Dietary oxysterols are incorporated in plasma triglyceride-rich lipoproteins, increase their susceptibility to oxidation and increase aortic cholesterol concentration of rabbits

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Abstract **Early fatty streaks and advanced lesions are characterized by the deposition of cholesterol and cholesterol oxidation products (oxysterols). Oxysterols have been shown to be cytotoxic and pro-atherogenic compared to cholesterol and are found in cholesterol-rich processed foods. The consumption of dietary oxysterols may be significant in the onset and development of vascular disease. In order to study the short term effects of low levels of ingested dietary oxysterols on lipoprotein and aortic cholesterol and oxysterol levels, rabbits were fed either standard chow, chow supplemented with 1.0% oxidized cholesterol (containing 6% oxysterols), or 1.0% purified cholesterol (control). To determine the distribution and uptake of oxysterols after a 2-week dietary period, triglyceride-rich plasma lipoproteins, low density lipoproteins and aorta were analyzed by GC–MS. The concentration of 7β-hydroxycholesterol was similar in all groups but the oxidized cholesterol-fed animals showed five times the concentration of 5α,6α-epoxycholesterol and double the level of 7-ketocholesterol in triglyceride-rich lipoproteins compared to the purified cholesterol-fed animals. The presence of 7-ketocholesterol in LDL was exclusive to animals fed the oxidized cholesterol diet. In addition, oxidation of triglyceride-rich lipoproteins was significantly greater in rabbits fed oxidized cholesterol compared to the pure cholesterol-fed animals. The oxidized cholesterol-fed animals also had a 64% increase in total aortic cholesterol, despite lower plasma cholesterol levels compared to the pure cholesterol control animals.—Taken together these results suggest that dietary oxysterols may substantially increase the atherogenicity of lipoproteins.**—Vine, D. F., J. C. L. Mamo, L. J. Beilin, T. A. Mori, and K. D. Croft. **Dietary oxysterols are incorporated in plasma triglyceride-rich lipoproteins, increase their susceptibility to oxidation, and increase aortic cholesterol concentration in rabbits.** ***J. Lipid Res.*** **1998.** 39: **1995–2004.**

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The presence of oxysterols in atherosclerotic lesions implicates the oxidation of cholesterol in the pathogenesis of this disease, despite the uncertainty of the origin of oxidatively modified sterols (1–3). Oxysterols are found in arterial foam cells and accumulate in advanced lesions (1, 2, 4). Oxysterols may be generated in the arterial wall after oxidation of lipoproteins by smooth muscle cells, endothelial cells, and macrophages (5) or alternatively may be derived from circulating lipoproteins. Oxidatively modified LDL is rich in oxysterols (6–8), and shows unabated uptake by macrophages and smooth muscle cells of the arterial wall, resulting in significant sterol loading in vitro. Consequently oxidatively modified LDL has been proposed to lead to the formation of the early fatty streak in vivo.

Oxysterols have been quantitated in cholesterol-rich processed foods (9) and so it is conceivable that oxidized cholesterol products in arterial lesions are partly derived from dietary sources. Consistent with this possibility are the findings of several laboratories which have shown that dietary oxysterols are incorporated into postprandial lymph chylomicrons. Oxysterols appear in postprandial plasma in man after a meal containing egg powder (10) and have been shown to be absorbed through the intestine into lymph chylomicrons in rats (11, 12). We have found that remnants of chylomicrons penetrate rapidly and efflux poorly from the arterial wall (13). In addition, chylomicron remnants serve as excellent substrate for macrophages (14) and are preferentially retained in arterial fatty streaks (15).

Oxysterols may represent the primary pro-atherogenic component of cholesterol-rich diets. Cholesterol per se has little atherogenic or cytotoxic affects, as shown by feeding (16, 17), venous infusion (18, 19), as well as in vitro studies, compared to cholesterol oxidation products (20, 21). In particular, rabbits given concentrates of autoxidized cholesterol products by gastric gavage show arterial

Abbreviations: TRL, triglyceride-rich lipoprotein; LDL, low density lipoprotein; GC–MS, gas chromatography–mass spectrometry; BHT, butylated hydroxytoluene; AAPH, 2.2′-azobis(2-aminopropane) hydrochloride.

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wall damage within 24 h, visualized by focal endothelial cell denudation and monocyte adherance, while atheromatous lesions develop with prolonged feeding. However, administration of purified cholesterol demonstrated none of these effects (16). The mechanisms of how oxysterols exert these atherogenic effects have been attributed to their uptake into the endothelium where, unlike normal cholesterol, they disturb cellular cholesterol homeostasis (22, 23) and membrane integrity (24, 25). Dietary oxysterols also have the potential to effect the metabolism of lipoproteins. They may, for example, exacerbate the atherogenicity of postprandial lipoproteins by delaying their clearance from plasma and enhancing arterial uptake (26, 27). Oxysterols could also influence the kinetics and arterial retention of hepatic-derived lipoproteins after transfer from chylomicrons (26, 27). Moreover, others have suggested that ingestion of lipid oxidation products may increase metabolic oxidative stress in vivo (28, 29).

Clearly, dietary oxysterols are potentially pro-atherogenic and there is evidence that they may also be mutagenic (24). Presently it is difficult to elucidate whether these putative properites are unique to oxidized cholesterol and/or cholesterol per se. There is also a paucity of knowledge with respect to the absorption, metabolism, and distribution of dietary oxysterols in vivo (30).

The objective of this study was to investigate the impact of dietary oxysterols on several factors which are thought to contribute to atherogenesis. In order to avoid the complexities in interpretation associated with chronic feeding regimes, we selected a feeding protocol of 2 weeks. We report the distribution of oxysterols in plasma lipoproteins and have assessed whether dietary oxysterols promote oxidative modification of pro-atherogenic lipoproteins in vitro. Despite the absence of gross arterial morphological changes, we have found that dietary oxysterols increase the arterial concentration of cholesterol after 2 weeks of feeding an oxysterol supplemented diet.

MATERIALS AND METHODS

Materials

Cholesterol (99.5%) was purchased from Sigma Chemical Company. Oxysterol standards were purchased from Steraloids (Wilton, NH) (5α-cholestane, 7α-hydroxycholesterol, 7β-hydroxycholesterol, 7-ketocholesterol, 5α,6α-epoxycholesterol, 5β,6β-epoxycholesterol, 3β,5α,6β-trihydroxy cholesterol). All organic solvents were redistilled before use and were of analytical grade. Double deionized water was used to make solutions and chemicals used were all of the highest grade. Cholesterol and triglyceride determinations were made using kits from Trace Scientific (Australia) and Wako (Japan), respectively. Oxidized cholesterol was prepared by heating cholesterol at 135°C for 4 h to produce approximately 6% cholesterol auto-oxidation products (31). The oxidized cholesterol mixture was analyzed by GC–MS in SCAN mode (6). Individual oxysterols were identified by mass spectral analysis and comparing spectra with standards and the mass spectra library (6). The percentage of individual sterols in the oxidized cholesterol mix was calculated by dividing individual peak areas by the total peak area. Table 1 lists the major products of cholesterol auto-oxidation. The oxidized cholesterol mixture contained <1 mg/100 g feed of 7-hydroperoxycholesterols as determined by HPLC analysis (courtesy of Dr. A. Brown, HRI Sydney). Purified cholesterol was prepared by recrystallization from ethanol to remove impurities and was 99.9% pure as determined by gas chromatography–mass spectrometry (GC–MS).

Experimental procedure

Twenty Semi-Lop rabbits aged 6 months with initial weights of 2.9 ± 0.1 kg were individually housed in stainless steel cages at the Royal Perth Hospital Animal House. Approval for experimental procedures was obtained from the Royal Perth Hospital Ethics Committee and procedures followed recommendations of the National Health and Medical Research Council of Australia. The rabbits were divided randomly into three dietary groups. Seven animals received rabbit chow supplemented with 1.0% auto-oxidized cholesterol (containing 6% oxysterols), 8 rabbits received 1.0% purified cholesterol supplemented chow (control diet), and 5 rabbits received standard rabbit chow. Cholesterol diets were prepared by mixing either oxidized or purified cholesterol with powdered standard rabbit chow (Glen Forrest Stock Feeds, Western Australia) and then pelleted. Care was taken to prevent oxidation of feed, which was packaged into daily individual portions, flushed with N_2, sealed and stored at 4°C in the dark.

Plasma lipid levels

Blood was drawn from the ear vein of non-fasted animals at baseline, 7 days and 14 days after feeding the respective diets. Blood was collected into 1.5 mg EDTA/ml of blood and immediately placed on ice. Plasma was prepared by centrifugation at 3000 rpm for 10 min at 4°C. Aliquots of plasma were flushed with N_2 and stored at −80°C for fatty acid and oxysterol analysis, and the remaining plasma was used to isolate lipoproteins. Plasma cholesterol and triglycerides were determined by enzymatic methods (Trace Scientific Pty Ltd, Baulkman Hills, NSW, Australia). HDL-cholesterol was determined after heparin/manganese chloride precipitation (32). LDL-cholesterol was derived by the method of Friedewald, as total cholesterol − (HDL-cholesterol) − (triglycerides × 0.46) (33).

Plasma lipoprotein preparation

Lipoproteins were separated by serial ultracentrifugation using a Beckman L-80 ultracentrifuge (34). Triglyceride-rich lipoprotein (TRL) was defined as lipoproteins with density <1.006 g/ml and remnant/LDL was isolated with density 1.006–<1.063 g/ml. To prevent oxidation of lipoproteins, density solutions were treated with EDTA and chelex-100 to remove trace amounts of transition metal ions. Aliquots of lipoprotein fractions were stored under N_2 at −80°C for lipid and oxysterol analysis. Lipoproteins were flushed with N_2 and kept at 4°C and used for oxidation experiments within 24 h.

Agarose gel electrophoresis

Plasma samples (4 μl) were loaded onto a 0.5% agarose gel (Beckman, Paragon, LIPO electrophoresis kit) and subjected to electrophoresis in 1.0% barbital buffer (pH 8.6) at 100 volts for 30 min. Lipoproteins were visualized by staining with Sudan Black B stain (7% w/v) in methanol.

Aorta preparation

The rabbits were killed (200 mg/kg sodium pentobarbitone) and the aortas were removed and placed immediately in ice-cold PBS containing 0.1% EDTA (treated with chelex-100), and the adventitia was dissected away. The aortas were then rinsed in fresh PBS, blotted dry between filter paper, snap frozen in liquid N_2, and stored at −80°C. For analysis aortas (approximately 0.5 g) were gradually thawed from −80° to −20°C and then placed on

ice for lipid extraction. The tissue was weighed and placed into a glass vial; 10 μg of 5α-cholestane was added as internal standard and aortas were minced finely with scissors in $CHCl_3/CH_3OH$ (2:1 (v/v) 0.01% BHT), flushed with N_2, vortexed vigorously, and sonicated at 4°C for 5 min (20 μm amplitude). Aortic tissue was filtered and the organic solvents were evaporated under N_2. Levels of esterified cholesterol were derived from total cholesterol (measured after saponification of the aortic lipid extract with 2 ml 1 M KOH in methanol at room temperature overnight) minus the unesterified cholesterol levels which were obtained from extracts without saponification. After saponification the aortic lipids were extracted into hexane and evaporated under N_2. The residue was derivatized and oxysterols were analyzed by GC–MS analysis as described below. Aortic cholesterol was quantitated by peak area ratio between cholesterol and the internal standard using GC–MS in the SCAN mode.

Oxysterol analysis

Plasma lipoprotein and aortic oxysterols were determined using a quantitative GC–MS method in Selective Ion Monitoring (SIM) as previously reported (6). Briefly, to 500 μl of plasma, 2 ml of 1 M KOH in methanol and 5 μg of 5α-cholestane (internal standard) were added. The tube was flushed with N_2 and samples were allowed to saponify at room temperature overnight. To the saponified sample 4 ml of H_2O was added and lipids were extracted twice with hexane (1 ml). The lipid extract was dried under N_2 and then derivatized with *bis*-(trimethyl-silyl)-trifluoroacetamide (BSTFA) (50 μl) and pyridine (50 μl) by heating samples at 60°C for 20 min under N_2. Samples were evaporated under N_2 and reconstituted in heptane (50 μl) for GC–MS analysis using a Hewlett-Packard HP 5890 gas chromatograph coupled with an HP 5970 Series mass-selective detector. An HP-1 crosslinked methyl silicone column (12 m × 0.2 mm, 0.33 μm film thickness, Hewlett-Packard) was used with helium as the carrier gas with an inlet pressure of 30 kPa. The mass spectrometer was operated in the electron impact mode (70 eV) with Selected Ion Monitoring (SIM). Four characteristic identifying ions were used for each oxysterol and the sensitivity of detection of oxysterols was 1 ng with a signal-to-noise ratio of 12:1 as previously described (6). To quantify the oxysterols calibration curves measuring the peak area of the oxysterol versus that of the internal standard were used. Peak identification was confirmed by relative retention time and mass spectral comparison with authentic standards, as well as with the HP MS Chemstation NBS Mass Spectral Data Library of compounds to give >98% match.

Oxidation of lipoproteins

Susceptibility of lipoproteins to oxidation was determined by exposure to the peroxyl radical generator 2.2′-azobis(2-aminopropane) hydrochloride (AAPH) (35) and lipid hydroperoxides were measured using ferrous oxidation of xylenol orange (FOX2) (36). The TRL and LDL fractions were dialyzed overnight with PBS at 4°C and then diluted to 0.1 mg protein/ml. Samples were incubated in PBS at 37°C with 3 mM AAPH and duplicate aliquots of the reaction solution were withdrawn every hour for 5 h. The FOX2 reagent was added and lipid hydroperoxides were measured at 560 nm as previously described (37).

Plasma isoprostanes

Plasma total (free plus esterified) F_2-isoprostanes were measured in base hydrolyzed (1 M KOH, 40°C for 30 min) plasma according to the method of Morrow and Roberts (38) with modification. Briefly, after C18 and silica Sep-Pak clean-up, samples were further purified by reverse phase HPLC prior to derivatization to the pentafluorobenzyl ester, TMS ether derivatives and analysis by negative ion chemical ionization GC–MS using a Hewlett-Packard MS Engine with methane as reagent gas. d_4-8-iso-$PGF_{2\alpha}$ (Cayman Chemical) was used as internal standard and samples were separated on a 30 m HP-5 MS capillary column heated from 160° to 300°C at 20°/min using helium as carrier gas at 0.7 ml/min.

Statistical methods

Dietary groups were compared using ANOVA with a Bonferroni correction for multiple comparisons (SPSS Inc., Chicago, IL). Area under the curve was used to assess differences between groups in the susceptibility of lipoproteins to oxidation. Results are expressed as mean ± SEM.

RESULTS

Diet preparation and influence on plasma lipids

The oxysterol content of the oxidized cholesterol diet is shown in **Table 1**. The major oxysterols generated on heating cholesterol at 135°C for 4 h were 7-ketocholesterol (41.8 ± 5.1%) and 7β-hydroxycholesterol (17.8 ± 2.2%), which are the primary oxidized cholesterols found in cholesterol-rich processed foods. Approximately 6% of cholesterol was converted to oxysterols.

Consumption of the oxidized cholesterol and the pure cholesterol-supplemented diets was similar to that for rabbits given standard chow alone, as were body weights at the end of the 2 weeks supplementation (**Table 2**). In contrast, there were substantial differences in the concentration of plasma cholesterol after sterol feeding. Oxidized cholesterol feeding increased total plasma cholesterol 8-fold, reflecting a greater proportion of apolipoprotein B containing lipoproteins, primarily remnants of TRL (**Fig. 1**). However, the increase in plasma cholesterol after supplementation with pure cholesterol was more than double that seen with the oxidized cholesterol-fed rabbits. Electrophoresis of plasma on agarose gel showed the increase in plasma cholesterol in the pure cholesterol-fed group was mainly attributed to a higher concentration of LDL rather than both LDL and TRL as observed in the rabbits given oxidized cholesterol (Fig. 1).

The HDL cholesterol concentration in rabbits given oxysterols or pure cholesterol was significantly increased above

TABLE 1. Cholesterol and oxysterol content of the oxidized cholesterol diet

Sterol	% of Oxysterol in Oxidized Cholesterol	mg/100 g feed
Cholesterol		941.4 ± 8.1
Oxysterols		
7-ketocholesterol	41.8 ± 5.1	25.1 ± 3.0
7β-hydroxycholesterol	17.8 ± 2.2	10.7 ± 1.3
5α,6α-epoxycholesterol	3.8 ± 0.5	2.3 ± 0.3
5β,6β-epoxycholesterol	3.8 ± 0.5	2.3 ± 0.3
Cholest-4-ene-3-one	7.2 ± 1.2	4.3 ± 0.7
25-hydroxycholesterol	4.1 ± 0.5	2.5 ± 0.3
Unknowns	21.3 ± 3.5	12.8 ± 2.0

Three separate determinations were made of the pooled oxdizied cholesterol sample and of the feed. Values given as mean ± SEM.

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TABLE 2. Plasma lipids of rabbits fed either an unsupplemented standard chow diet, a 1.0% oxidized cholesterol (containing 6% oxysterols) diets, or a 1.0% purified cholesterol-supplemented diet

	Unsupplemented (n = 5)	Purified (n = 8)	Oxidized (n = 7)
Body weight (kg)	3.09 ± 0.19	3.22 ± 0.29	3.35 ± 0.29
Consumption feed (g/day)	94 ± 9	82 ± 5	94 ± 10
Total cholesterol (mmol/l)	0.58 ± 0.04[b]	11.91 ± 2.21	4.82 ± 0.64[a]
LDL-cholesterol (mmol/l)	0.20 ± 0.05[b]	10.66 ± 2.03	3.84 ± 0.63[a]
HDL-cholesterol (mmol/l)	0.25 ± 0.03[b]	1.02 ± 0.36	0.74 ± 0.16
Triglyceride (mmol/l)	0.62 ± 0.14	0.44 ± 0.08	0.84 ± 0.18

Values given as mean ± SEM.
[a] Oxidized and purified groups significantly different from each other, $P < 0.05$.
[b] Unsupplemented group compared to cholesterol-fed groups, $P < 0.05$.

standard chow-fed animals (Table 2). In unsupplemented rabbits, half of the plasma cholesterol was HDL-associated, whereas in oxidized and purified cholesterol-fed rabbits, the HDL component represented 10–17% of the total. Plasma triglycerides were not significantly altered after cholesterol feeding.

Oxysterol distribution in plasma and lipoprotein fractions

Total plasma oxysterols were significantly elevated in both cholesterol-supplemented groups (**Fig. 2**). 7β-Hydroxycholesterol was the only oxysterol identified in plasma from unsupplemented rabbits, suggestive of basal endogenous production. Rabbits given dietary oxysterols had substantially greater plasma concentrations of 7β-hydroxycholesterol, 7-ketocholesterol, and 5α,6α-epoxycholesterol compared to standard chow-fed animals. The oxysterols 5β,6β-epoxide, cholest-4-ene-3-one and 25-hydroxycholesterol were not detected in the plasma or lipoproteins of the oxidized cholesterol-fed group despite the presence of these oxysterols in the diet. Rabbits fed pure cholesterol also showed an increase in the total concentration of oxysterols compared to the unsupplemented group and this was comprised mainly of 7β-hydroxycholesterol. 5α,6α-Epoxycholesterol was present in the plasma of purified cholesterol-fed animals but only in trace amounts.

The total oxysterols associated with TRL particles (density less than 1.006 g/ml) in oxidized cholesterol-fed animals was 2-fold greater than in unsupplemented rabbits and rabbits given pure cholesterol (**Fig. 3**). 7-Ketocholesterol and 5α,6α-epoxycholesterol were increased in both cholesterol-fed groups, although significantly more so in rabbits given dietary oxysterols. Oxysterol levels were expressed as nmol/unit protein to represent the concentration of oxysterols/lipoprotein particle. Feeding oxidized cholesterol induced a 5-fold increase in 5α,6α-epoxycholesterol (5.39 ± 2.62 nmol/mg protein and 1.28 ± 0.43 nmol/mg protein, respectively, $P < 0.001$) and a 2-fold rise in 7-ketocholesterol (8.10 ± 1.43 nmol/mg protein and 4.22 ± 0.78 nmol/mg protein, respectively, $P < 0.001$) compared to rabbits fed purified cholesterol. 7β-Hydroxycholesterol did not differ among the three groups of rabbits.

In the remnant/LDL lipoprotein fraction, 7β-hydroxycholesterol levels were over 30% greater in the oxidized cholesterol-fed rabbits (14.91 ± 0.92 nmol/mg protein) compared to the unsupplemented (9.71 ± 1.27 nmol/mg protein, $P < 0.01$) and purified cholesterol groups (10.96 ±

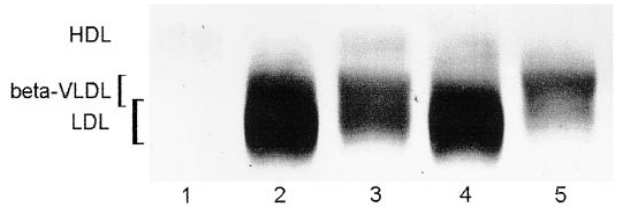

Fig. 1. Agarose gel electrophoresis of plasma from rabbits fed normal chow, a 1.0% oxidized cholesterol diet or a 1.0% purified cholesterol-supplemented diet for 14 days. Lane 1, standard chow; lanes 2,4, purified cholesterol; lanes 3,5, oxidized cholesterol. The oxidized cholesterol-fed group displayed an increase in both LDL and TRL (β-VLDL) compared to the purified cholesterol-fed rabbits, which showed an increase primarily in the concentration of LDL.

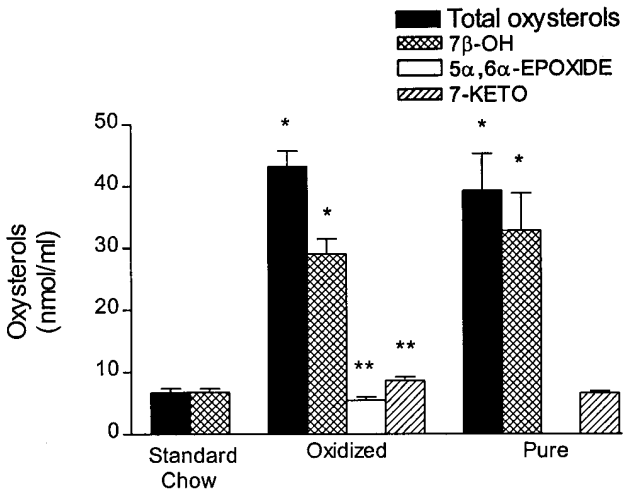

Fig. 2. Total oxysterol content of plasma (nmol/ml) in rabbits fed a unsupplemented diet of standard chow (n = 5), a 1.0% oxidized cholesterol diet (n = 7), or a 1.0% purified cholesterol-supplemented diet (n = 8) for 14 days. Values are expressed as mean ± SEM. *$P < 0.001$, for total oxysterols and 7β-hydroxycholesterol levels in oxidized cholesterol and purified cholesterol groups compared to the standard chow group. **$P < 0.01$, for 5α,6α-epoxycholesterol and 7-ketocholesterol in oxidized cholesterol group compared to purified cholesterol group.

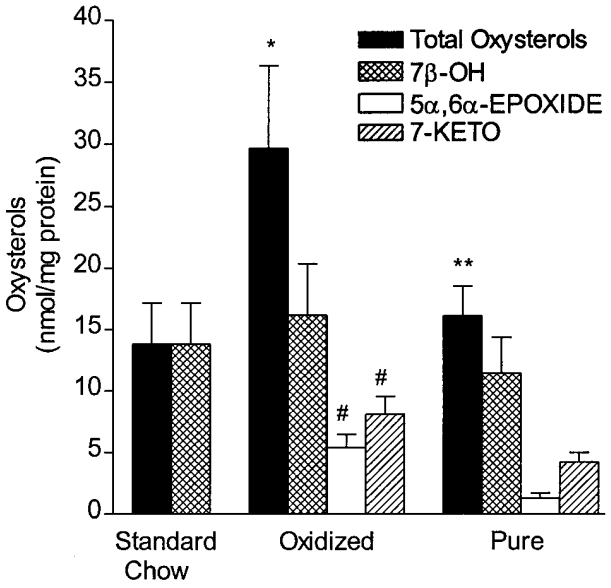

Fig. 3. Oxysterols in the postprandial triglyceride-rich plasma fraction (TRL) of rabbits fed a diet of standard chow (n = 5), a 1.0% oxidized cholesterol diet (n = 7), or a 1.0% purified cholesterol-supplemented diet (n = 8) for 14 days. Values are expressed as mean ± SEM in nmol/mg protein. *$P < 0.01$, total oxysterols of oxidized cholesterol group compared to the standard chow and purified cholesterol group. **$P < 0.01$, total oxysterols of purified cholesterol group compared to the standard chow group. # $P < 0.05$, 7-keto and 5α,6α-epoxycholesterol levels of the oxidized cholesterol group compared to the purified cholesterol group.

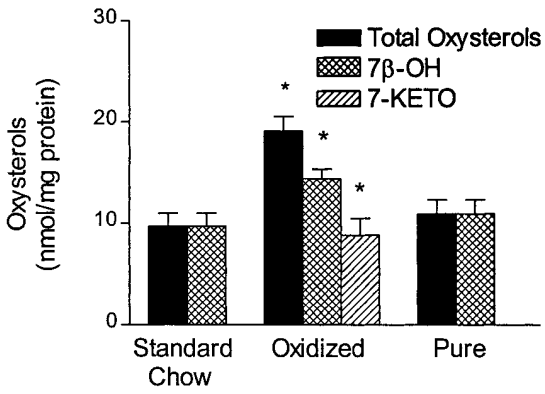

Fig. 4. Oxysterols in the remnant/LDL fraction of rabbits fed a diet of standard rabbit chow (n = 5), a 1.0% oxidized cholesterol diet (n = 7), or a 1.0% purified cholesterol diet (n = 8) for 14 days. Values are expressed as mean ± SEM in nmol/mg protein. *$P < 0.001$, for total oxysterols, 7β-hydroxy and 7-keto cholesterol levels of oxidized cholesterol compared to standard chow and purified cholesterol-supplemented groups.

1.39 nmol/mg protein, $P < 0.01$) (**Fig. 4**). Although 7-ketocholesterol levels were similar to that of the TRL fraction, 5α,6α-epoxycholesterol was not detected. The oxysterol profile of the remnant/LDL plasma fraction from rabbits given pure cholesterol was similar to that of unsupplemented animals.

Lipoprotein oxidation and oxidative stress

The lipid compositions of plasma lipoprotein fractions subsequently exposed to AAPH peroxidation were different among groups (**Table 3**). The cholesterol/triglyceride ratio of the TRL and remnant/LDL fractions was greater in rabbits fed oxidized and purified cholesterol compared to unsupplemented animals. In addition, the ratio of cholesterol to protein per particle was higher in the TRL and remnant/LDL fractions in both cholesterol-fed groups, and the triglyceride/protein ratio was reduced due to dilution of samples to a standard concentration of protein (100 μg/ml) prior to exposure to AAPH. The polyunsaturated fatty acid content of the lipoprotein fractions was measured as they are the primary substrate for lipid peroxidation; however, there was no difference in the concentration of total polyunsaturated fatty acids among groups for either the TRL or remnant/LDL fractions. The P:S ratio remained unaltered in the TRL fraction of all groups; however, in the remnant/LDL fraction the P:S ratio was lower for the standard chow group compared to the cholesterol-fed groups. The susceptibility of lipoprotein fractions from both supplemented groups to oxidation with AAPH is shown in **Fig. 5**. The total lipid hydroperoxides produced on oxidation of TRLs from oxysterol-fed rabbits was nearly double that for rabbits fed pure cholesterol as determined by area under the curve analysis (294 ± 45 versus 154 ± 70, $P < 0.05$). In contrast, there was no difference in lipid hydroperoxide production between the LDL fractions isolated from pure cholesterol and oxidized cholesterol-fed rabbits.

To determine whether dietary cholesterol supplementation leads to in vivo oxidative stress as indicated by the plasma total oxysterols (Fig. 2), we measured plasma total F_2-isoprostanes. The differences among groups were not statistically significant (412 ± 28 pg/ml, 530 ± 82 pg/ml, and 520 ± 99 pg/ml for the standard chow, oxidized, and pure cholesterol-supplemented groups, respectively).

Aortic cholesterol concentration

To elucidate whether dietary oxysterols were potentially atherogenic after short term feeding, we monitored aortic lesion formation and arterial cholesterol concentration. After 2 weeks of supplementation with oxysterols or cholesterol, there were no visible lipid lesions on the abdominal or carotid vessels in either group. However, the concentration of aortic total cholesterol in rabbits fed oxidized cholesterol was increased more than 2-fold (653 ± 131 μg/g versus 278 ± 39 μg/g aorta, respectively) compared to unsupplemented and purified cholesterol-fed rabbits (**Fig. 6**). Supplementation of the diet with pure cholesterol caused no significant increase in arterial cholesterol concentration (398 ± 41 μg/g aorta) compared to the unsupplemented group. There was no significant difference in the level of unesterified cholesterol among the three groups. The increase in aortic total cholesterol in the oxidized cholesterol-fed group was associated with an increase in the esterified cholesterol. Moreover, despite a 50% lower exposure of the aorta to circulating cholesterol, as determined by area under the curve analysis (**Fig. 7**), the oxidized cholesterol-fed group had greater choles-

TABLE 3. Lipid composition of TRL and remnant/LDL plasma fractions (diluted to a standard 100 μg protein/ml) subsequently exposed to AAPH peroxidation

	Cholesterol	Triglyceride	Chol/Trig	Chol/Protein	Trig/Protein	PUFA	P:S
	mg	*mg*				*mg*	
TRL							
Std chow (n = 5)	7.6 ± 0.5[a]	32.1 ± 4.3[a]	0.19 ± 0.08[a]	0.76 ± 0.05[a]	2.94 ± 0.58[a]	0.18 ± 0.04	2.13 ± 0.1
Oxidized (n = 7)	26.3 ± 7.2	13.8 ± 2.8	2.14 ± 0.55	2.63 ± 0.72	1.29 ± 0.25	0.12 ± 0.01	2.08 ± 0.4
Pure (n = 8)	37.3 ± 5.8	13.9 ± 3.3	3.65 ± 1.10	3.58 ± 1.62	1.39 ± 0.33	0.10 ± 0.01	2.08 ± 0.1
Remnant/LDL							
Std chow (n = 5)	3.0 ± 1.4	15.3 ± 0.4[a]	0.14 ± 0.07[a]	0.22 ± 0.11[a]	1.52 ± 0.03[a]	0.10 ± 0.01	1.59 ± 0.01[a]
Oxidized (n = 7)	16.4 ± 3.6	3.1 ± 1.3	6.68 ± 2.27	1.64 ± 0.36	0.31 ± 0.06	0.08 ± 0.01	2.34 ± 0.07
Pure (n = 8)	28.6 ± 5.9	3.6 ± 1.8	10.14 ± 3.06	2.86 ± 0.59	0.35 ± 0.07	0.07 ± 0.01	1.97 ± 0.02

Values given as mean ± SEM.
[a] $P < 0.05$ for the unsupplemented standard chow group compared to both the oxidized and pure cholesterol-fed groups.

terol accumulation in the vessel wall. Oxysterols could not be detected at quantifiable levels in aortic lipid extracts from any dietary groups.

DISCUSSION

Arterial fatty streaks are characterized by cholesterol and oxysterol deposition (1, 2) which are of unknown origin but known to be associated with a Western diet rich in saturated fat and with elevated blood cholesterol levels. There is no causal evidence that has unequivocally established a relationship between dietary intake of oxysterols and their accumulation in blood vessels. Nevertheless, ingestion of oxysterols has been proposed to be proatherogenic (20, 21, 30). An increased incidence of cardiovascular disease in Indian immigrants to the United Kingdom was linked to a greater consumption of ghee, a clarified butter oil which contains significant amounts of oxysterols (39). In addition, it has been suggested that arterial fatty lesions in cholesterol-fed rabbits are due to oxysterols associated with USP-grade cholesterol (16). However, epidemiological studies in human and rabbit models of cholesterol-induced atherogenesis are confounded by variables associated with long term lifestyle and feeding patterns. To explore whether dietary oxysterols might be uniquely atherogenic and/or amplify atherogenic properties associated with cholesterol consumption, we investigated the effects of short term feeding of dietary cholesterol containing a small percentage of oxysterols, compared to a control diet containing pure cholesterol.

The oxysterols produced by heating cholesterol at 135°C for 4 h were qualitatively similar to those found in common cholesterol-rich food products (9, 10), with significant amounts of 7-ketocholesterol, 7β-hydroxycholesterol, and 5α,6α-epoxycholesterol.

We and others have shown that oxysterols are absorbed and secreted in lymph chylomicrons (11, 12). However,

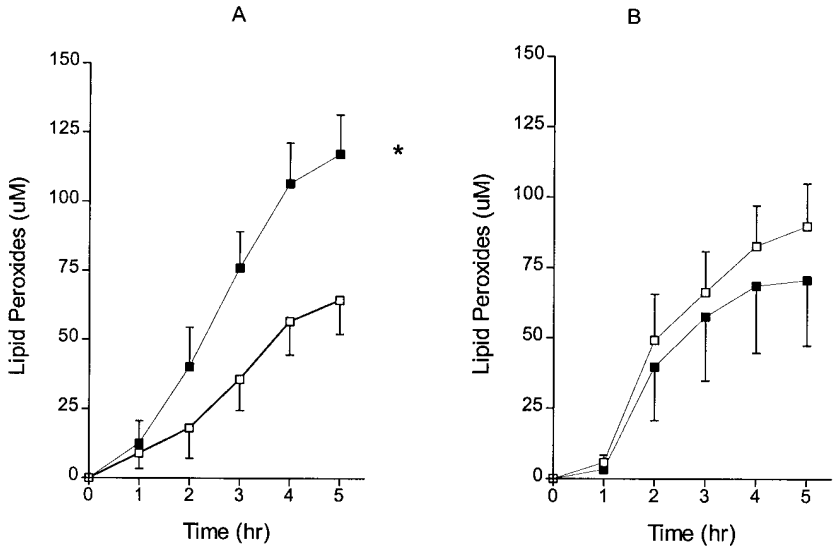

Fig. 5. Lipid peroxidation of the (A) TRL fraction (100 μg protein/ml) and (B) remnant/LDL fraction (100 μg protein/ml). Lipoproteins from rabbits fed a 1.0% oxidized cholesterol diet (n = 7) (■) or a 1.0% purified cholesterol control diet (n = 8) (□) for 14 days were exposed to 3 mm AAPH at 37°C and lipid peroxides were measured every hour using the ferrous oxidation–xylenol orange assay. Results are mean ± SEM; *$P < 0.05$ for area under the curve analysis between the two dietary groups.

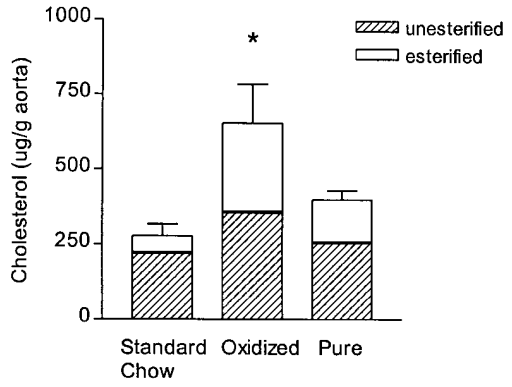

Fig. 6. Aortic total cholesterol, esterified and unesterified, in rabbits after 14 days of feeding standard chow, a 1.0% oxidized cholesterol diet or a 1.0% purified cholesterol diet. Data are expressed as μg cholesterol per g of aortic tissue (wet weight) mean ± SEM. *$P <$ 0.05 for total cholesterol of the oxidized cholesterol compared to the standard chow and purified cholesterol-fed animals.

the plasma transport of oxysterols is more difficult to determine because the clearance of chylomicrons and their remnants differs substantially from hepatic-derived lipoproteins. Despite this limitation, the oxysterol distribution among plasma lipoproteins does provide useful information as to their likely kinetics. In this study non-fasted plasma was collected. After 2 weeks of feeding a diet enriched with oxidized sterol products, the plasma concentration of oxysterols was significantly increased. There was also an increase in plasma oxysterol concentrations in rabbits given pure cholesterol, although the composition of specific oxysterols differed. The main difference was the amount of 5α,6α-epoxycholesterol which appeared almost exclusively in animals fed the oxysterol diet. Both cholesterol-fed groups had elevated 7β-hydroxycholesterol and

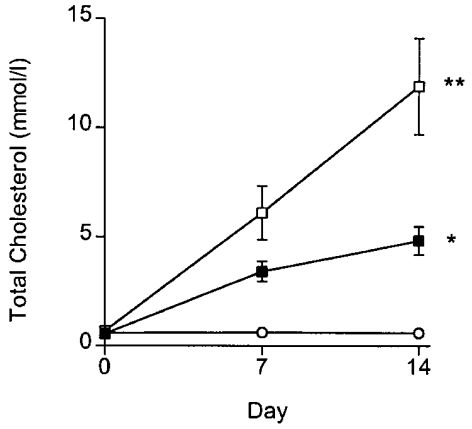

Fig. 7. Plasma total cholesterol determined just prior to (day 0) and 7 and 14 days after dietary supplementation of rabbits with standard chow (○), 1.0% oxidized cholesterol (■), or 1.0% purified cholesterol diets (□). To determine the exposure of the arterial wall to cholesterol over the 14-day dietary period, area under the curve analysis of plasma cholesterol over time was used. **P = 0.017 for the purified cholesterol compared to the oxidized cholesterol-fed animals; *P = 0.001 for the oxidized cholesterol-fed animals compared to the standard chow group.

7-ketocholesterol levels, suggesting that a cholesterol-supplemented diet increases endogenous oxidative stress, which results in increased formation of oxysterols as previously suggested (28, 29). The level of 7β-hydroxycholesterol observed in all groups was comparable to results from other studies reporting oxysterols in rabbit plasma (29). However, specific oxysterol compounds in plasma may also be derived directly from the diet as reflected in the increased levels of 7-ketocholesterol and 5α,6α-epoxide in the oxysterol-fed group. In a previous study we have shown that there is selective absorption and incorporation of dietary oxysterols into lymph chylomicrons; in particular 5β,6β-epoxide did not appear to be absorbed compared to the 5α,6α-epoxide (12). In this study 5β,6β-epoxide, cholest-4-ene-3-one and 25-hydroxycholesterol were not observed in the plasma of the oxidized cholesterol-fed rabbits although these oxysterols were in equal if not greater amounts in the feed compared to the 5α,6α-epoxide. This suggested that these oxysterols may not have been absorbed or had very low absorption from the diet.

There were significant differences in the plasma distribution of oxysterols. The levels of oxysterols in the TRL fraction were significantly elevated in the oxidized cholesterol-fed animals. Significant quantities of 7-ketocholesterol and 5α,6α-epoxycholesterol were found in the TRL fraction; however, there was no 5α,6α-epoxycholesterol in the remnants/LDL fraction. Furthermore, the increased level of 7β-hydroxycholesterol after oxysterol consumption was exclusively associated with the non-TRL fraction. Interestingly, in the remnant/LDL fraction, only the oxysterol-fed rabbits contained significant quantities of 7-ketocholesterol. These observations suggest that dietary oxysterols are also transported in remnants of triglyceride-rich lipoproteins after conversion to the remnant form and/or transfer to more dense lipoproteins. It may be significant that 7-ketocholesterol is also a major oxysterol constituent of human and rabbit atherosclerotic foam cells (40).

The presence of 7-ketocholesterol and 5α,6α-epoxycholesterol in the TRL fraction from pure cholesterol-fed animals may reflect an increase in oxidative stress in vivo. However, we were unable to show any significant difference in plasma isoprostanes, a marker of in vivo oxidative stress, among the dietary groups (41).

Oxidatively modified lipoproteins have been proposed to be the source of sterols in lipid-laden arterial macrophages. The identification of dietary oxysterols in plasma lipoproteins coupled with the observations that postprandial remnants can induce foam cell formation in vitro is consistent with a causal role in atherogenesis (14). However, dietary oxysterols may exacerbate uptake of cholesterol-containing lipoproteins by macrophages if they potentiate oxidative damage in vivo. To explore this possibility, we monitored the rate of lipid hydroperoxide production in the presence of controlled free radical attack. The TRL susceptibility to oxidation in the presence of AAPH was markedly enhanced in rabbits given dietary oxysterols compared to rabbits given pure cholesterol. Several factors could have contributed to the greater rates of oxidation of TRL from oxysterol-fed rabbits. In a previous

study, lymph chylomicrons isolated from oxysterol-fed animals were substantially larger in size due to increased triglyceride and cholesterol content compared to chylomicrons from unsupplemented or purified cholesterol-fed animals (12). The larger size may make lipoproteins more susceptible to peroxyl radical-induced lipid oxidation due to more triglyceride available and greater surface exposure of lipid for free radical attack. However, the cholesterol/protein ratio and the triglyceride/protein ratio of the TRL in this study indicated there was no difference in the size or triglyceride content of the particles isolated from the oxidized or the purified cholesterol-fed groups. Although polyunsaturated fatty acids are the major substrate for lipid peroxidation in lipoproteins, we found no difference in the concentration of polyunsaturated fatty acids among the cholesterol-fed groups. However, the oxysterol content of the TRL may have altered the structural packing of lipid in the core and surface of the lipoproteins making them more susceptible to peroxyl radical oxidation. Oxysterols appear to be predominately esterified in lipoproteins (6) and reside mainly in the core of the particle. Indeed, decreased packing of phospholipid nonpolar chains was observed in egg yolk liposomes and dipalmitoylphosphatidylcholine bilayers due to incorporation of 7-hydroxycholesterols (42, 43). In addition, angle-resolved fluorescence depolarization spectroscopy has shown that membranes containing the oxysterols 7β-hydroxycholesterol, 7-ketocholesterol, and 5α,6α-epoxycholesterol have altered membrane molecular order and dynamics (25). The TRL isolated from the oxidized cholesterol-fed group had increased 7-ketocholesterol and 5α,6α-epoxide which may have made these particles more susceptible to oxidation compared to the TRL from purified cholesterol-fed animals.

The AAPH-initiated production of lipid hydroperoxides in the remnant/LDL fraction from rabbits given pure cholesterol or the oxysterol supplemented diet were similar to each other. The reasons for this difference in behavior of the two lipoprotein fractions toward oxidation are unclear. However our data do suggest that dietary oxysterols may exacerbate atherogenesis as a consequence of increased susceptibility of triglyceride-rich lipoproteins to oxidative modification in vivo.

A striking result from this study was that the aortas of the oxidized cholesterol-fed rabbits had 64% more cholesterol than animals fed purified cholesterol at the end of the 2-week dietary period and this was mainly attributable to an increase in esterified cholesterol. Moreover this occurred despite the pure cholesterol-fed group having substantially greater arterial exposure to circulating cholesterol compared to the oxidized cholesterol fed rabbits. The esterification of cholesterol has been shown to be enhanced in the presence of oxysterols in macrophages (44), smooth muscle cells (45), and fibroblasts (46). Exposure of arterial wall cells and macrophages to oxidiatively modified LDL which is rich in oxysterols leads to macrophage foam cell formation (47). Indeed TRL, in particular chylomicron remnants, enriched with dietary oxysterols may lead to increased accumulation of arterial wall cholesterol. It has been demonstrated that chylomicron remnants penetrate and are retained by the arterial wall (13, 15) and induce lipid loading in human monocyte-macrophages (14). In addition we have previously observed that chylomicron remnants containing oxysterols have delayed clearance from plasma compared to those prepared with purified cholesterol (27). Mortimer et al. (26) also reported that chylomicron remnants have reduced hepatic uptake when they contain 7-hydroxycholesterols compared to native cholesterol. The delayed clearance of chylomicron remnants is associated with the early onset of atherosclerosis as in patients with familial hypercholesterolemia (48) and type III hyperlipoproteinemia (49). Therefore, the elevated aortic cholesterol seen in the oxidized cholesterol-fed rabbits may be associated with dietary oxysterols enhancing accumulation of cholesterol delivered by chylomicron remnants to the arterial wall. We are investigating this further. The increased arterial cholesterol was not simply a consequence of hypercholesterolemia per se, because in rabbits given pure cholesterol there was no significant increase in arterial cholesterol. Rather the increased concentration of 7-ketocholesterol and 5α,6α-epoxide in plasma, LDL, and in particular the TRL, in the oxidized cholesterol-fed group appears to have been associated with the elevated arterial cholesterol deposition observed. Oxysterols have been shown to affect cellular cholesterol efflux which may offer a further explanation for the increased cholesterol accumulation observed in these animals. The efflux of cholesterol has been shown to be inhibited in the presence of oxysterols, including 7-ketocholesterol, 7-hydroxycholesterols and 5,6-epoxides, in macrophages (50, 51) and fibroblasts (50, 52). In addition, the intravenous injection of 7-ketocholesterol in the rabbit appears to lead to impaired tissue efflux of cholesterol (53).

In the presence of increased TRL oxysterols we may have predicted a parallel increase in arterial oxysterol concentration but there were only trace levels of oxysterols associated with aortic tissue. The absence of oxysterols in the arterial wall of rabbits given oxidized sterols may have been a consequence of several factors. Extraction of the entire tissue may have limited our ability to detect very low levels of oxysterols, particularly in the absence of focal sites of atherogenesis which characterize the early stages of atherosclerosis. Studies in endothelial monolayers have shown that radiolabeled oxysterols efflux more rapidly and to a greater extent than unoxidized cholesterol (54). Therefore, oxysterols may have effluxed out of the arterial wall at a faster rate than unoxidized cholesterol, limiting their detection by GC–MS. Furthermore, feeding 7-ketocholesterol in the diet of chicks has been shown to cause focal sites of endothelial damage (17) and in smooth muscle cell culture oxysterols promote focal sites of necrosis (55). Consistent with the response to injury hypothesis is the observation that dietary oxysterols had a marked effect on arterial cholesterol concentration, which may demonstrate increased delivery and retention of cholesterol rich lipoproteins by the arterial wall.

In conclusion, we have shown in a short dietary period

that rabbits fed an oxidized cholesterol diet containing low levels of oxysterols have higher concentrations of oxysterols in triglyceride-rich lipoproteins than animals fed either standard chow or a purified cholesterol-supplemented diet. Furthermore, the oxysterol diet led to the formation of a TRL fraction that was also more susceptible to oxidation and these animals had significantly increased arterial cholesterol content. The elevation of plasma cholesterol levels was substantially less with the oxysterol diet compared to the pure cholesterol-supplemented diet. On the other hand, the pure cholesterol-supplemented diet also increased plasma oxysterol levels but did not increase the arterial concentration of cholesterol. Collectively, the data suggest that dietary oxysterols are pro-atherogenic compared to unoxidized cholesterol and that TRL enriched with dietary oxysterols may be a key atherogenic component. It is possible that the atherogenic basis of cholesterol-fed diets is positively related to the level of oxidized sterol products.

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