < 0.01, †P < 0.05, and ‡P < 0.07 vs the control.
§Data in parentheses represent the percentage of CEs in TC.
study, we administered the same mixture under similar conditions, except that the animals were fed a cholesterol-containing diet that produced hypercholesterolemia under controlled conditions. This led to a significant elevation of circulating ChOx levels. Although the relevance of the ChOx levels achieved in the present study to the ChOx levels under real physiological conditions is not clear, our results indicate that elevations in plasma ChOx levels lead to a significant increase in lesion area and cholesterol accumulation in the arterial wall under moderate hypercholesterolemic conditions.

Rabbits fed a low cholesterol–containing diet (<0.5% by weight) have been studied more frequently in recent years as an atherosclerotic animal model.32–34 In this model, plasma cholesterol levels comparable to those found in human hypercholesterolemia can be achieved.32–34 The early foam cell lesions more closely resemble the human fatty streak than lesions in animals fed a high-cholesterol diet (≥0.5% by weight), and the advanced lesions consist primarily of smooth muscle cells, lipid-laden foam cells, and a fibromuscular cap covering a core composed of extracellular lipid and necrotic debris,35,36 which is a hallmark of advanced atherosclerosis in humans. There have been a number of attempts to define the relationship between atherosclerotic lesion formation and diet-induced hypercholesterolemia35,37–39 in low cholesterol–fed rabbits. Bocan et al57 reported that plasma cholesterol levels >700 mg/dL, ie, a total plasma cholesterol exposure >31 868 mg/dL day, represented a threshold level necessary for consistent development of foam cell lesions. Similarly, Kolodgie et al39 reported a total plasma cholesterol exposure threshold of 5000 mg/dL×week (35 000 mg/dL×day), below which lesions were minimal but above which the extent of sudanophilic lesions was correlated with total plasma cholesterol exposure. Consistent with these findings, our studies showed that a total plasma cholesterol exposure in the range of 27 000 to 34 000 mg/dL×day was sufficient to produce a small number of early sudanophilic lesions. When rabbits were fed an adjusted diet containing 0.1% to 0.3% cholesterol, final plasma cholesterol levels and total plasma exposure were controlled at 470±80 mg/dL and 30 600±900 mg/dL×day, respectively (Figure 3A and 3B). Under these circumstances, a poor correlation (r=0.28, P<0.44) between total plasma cholesterol exposure and percentage of sudanophilic lesion area was observed (Figure 9A). This is consistent with the findings by Kolodgie et al39 who classified cholesterol exposure according to different dietary cholesterol levels and reported that under 35 000 mg/dL×day, no correlation could be found, although others57,58 have reported that foam cell lesions in rabbit aortas are correlated with total plasma cholesterol exposure over a wide range.

Total plasma ChOx exposure has not been taken into consideration in previous cholesterol feeding studies, although it has been shown that cholesterol feeding leads to a substantial increase in circulating ChOx levels.21,22,24,47 This increase may result from direct intake of ChOx in cholesterol-supplemented diets21,22,24 and/or from endogenous ChOx production associated with hypercholesterolemia.47 In the present study, plasma total ChOx levels in both groups increased with cholesterol feeding. Although the ChOx levels in the diet were below the limits of detection, large intakes of dietary cholesterol with minimal oxidation could lead to substantial ChOx accumulation in the gut. Though with controversy,48 rapid59 and continuous60 absorption of ChOx has been reported. In addition, cholesterol in the gastrointestinal tract could also be oxidized like other lipids.51 Alternatively, the increase can also be due to postabsorption cholesterol oxidation. The liver has been implicated in this process by other investigators,13,52 and the liver ChOx content in these rabbits was substantially higher than that in normocholesterolemic rabbits.25 In addition, in situ cholesterol oxidation by vascular cells or local oxidants may also be possible. ChOx levels in the lecithin-injected control group reached a plateau when plasma cholesterol levels were controlled, whereas the ChOx levels in ChOx-injected rabbits continued to rise (Figure 4A). The significantly greater ChOx content in livers from ChOx-injected rabbits indicate the involvement of this organ in clearing injected ChOx. However, the liver may also contribute to the release of ChOx into the circulation. Lipid oxidation products have been reported to be processed and repackaged into VLDL by the liver ex vivo.53 This is consistent with our previous findings25 in which ChOx were administered to normocholesterolemic rabbits. Under these conditions, there was a rapid clearance of plasma ChOx, followed by a gradual increase after multiple ChOx injections, and the increased ChOx content in the circulation was mainly associated with the LDL–VLDL fraction. In the present study, after 7 weeks there was a marked increase in total plasma ChOx exposure in the ChOx-injected rabbits (Figure 4B). This increase is considered an important criterion for exposure to and toxicity resulting from treatments with many toxic agents, and the continuous exposure to elevated ChOx is postulated to cause injury to the vessel wall as well as perturbations in cholesterol metabolism.

Evidence demonstrating the effects of ChOx on vascular lesions in vivo varies considerably. Cook et al54 fed male NZW rabbits cholestane triol at 0.1% diet weight and observed sudanophilic lesions in the aorta after only 27 days. Matthias et al55 failed to reproduce Cook’s findings in rats but reported angiotoxity resulting from cholestane triol ingestion. Jacobson et al56 found a significant increase in coronary luminal stenosis in pure cholesterol plus cholestane triol–fed White Carneau pigeons compared with pure cholesterol–fed controls, but no difference in aortic lipids was found. Imai et al57 reported grossly visible thickening in the major branches of rabbit pulmonary arteries after 3 consecutive intravenous injections of cholestane triol or 25-hydroxycholesterol. By increasing plasma cholesterol levels to ≥1100 mg/dL during a 12-week period, Staprans et al24 found that dietary ChOx accelerated the development of aortic atherosclerosis in cholesterol-fed rabbits. Similarly, Mahfouz et al60 reported that dietary ChOx caused more severe atherosclerotic lesions than did pure cholesterol at plasma cholesterol levels >1400 mg/dL over 11 weeks. By contrast, Higley et al58 found that cholesterol-fed NZW rabbits exhibited 6-fold more arterial lesions than did rabbits fed a cholesterol-free ChOx mixture. The discrepancy between these
studies may be due to different responses to ChOx in different animal species and/or to different biological effects and potencies of the various ChOx mixtures versus individual ChOx.1,59,60

Based on these findings, a ChOx mixture resembling that found in hypercholesterolemic rabbits was used in the present study, and this mixture represented the ChOx found in plasma,21 atherosclerotic lesions,61 and in vivo circulating ox-LDL.30 Injections of the ChOx mixture led to significantly greater foam cell lesion formation in these moderately hypercholesterolemic animals. This is consistent with the findings by Mahfouz et al.23 and Stappans et al.24 who used very high plasma cholesterol levels. Although most previous studies reported that ChOx administration caused vascular lesions resulting from chronic or prolonged exposure,34–37 no attempt was made to define the relationship between the lesion and plasma ChOx levels or total plasma ChOx exposure. The present findings show that the increased ChOx level and exposure in ChOx-injected rabbits are associated with increased sudanophilic lesion formation (Figure 8). The relatively small total aortic coverage (5.6%) indicates very early stage lesions. The positive and strong correlation between the sudanophilic lesion area and total plasma ChOx exposure (Figure 9B) verifies that when plasma cholesterol concentrations approximate the threshold levels required for fatty streak formation, ChOx levels are better determinants for the progression of lesion formation.

The effects of ChOx on lesion formation are further demonstrated by the TC contents in aortic tissues (Table 2). Due to hypercholesterolemia, aortic TC contents in both groups increased substantially; however, the aortic segments from ChOx-injected rabbits contained 1.3 to 1.7-fold more TC than in controls. This is supported by our previous findings25 that ChOx injection led to aortic cholesterol accumulation in normcholesterolemic rabbits. The positive and significant correlation between TC in the aortic arch and ChOx exposure (Figure 10B) indicates that ChOx play an important role in facilitating the accumulation of aortic cholesterol under mild to moderate hypercholesterolemic conditions.

The mechanisms by which ChOx facilitate vascular cholesterol accumulation remain to be elucidated. ChOx can induce endothelial dysfunction12 or endothelial injury.4 Previous studies have shown that certain ChOx and ChOx-enriched LDL decreased vascular endothelial barrier function, leading to increased transendothelial macromolecule transfer,9,10,62,63 and that some ChOx strongly inhibit gap junction formation in fibroblasts.64 Alternatively, apoptosis induced by ChOx leads to increased endothelial cell turnover, which is associated with increased endothelial transcytosis or permeability.65,66 Our previous studies with rabbits25 demonstrated that ChOx injection led to increased vascular permeability. Therefore, circulating ChOx may increase arterial wall permeability and retention of particles such as LDL and VLDL, thus depositing more cholesterol and CE into the vessel walls. This is consistent with the finding that the increased aortic cholesterol content in ChOx-injected rabbits was mostly in the form of CEs (Table 2). Another possible mechanism could involve increased uptake6 or retention15,67 of cholesterol by vascular cells associated with activation of ACAT,17 analogous to ACAT activation by hypercholesterolemia.36,39,68 Recent studies in our laboratory (A.S., personal communication) as well as in others17 have shown that ChOx inhibit cholesterol efflux, either by disrupting intracellular cholesterol trafficking16 or by inhibiting cholesterol transfer to HDL.15 In addition, ChOx have been shown to damage lysosomes, similar to the effects of ox-LDL and core aldehydes,69 which could inhibit CE hydrolyses and other lysosomal enzymes and facilitate CE accumulation.70

Because ChOx are invariably present in cholesterol-containing diets, foam cell formation may be substantially determined by ChOx content rather than by cholesterol. Our findings indicate that whereas elevated cholesterol levels are required for the development of atherosclerotic foam cell lesions, the ChOx associated with hypercholesterolemia mediate lesion formation.

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