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Selective Distribution of Oxysterols in Atherosclerotic Lesions and Human Plasma Lipoproteins

JACOB VAYA[a], MICHAEL AVIRAM[b,c,*], SAEED MAHMOOD[a], TONY HAYEK[b], EHUD GRENADIR[b], AARON HOFFMAN[b] and SIMCHA MILO[b]

[a]*Laboratory of Natural Medicinal Compounds, Migal-Galilee Technological Center, Kiryat Shmona 10200, Israel,* [b]*Dept. of Medicine, Rambam Medical Center, Haifa and* [c]*Lipid Research Laboratory, Rambam Medical Center, Faculty of Medicine and Rappaport Institute for Research in the Medical Sciences, Technion, Haifa, Israel*

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The presence of oxidized sterols (oxysterols) in human serum and lesions has been linked to the initiation and progression of atherosclerosis. Data concerning the origin, identity and quantity of oxysterols in biological samples are controversial and inconsistent. This inconsistency may arise from different analytical methods or handling conditions used by different investigators. In the present study, oxysterol levels and distribution were analyzed by an optimized GC-MS method, in human atherosclerotic coronary and carotid lesions, in atherosclerotic apolipoprotein E deficient mice (E° mice) and in native and *in vitro* oxidized human low and high density lipoproteins. Oxysterol levels were analyzed with a limit of detection of 0.06 – 0.24 ng, with 25-hydroxycholesterol (25-OH) being the least sensitive. In human coronary and carotid lesions, obtained from endatherectomic samples, 27-hydroxycholesterol (27-OH) was the major oxysterol, with about 85% as sterols esterified to fatty acids. While total cholesterol and oxysterols levels were similar in both kinds of human lesions, oxysterol distribution was significantly different. In coronary lesions the mean levels of 27-OH and 7β-hydroxycholesterol (7β-OH) were 38% and 20% of total oxysterols, whereas in carotid lesions their mean levels were 66% and 5%, respectively. Unlike in human aortic lesions, 27-OH was entirely absent in E° mice, whereas the level of 7α-hydroxycholesterol (7α-OH) was 28% of the total oxysterols, vs. 5% in human coronary lesions. As 27-OH is an enzymatic product of cholesterol oxidation, this finding may indicate that such an enzymatic process does not take place in E° mice.

Keywords: Oxysterols; atherosclerosis; human lesion; E° mice; LDL; HDL

INTRODUCTION

Atherosclerosis is a major cause of morbidity and mortality in the Western world [1]. Evidence suggests that some oxidized cholesterol products (oxysterols) present in human and animals tissues and blood are involved in the development of atherosclerosis [2], as well as of other degenerative diseases [3]. Oxysterols have been shown to affect cholesterol biosynthesis, plasma membrane structure, and cell proliferation [3]. Oxysterols also exert cytotoxicity to cells [3–8], induce apoptosis [9–11], act as chemo-attractants [12] and can stimulate formation of foam cells [13] and advanced atherosclerotic plaque [3, 14–

* Correspondence to: Prof'. Michael Aviram, Head, Lipid Research Laboratory, Rambam Medical Center, Bat-Galim, Haifa, Israel, 31096. Tel: 972–4–8542970, Fax: 972–4–8542130, E. Mail: aviram@tx.technion.ac.il

16]. Oxysterol levels in lipoprotein [17], plasma [18] and atherosclerotic lesions [14, 15] may be linked to atherosclerosis development. The attribution of oxysterol levels or types in mammals, as indicators of oxidative damage, continues to attract investigators [19–21].

The presence of oxidized sterols in serum and tissues could have a dietary origin. It is assumed that about 1% of the cholesterol consumed in the human diet is present in an oxidized form [22], mainly as 7-ketocholesterol (7-keto), 7α and 7β-hydroxycholesterol (7α-OH, and 7β-OH), and 5α, 6α *and* 5β,6β-epoxycholesterol (α-epoxy and β-epoxy). Oxysterols such as 7α-OH, 27-hydroxycholesterol (27-OH) and 24-hydroxycholesterol (24-OH) can be formed enzymatically during reverse cholesterol transport from peripheral tissues to the liver, or during bile acid biosynthesis in the liver [23]. Evidence suggests that oxysterols could also be formed *in vivo* by cholesterol autoxidation [24–26].

Several oxysterols have been detected in human tissues, in plasma lipoproteins and in atherosclerotic lesions. The type and amount found vary widely, according to detection method used and experimental handling conditions. These methods include thin layer chromatography (TLC), high performance liquid chromatography (HPLC), liquid chromatography coupled to mass spectrometry (LC-MS), gas chromatography (GC), and gas chromatography coupled to mass spectrometry (GC-MS). Whereas 27-OH was found to be the major oxysterol in advanced human lesions, 7β-OH was dominant in fatty streaks [14]. In plasma, some studies reported that 27-OH, 7α-OH, 7β-OH and 7-keto cholesterol are the major oxysterol constituents [27, 28], whereas another study has found α-epoxy and β-epoxy cholesterol to be the major ones [29].

In the present study we optimized a GC-MS analytical method and work-up procedure to determine the levels and types of oxysterols, before and after alkaline saponification. Oxysterol selectivity and specificity patterns were studied in human coronary and carotid lesions *vs*. aortas obtained from atherosclerotic, apolipoprotein E deficient (E°) mice and in native and oxidized human lipoproteins. Comparison of human lesions to lesions from E° mice may help evaluate the suitability and limitation of this *in vivo* model system for human lesion research.

MATERIALS AND METHODS

Apparatus and Materials

GC-MS analysis was performed by means of an HP gas chromatograph, Model 5890 Series II (Waldbronn, Germany), fitted with a HP-5 trace analysis column (5% phenyl methyl siloxane), with a mass selective detector, Model 5972, (Waldbronn, Germany). *N,O*-bis(trimethylsilyl) acetamide (BSA), and cholesterol were purchased from Sigma Chemical Co. (St. Louis, MO), and 2,2′-azobis (2-amidino-propane) dihydrochloride (AAPH) was purchased from Polysciences, Inc. (Warrington, PA). 7-Ketocholesterol (7-keto), 7α and 7β-hydroxycholesterol (7α-OH, 7β-OH), 5α,6α and 5β,6β-epoxycholesterol (α-epoxy, β-epoxy), 27-hydroxycholesterol (27-OH), 4β-hydroxycholesterol (4β-OH), cholestane and 19-hydroxycholesterol (19-OH), used as internal standards, were purchased from Steraloids Inc. Wilton N.H. USA. All solvents were of either spectrophotometric or HPLC grade.

Sampling from Coronary & Carotid Lesions for Oxysterol Determination

Patients undergoing routine coronary artery bypass surgery in the Dept. of Cardiac Surgery at the Rambam Medical Center in Haifa, Israel, underwent additional right coronary artery endarterectomy. The freshly removed atherosclerotic plaques were immersed in normal saline. In the process of construction of the prox-

imal venous graft anastomosis of the same patients, holes were punched in the ascending aortic walls. The pieces removed, which measured 4.5 mm in diameter, served as control. Carotid atherosclerotic lesions were obtained from patients undergoing carotid endarterectomy for severe carotic stenosis (of over 70%). These patients, whether symptomatic or asymptomatic, underwent surgery under regional anesthesia. Complete atherosclerotic plaques were removed, including the common internal and external carotid parts of the plaque. The plaques were immediately placed in saline. The samples were put on filter paper to absorb liquid, and then weighed and ground to a powder (in liquid nitrogen) before extraction. On average, the weight of samples of lesion dried by lyophilization was about 1/3 the weight of wet samples. The powder obtained from each sample was extracted by diethyl ether, together with the internal standard (cholestane and 19-OH). Samples were divided into two, to determine free and total sterols, as with lipoprotein extracts.

Mice were fed a regular mouse chow diet (Purina chow containing 4% fat). Aortas from apolipoprotein E-deficient mice (E° mice) were prepared from specimens at 6 months of age. Mice were sacrificed, and entire aortas were removed and placed in cold Hank's Balanced Salt Solution (HBSS). Aortas from E° mice were worked up and oxysterols extracted, as described for human aortas.

Isolation and Oxidation of Lipoproteins

Serum low density lipoprotein (LDL) and high density lipoprotein (HDL) were isolated from plasma derived from fasting normolipidemic volunteers from the Rambam Medical Center, Haifa, Israel [30]. Prior to oxidation of the lipoproteins, HDL and LDL were dialyzed under nitrogen against phosphate buffered saline (PBS), EDTA-free solution, pH 7.4, at 4°C. Human plasma LDL and HDL (0.5 mg protein/ml) were oxidized in a shaking water bath at 37°C under air. One ml LDL was incubated with AAPH (5 mM) or with copper ions (10 μM) of $CuSO_4$ for 3 and 6 h at 37°C. Lipoprotein oxidation was terminated by the addition of 10 μM butylated hydroxytoluene (BHT) and 1 mM Na_2EDTA and refrigerated at 4°C. LDL and HDL protein concentrations were determined by Folin phenol reagent [31]. The degree of lipoprotein oxidation was verified by the thiobarbituric acid reactive substances (TBARS) assay [32] and ranged between 20–32 nmol TBARS/mg lipoprotein.

Extraction and Determination of Oxysterols from Plasma Lipoproteins

Oxysterols are present in lipoproteins and in lesions in free form and as esters with various fatty acids. Conditions for their extraction, hydrolysis and stability were optimized, using standards of cholesteryl linoleate and three oxysterol compounds, β-epoxy, 7-keto and cholestane-3,5,6-triol. The following procedure for LDL was also employed for samples of HDL:

Plasma cholesterol and oxysterols were extracted three times (2 ml each) from oxidized LDL (0.5 mg LDL protein/ml) by diethyl ether, which was treated previously with aluminum oxide to eliminate possible peroxide content. Prior to extraction, 50 μl 5α-cholestane and 50 μl 19-OH (50 μg/ml) were added to each sample as internal standards. In some instances, especially in hydrolyzed samples of LDL, where the amount of cholesterol is very high, the cholesterol peak in the GC-MS partially overlaps that of 19-OH. In those cases 5α-cholestane was used predominantly as internal standard. The upper phases were collected, divided into two portions and evaporated to dryness under nitrogen. One part of the sample was then silylated, as described below, to determine free sterol content, and the second part was saponified, to determine total sterol content in samples. The amount of sterol esters in samples was calcu-

lated by subtracting the measured free sterol content (unsaponified) from the total sterol content measured in saponified samples.

Saponification of samples was performed as follows: the dry residue of the extracted sample was dissolved in 2 ml diethyl ether, and 2 ml 20% KOH in methanol (w/v) was added. The remaining head space of the vial was filled with nitrogen and the reaction mixture was left in the dark at ambient temperature for 3 h. The mixture was then neutralized by adding 2 ml 25% citric acid in water, and the upper organic phase was removed. The remaining aqueous layer was washed three more times with 1.5 ml diethyl ether, and the organic layers were collected, combined, dried (sodium sulfate), filtered, and evaporated to dryness under nitrogen. A standard of cholesteryl linoleate was subjected to identical saponification and extraction conditions, and cholesterol was recovered with a 98% (± 5%) yield. The stability of the oxysterols under the experimental conditions was also verified (extraction, hydrolysis, re-extraction and silylation). Thus, β -epoxy, 7-keto and cholestane-3,5,6-triol (triol) were subjected to saponification under identical conditions, following extraction and GC-MS analysis, using the external standard method. The recovery was almost quantitative (RSD ± 4.6%-6.8%). Silylating conditions: silylation with BSA in 1,4-dioxane was found to be milder and superior, in terms of reaction conversion, reproducibility and suitability to GC-MS, to other reagents examined, such as trimethylsilylchloride in pyridine and BSA in other solvents (diethyl ether, DMF, $CHCl_3$).

Oxysterol Detection by GC – MS

Standards or the dried extracts were subjected to a silylating reagent, N,O-bis(thrimethylsilyl)acetamide (BSA) (200 μl), followed by the addition of 1,4-dioxane (dried on 4Å molecular sieves and passed through aluminum oxide) (200 μl) and heated to 70°C for 30 min. Samples were analyzed on GC-MS coupled to a HP 5972 quadrupole mass spectrometer and linked to a HP ChemStation data system. The GC was fitted with a 30-meter HP-5 trace analysis capillary column (0.32 mm I.D., 0.25 μm film thickness, 5% phenyl methyl silicone), operated in splitless mode for 0.8 min and then in split ratio of 1:1. Helium was used as carrier gas, at a flow rate of 0.656 ml/min, pressure 10.4 psi and at a linear velocity of 31 cm/s. The MS transfer line was maintained at 280°C. The injector was set at 300°C, the detector at 330°C and the column heated at a gradient starting at 200°C, increasing to 250°C at 10°/min and then at 5°/min to 300° and maintained for an additional 15 min at 300°C. Samples were detected in the GC-MS in total ion monitor (TIM) from which 2–4 most representative ions were selected for re-injection in single ion monitoring mode (SIM). Aortas from E° mice were detected in SIM mode due to the small size of samples and hence the need for a lower limit of detection. The mean quantity of each oxysterol was calculated from calibration curves of its standards. Under the above conditions, the limit of detection for each oxysterol was determined with deviations of less than 6% of the mean. Corresponding areas equal to 10 times the area measured in the blanks were set as the limit of detection. Under the above conditions, the oxysterols shown in Table I were separated and their quantities determined. Cholestane and 19-OH were used as internal standards (IS). The selection of the above oxysterols was based on literature data, which presented them as candidates in biological systems.

Statistic analysis

The student t-test was used in order to analyze the significance of the results. Results are given as mean ± S.D.

RESULTS

Determination of Oxysterols by GC-MS

In the present study, identical GC-MS analysis and optimized extraction, saponification and silylation conditions were used to determined the type and level of oxysterols in human coronary and carotid lesions *vs.* oxysterols in lesions from E° mice, a commonly used *in vivo* model system for atherosclerosis studies.

Samples were injected to GC-MS in TIM, from which the most representative ions were selected for re-injection in SIM. For maximum sensitivity, the oxysterols were injected as their silyl ether derivatives and the limits of detection obtained were in a range between 0.06 ng (7α-OH, 7β-OH, 7-keto, 27-OH) and 0.24 ng (25-OH). Response factors for each oxysterol under the analytical conditions were calculated from the peak area ratio obtained for 10 μg/ml of each oxysterol to 10 μg/ml of 19-OH area (Table I). A representative GC-MS spectrum of a mixture of oxysterol standards (25 μg/ml each) is shown in Figure 1A, together with a representative sample of human coronary (Fig 1B), and human carotid lesion (Fig. 1C) taken in TIM mode, and of representative apoE deficient mouse aorta taken in SIM mode. Table I and Fig. 1A shows, in agreement with others [27], that the response factors of 25-OH and 7α-OH (both about 0.2) are significantly lower in GC-MS analysis than the response factors of the other oxysterols detected (0.84–1.72). A spectrum of the human coronary lesion shows the presence of most of the oxysterols, together with many other unidentified compounds, but with no 4-OH or triol. Human carotid lesions also contained no 7α-OH. A spectrum of E° mouse (Fig. 1D) showing a high level of 7α-OH and a total absence of 27-OH, was taken in SIM mode, due to the small size of the sample, and hence the need for higher sensitivity.

Oxysterols in Human Atherosclerotic Lesions

The oxysterol 27-OH was found to be the major oxysterol in human coronary lesions (0.25±0.1 μg/mg mean wet weight), with a level more than ten times higher than the content of an aortic sample taken from the same patient as control expressed in terms of μg oxysterol/mg mean wet weight lesion (0.02±0.01), or in terms of μg oxysterol/100 μg cholesterol (Table II). The next most abundant oxysterols in coronary lesion were 7β-OH and β-epoxy (0.14 ± 0.09 and 0.12 ± 0.07 μg/mg means wet weight, respectively). Both these oxysterols were present in lesions at higher levels than 7-keto (0.08±0.04 μg/mg means wet weight). The total cholesterol content in coronary lesions was found to be more than ten times that formed in the control non-lesion aortic samples obtained from the same patients (50.3 vs. 4.5 μg/mg means wet weight). Presenting the level of oxysterols per amount of the substrate cholesterol (Table II, μg oxysterol/100 μg cholesterol), shows that in coronary human lesion, although the mean total amounts of α and β epoxy cholesterol in the lesions/sample wet weight are three times higher than in control, the ratios per substrate are lower than in control. This could arise from an increased level of cholesterol by more than 10 times in the lesion compared with control, while the increased level of the two epoxies is only by three times in the lesion. Samples from human carotid plaques were also tested (Table II). The levels of cholesterol and 27-OH were approximately the same as those found in coronary lesions (51.2 and 0.25 μg/mg means wet weight, respectively). However, oxysterol distribution between coronary and carotid lesions was different ($p<0.2$). While the mean level of 27-OH in coronary plaques was 38%±14.5% of total oxysterols, in carotid plaques it contributed as much as 66%±28.9% to total oxysterols. The percentage of 7β-OH in the carotid plaques however, was much lower than in coronary plaques (5% ± 2.6% *vs.* 20% ± 13.0% out of total oxysterol, respectively, $p<0.2$).

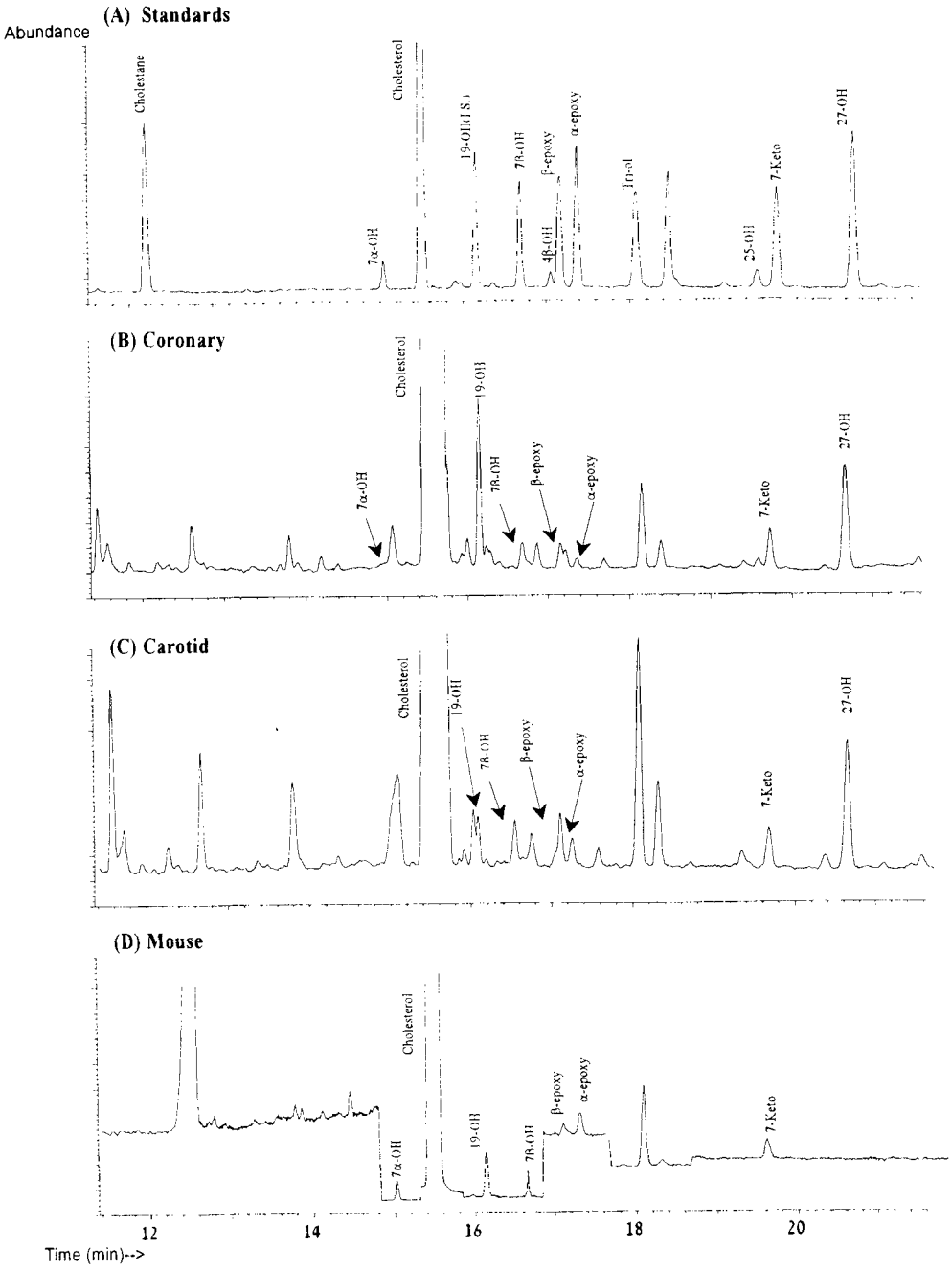
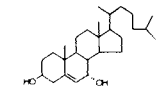
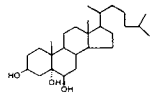
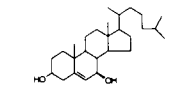
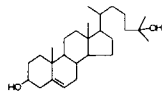
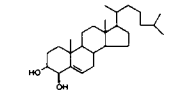
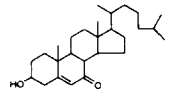
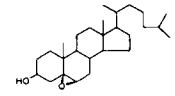
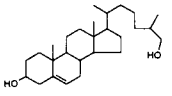
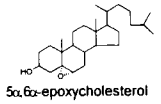
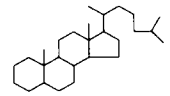
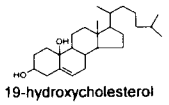


FIGURE 1 (A) Standards of silylether derivative of cholesterol and oxysterols in concentrations of 100 μg/ml and of 25 μg/ml, respectively, were injected (1 μl) to GC-MS taken in TIM mode and using cholestane and 19-OH as internal standards (25 μg/ml). The following oxysterols were injected and separated: (7α-OH), (7β-OH), (4β-OH), α -epoxy and β-epoxy, triol, 25-OH, 7-keto and 27-OH. (B) Spectrum of representative human coronary lesion and of human carotid lesion (C) analyzed in TIM mode with 19-OH as internal standards showing total oxysterol levels. (D) Spectrum of representative apolipoprotein E-deficient mice aorta analyzed in SIM mode

TABLE I Structure, selected ions, response factors and limit of detection of oxysterols

Structure	*Selected ions*	*Response factor*	*[a]Limit of detection*	*Structure*	*Selectee ions*	*Response factor*	*[a]Limit of detection*
 7α-hydroxycholesterol	546,456	0.21	0.06	 3,5,6-trihydroxycholesterol	546,456 403	1.09	0.12
 7β-hydroxycholesterol	546,456	0.84	0.06	 25-hydroxycholesterol	546,456 382,472	0.19	0.24
 4β-hydroxycholesterol	546,456 474,384	0.12	0.12	 7-ketocholesterol	546,456 382,472	1.07	0.06
 5β,6β-epoxycholesterol	546,456 474,384	1.01	0.12	 27-hydroxycholesterol	546,456 417	1.72	0.06
 5α,6α-epoxycholesterol	546,456 474,384	1.21	0.12	 5α-cholestane internal standard (I.S.)	372,357	1.47	----
 19-hydroxycholesterol	465,366 356	1.00	----				

The selected ions were deduced from their MS spectra in TIM mode. Measurement deviations < 8% of the mean. Response factor was calculated from the ratio of the peak area obtained for 10 μg/ml of each oxysterol to 10 μg/ml of 19-hydroxycholesterol (19-OH) area.

a. Units for limit of detection were ng/injection.

The amount of oxysterols in the above samples was also analyzed for free and esterified sterols (Fig 2). Cholesterol was found mainly in its free form in advanced coronary human lesions (about 55%), compared with only 15% free 27-OH cholesterol. All 7-oxygenated sterols (7-keto, 7α-OH and 7β-OH) were present in about the same amount in their free forms (approx. 25%), while the levels of free sterols in the two epoxy compounds ranged from 31% to 36%.

Oxysterols in Lesions from E° Mice

E° mice are commonly used as an *in vivo* model system for atherosclerosis, in relation to oxidation stress. In aortas from E° mice, 27-OH cholesterol was completely absent (Fig. 1D and Table II), in contrast to its abundant presence in coronary and carotid human lesions. Another major characteristic of E° mice lesions is the relatively high level of 7α-OH (28% of total oxysterols), in comparison to human lesions (5% or not

TABLE II Total oxysterols in human coronary and carotid lesions and in E° mice aortas

				cholest	27-OH	7-keto	α-epoxy	β-epoxy	7α-OH	7β-OH
coronary	lesion (n=8)	a	mean	50.3**	0.25**	0.08**	0.06**	0.12*	0.04**	0.14**
			sd	15.60	0.10	0.04	0.03	0.07	0.01	0.09
		b	mean	100.00	0.50	0.16	0.11	0.25	0.07	0.28
	control (n=6)	a	mean	4.50	0.02	0.02	0.02	0.04	nd	0.01
			sd	1.00	0.01	0.01	0.01	0.03	`	0.02
		b	mean	100.00	0.04	0.04	0.35	0.82	nd	0.24
carotid	lesion (n=5)	a	mean	51.2*	0.25**	0.07**	0.01	0.03*	nd	0.02**
			sd	11.20	0.11	0.03	0.01	0.01	`	0.01
		b	mean	100.00	0.50	0.14	0.01	0.06	nd	0.04
	control (n=4)	a	mean	37.60	0.03	0.01	0.01	0.01	nd	nd
			sd	6.40	0.01	0.01	0.01	0.01	`	`
		b	mean	100.00	0.07	0.02	0.01	0.02	nd	nd
E(0) mice	Aorta (n=15)	a	mean	5.15	nd	0.16	0.07	0.06	0.16	0.11
			sd	1.80	`	0.06	0.04	0.03	0.05	0.05
		b	mean	100.00	nd	3.17	1.42	1.09	3.06	2.13

P*<0.05, p**<0.01

(a). μg of total oxysterol/mg wet weight lesion. (b). μg of total oxysterol/100 μg cholesterol in sample. nd- not detected (not found). Coronary and carotid lesions were taken from 8 and 5 patients, respectively. Samples of ascending aortic walls were taken as control. Results of human subjects are presented as mean±S.D. of three separate analysis of each sample analyzed in triplicates. Aortas from 15 apolipoprotein E-deficient mice were prepared as described in Materials and Methods. Each aorta was analyzed twice in triplicates and data represent mean±S.D. of the two separate analysis of the 15 aortas.

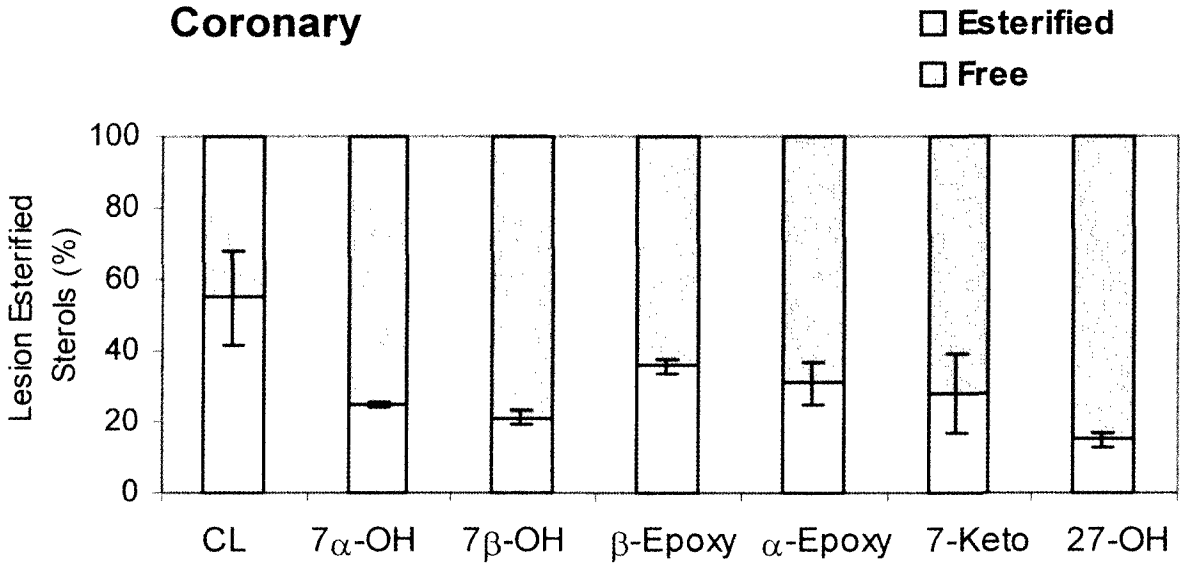


FIGURE 2 Free and esterified distribution of coronary lesion's oxysterols. Samples were divided into two parts: one part was silylated and analyzed to determine free sterols, and the second part was saponified, extracted, and silylated with BSA to determine total sterols. Results are given as mean ± S.D. of three separate experiments, each performed in triplicate

detected in coronary and carotid human lesions, respectively). The three 7-oxygenated sterols in the E° mice lesion (7α-OH, 7β -OH and 7-keto) make up to about 80% of total oxysterols detected. Table II also shows that in E° mice lesions, the relative levels of oxysterols to cholesterol (1.09–3.17 μg/100 μg cholesterol) are significantly higher than in human lesions (0.06–0.5 μg/100 μg cholesterol).

Oxysterols in Native and Oxidized Human LDL and HDL

We next investigated the oxysterol patterns of LDL and HDL, in their native conditions, as well as after copper ion- or AAPH-induced lipoprotein oxidation. Results obtained by GC-MS are shown in Table III. These results demonstrate that native LDL was almost free of oxysterols, with a minor amount of 7β-OH (0.19 nmol/mg LDL protein), while HDL had slightly more oxysterols in its native status (Table IIIB). During copper ion-induced LDL oxidation, oxysterols were formed, and their amount increased with time of incubation. The predominant formation of 7-keto, 7β-OH and β-epoxy (77.1, 41.4 and 41.7 nmol/mg LDL protein after 6 h incubation, respectively), was noted, while 27-OH, the enzymatic product of cholesterol catabolism, could not be detected. In AAPH-induced LDL oxidation, less oxysterol formed at the same time of incubation, but 7-keto and 7β-OH were still the major oxysterols detected (43.6 and 42.5 nmol/mg LDL protein, respectively), with no detection of 27-OH (Table IIIA). The domination of oxygenated 7-position (7-keto, and 7β-OH) during copper ion-induced LDL oxidation was previously reported [33], but in the present study, β-epoxy cholesterol was also formed to a similar level as that of 7β-OH (41.7 nmol/mg LDL protein).

TABLE III Total oxysterol content of human LDL and HDL

		(A) nmol/1mg LDL protein Mean (± SE)				*(B) nmol/1mg HDL protein*	
		$CuSO_4$		*AAPH*		*$CuSO_4$*	
Oxysterol/time: zero		*3 h*	*6 h*	*3 h*	*6 h*	*zero*	*3 h*
Cholesterol	818±49	526±32	535±43	521±29	536±43	232±21	248±26
7α-OH	0.05±0.017	4.48±0.58	10.05±0.93	2.27±0.32	7.97±0.81	0.4±0.07	3.61±0.32
7β-OH	0.19±0.025	24.78±2.63	41.4±3.71	10.62±1.54	42.48±6.20	0.26±0.03	4.00±0.64
β-Epoxy	nd	8.45±1.09	41.7±4.59	2.93±0.56	25.11±3.01	nd	2.08±0.37
α-Epoxy	nd	1.37±0.22	3.82±0.39	0.28±0.05	2.03±0.18	nd	0.18±0.04
7-Keto	nd	23.69±2.07	77.12±6.93	6.99±0.77	43.61±4.47	1.30±0.18	9.50±0.08
25-OH	nd	0.38±0.08	6.47±1.03	nd	0.19±0.06	0.44±0.12	1.01±0.22
27-OH	nd	nd	nd	nd	nd	nd	nd

Human plasma LDL and HDL (0.5 mg protein/ml) were oxidized at 37°C. (A) LDL (1 ml) was incubated with AAPH (5 mM) or with copper ions (10 μM $CuSO_4$) for 3 or 6 h at 37°C. (B) HDL (1 ml) was incubated for 3 h with copper ions as described for LDL. Total oxysterol content was determined by GC-MS. Results are given as mean ± S.D (n=3).

While there are many reports on the amount of oxysterol formed during copper ion-induced LDL oxidation, very little is known about the formation of oxysterols in oxidized HDL. In the present study, the HDL and LDL oxysterol contents were compared, using identical oxidation conditions. As expected, the amount of cholesterol in native HDL is about one fourth that of the cholesterol contents in LDL (232 *vs.* 818 nmol/mg lipoproteins). This low cholesterol level in HDL resulted also in less oxysterol formation after HDL oxidation, in comparison with LDL oxidation (Tables IIIB and IIIA). The distribution of oxysterols also differed. While 7-keto remained the dominant oxysterol formed in both oxidized LDL and HDL, the decrease in the levels of 7α-OH and of 25-OH in HDL (3.6 and 1.0 nmol/mg, respectively) was less marked. In addition, less of both epoxy isomers was formed in oxidized HDL (2.1 and 0.2 nmol/1 mg) than in oxidized LDL.

DISCUSSION

In the present study, a single optimized GC-MS analytical technique and handling was employed to determine the type and level of ten different sterols in major oxysterol origins; lesions and lipoproteins. Oxysterols were separated and their levels tested with low limits of detection, in the range of 0.06–0.24 ng/injection. The present study demonstrated that the distribution of 27-OH and 7α-OH, both of enzymatic origin, differs in human and E° mice lesions: 27-OH is the main oxysterol in humans, while in E° mice it is totally absent. Instead, the level of 7α-OH, a minor sterol in human lesions, is a major oxysterol in E° mice. This suggests that the pathway of oxysterol biosynthesis differs in the two systems.

27-OH is an enzymatic product of cholesterol. It is formed from cholesterol by the mitochondrial enzyme 27-hydroxylase in the liver, and it was shown to co-localize with macrophages in human carotid lesions [47]. 27-OH functions in humans as an alternative pathway to bile acid biosynthesis and as a regulator of cholesterol metabolism *in vivo* [33], especially in the liver. Involvement of 27-OH in the elimination of cholesterol from extrahepatic cells has been also demonstrated [48]. In the present study, the level and distribution of different oxysterols were

tested in human coronary and carotid lesions, in their free and esterified form. In agreement with previous findings, 27-OH was the major oxysterol in human lesions. The present study showed that atherosclerotic plaques originating from carotid or coronary lesions, contained about the same amount of cholesterol and oxysterols, but that their oxysterol distribution was different, with 27-OH contributing up to 66% of the total oxysterols in carotid lesion and only up to 38% in coronary lesions.

E° mice are hypercholestrolemic under oxidative stress, and develop atherosclerosis within a few months. Hence, they are useful in research relating lipoprotein oxidation to atherosclerosis, or where anti-atherogenicity of antioxidants is under investigation. Results obtained with lesions from E° mice showed significantly different oxysterol distribution and level from that shown in human lesions: in E° mice we found 22 times more 7-keto, 140 times more α-epoxy, 18 times more β-epoxy and 50 times more 7β-OH/100 μg cholesterol than in human carotid lesions with total absence of 27-OH in E° mice lesions. As 27-OH is an enzymatic product of cholesterol oxidation, this finding may indicate that such an enzymatic process does not take place in E° mice. Our results also showed that, while the relative amount of 7α-OH was only 5% of the total oxysterols in human lesions, it was as high as 28% of total oxysterols in E° mice lesions. 7α-hydroxylase is the first and the rate-limiting enzyme in bile acid biosynthesis in human liver, and the formation of 27-OH, among others, is probably involved in the elimination of cholesterol from extrahepatic cells. One may thus speculate that, as normal mice are not prone to atherosclerosis, a process that eliminates cholesterol from extrahepatic cells is not necessary. It is also possible that, unlike in humans, 7α-OH formation is not a rate-limiting enzyme in cholesterol biosynthesis in mice.

Data concerning the level and identity of the major oxysterols formed during copper ion-induced LDL oxidation are controversial. Studies showed the major oxysterol to be 7-keto [17, 34, 35], or cholest-3,5-dien-7-one [36] or 7β-OH [37, 38]. This inconsistency could be possibly explained by the finding that 7-OOH (7α-OOH + 7β -OOH) is prevalent at an early stage of copper ion- or AAPH-induced LDL oxidation, and decomposes with time to form 7-OH and cholest-3,5-dien-7-one [39, 40]. Both 7-OH and 7-keto are partly formed from the same 7-OOH, and their formation ratio varies under different conditions. Cholest-3,5-dien-7-one, on the other hand, is obtained from 7-keto, after elimination of water during the GC analysis. In the present study, when oxysterol formation after copper ion- or AAPH-induced LDL oxidation was examined, 7-keto was the principal oxysterol detected after 6 h of copper ion-induced LDL oxidation, while 7-keto and 7β-OH were found in equal amounts when AAPH was used as the inducer of oxidation. In both oxidation reactions, the enzymatic product of cholesterol oxidation, 27-OH, was not detected. When HDL was incubated with copper ions, all oxysterols (except 25-OH and 7α-OH) were significantly lower in HDL than in LDL ($p<0.003$). The main oxysterols formed in oxidized HDL were also 7-keto and 7β-OH, with the former predominant, and with a higher level of 7α-OH than that formed in LDL. The differences in oxysterol distribution among the lipoproteins (LDL *vs.* HDL), as a result of oxidation induced by copper ions, may be due to differences in their composition, beyond that of their cholesterol content. We have shown elsewhere [41] that, under copper ion-induced LDL oxidation, the addition of a lipophilic antioxidant (glabridin) inhibited formation of 7-keto to a greater extent than the formation of 7-OH. As LDL consists of several lipophilic antioxidants which differ in their relative content from HDL (tocopherol, β-carotene, lycopene and ubiquinol), these antioxidants may be responsible for the differences in the formation of oxysterols and their distribution in LDL and HDL exposed to oxidation stress.

In summary, the present study showed, using optimized sensitive GC-MS methodology, that the level and distribution of oxysterols in human atherosclerotic lesions, coronary and carotid, are different to those in E° mice and plasma lipoproteins.

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