Measurement of Oxidative Interactions of Cholesterol

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A simple analytical technique, using a silicic acid mini-column and capillary gas chromatography, was used for measuring the oxidative interactions of cholesterol with other compounds. When triacylglycerols were added to cholesterol before heating at 180°C, the latter oxidized faster than cholesterol heated alone, and a relatively high amount of epoxides was found.

Dipalmitoylphosphatidylethanolamine and all the amino acids tested showed a protective effect, with cysteine and alanine being the most effective. The results of this study indicate that the varius compounds added not only influenced the rate of cholesterol oxidation, but also exerted different influences on its oxidative pathway.

KEY WORDS: Cholesterol, interactions, measurement, oxidation.

Certain products of cholesterol oxidation have been reported to produce cytotoxic, angiotoxic and carcinogenic effects (1). In food, cholesterol exists in close proximity to other lipid and non-lipid molecules. Interactions between these compounds and cholesterol may affect both its oxidative stability and its oxidation pathway. The purpose of this work was to monitor the influence of these neighboring molecules on the extent and the products of cholesterol oxidation.

EXPERIMENTAL PROCEDURES

Materials. Cholesterol, phospholipids, triacylglycerols and silicic acid were purchased from Sigma Chemical Co. (St. Louis, MO), and cholesterol oxide standards were from Steraloid Inc. (Wilton, NH). Silylating agents were obtained from Pierce Chemical Co. (Rockford, IL) and Supelco Inc. (Bellefonte, PA). The solvents used in this study were reagent or high-performance liquid chromatography (HPLC) grade from various sources.

Separation of steroids from other lipids. Although size exclusion chromatography (SEC) on Styragel columns (8′ of 100 Å and 16′ of 60 Å; Waters Assoc., Milford, MA) showed complete separation of steroids from other lipid classes, it took more than 2 hr for one sample. Therefore, an alternative method of silicic acid column chromatography was tested. Silicic acid (60–200 mesh) was activated for 3 hr at 121°C and packed in a mini-column (6 mm in diameter and 5 cm long). Solvent systems of 5–100% diethyl ether in hexane were applied. Composition of the eluates was tested by thin-layer chromatography (precoated silica gel 60G plates, 250 μm thick). Developing solvent mixtures consisted of benzene/diethyl ether/ethyl acetate/acetic acid (80:10:10:0.2, v/v/v/v) (2).

The non-polar lipids, mainly triacylglycerols, were eluted with hexane/diethyl ether mixture (95:5, v/v), followed by cholesterol and its oxidation products with 100% diethyl ether. The ether fraction was collected and stored at −20°C until further analysis.

Analysis of cholesterol and its oxides. The advantage of liquid chromatography (LC) for steroid analysis is that it does not require high temperature for separation and derivatization of the sample. However, in our laboratory separation of cholesterol and its oxides by gas chromatography (GC) was more successful. The procedure is as follows:

Silylation of cholesterol and its oxides. Free sterols have been reported to show significant thermal decomposition and poor resolutions during GC separation. These problems can be alleviated by derivatization. Thermal decomposition was minimized and peak symmetry was improved by silylation (3,4). To set the optimum silylating conditions for cholesterol and its oxides, three silylating agents, N,O-*bis*-[trimethylsilyl]trifluoroacetamide (BSTFA), BSTFA 1% trimethylchlorosilane (TMCS), sylon BTZ (N-trimethylsilylimidazole + N,O-*bis*-[trimethylsilyl] acetamide + TMCS, 3:3:2, v/v/v), and different time-temperature relationships (room temperature, 80°C and 120°C for 0.5, 1, 2 and 24 hr) were tested for maximum conversion of cholesterol and its oxides to their silylated forms (5–7).

BSTFA is a popular silylating agent for cholesterol, but it showed poor reproducibility when heated at 80°C for 2 hr. Cholesterol and its oxide peaks reached their maxima after 20 hr heating with BSTFA at 80°C or 0.5 hr at 120°C. Sylon BTZ is known to be one of the most powerful silylating agents. However, it showed low levels of cholesterol and its oxide peaks. During addition of water and extraction of silylated compounds, some of the trimethylsilyl ethers may be destroyed. BSTFA with 1% TMCS as an acid catalyst showed maximum values for cholesterol and its oxides within 1 hr at 80°C, and provided better silylation of 7α- and 7β-diols than BSTFA only.

Internal standard. Cholest-5-en-3α-ol, 5α-cholestane, 5α-androstan-3β-ol, 5α-androstan-3β-ol-17-one and its acetate form were tested as internal standard for the analysis of cholesterol and its oxides. Cholest-5-en-3α-ol, 5α-androstan-3β-ol and -17-one caused overlapping with some other peaks. 5α-Cholestane was eluted with the non-polar fraction during silicic acid column chromatography. On the other hand, 5α-androstan-3β-ol-17-one acetate was stable during silylation. It did not overlap with other peaks and showed complete migration to the steroid fraction during preparative LC.

Separation of cholesterol and its oxides by GC. A Varian 3700 (Varian Assoc., Palo Alto, CA) equipped with a polymethylsilane-coated capillary column (DB-1, 60 m × 0.253 mm i.d. and 0.25 μm film thickness; or Ultra-1, 50 m × 0.2 mm i.d. and 0.33 μm film thickness) was used to separate silylated cholesterol and its oxidation products. Temperature programming from 100°C to 300°C at the rate of 10°C/min and 295°C isothermal condition produced the best separation. Optimum GC conditions for the separation of cholesterol and its oxides were established by using a heated mixture of cholesterol and sphingomyelin (Fig. 1). Peaks were identified by comparison of their GC retention times and mass spectra with those of authentic compounds. For quantitation, correc-

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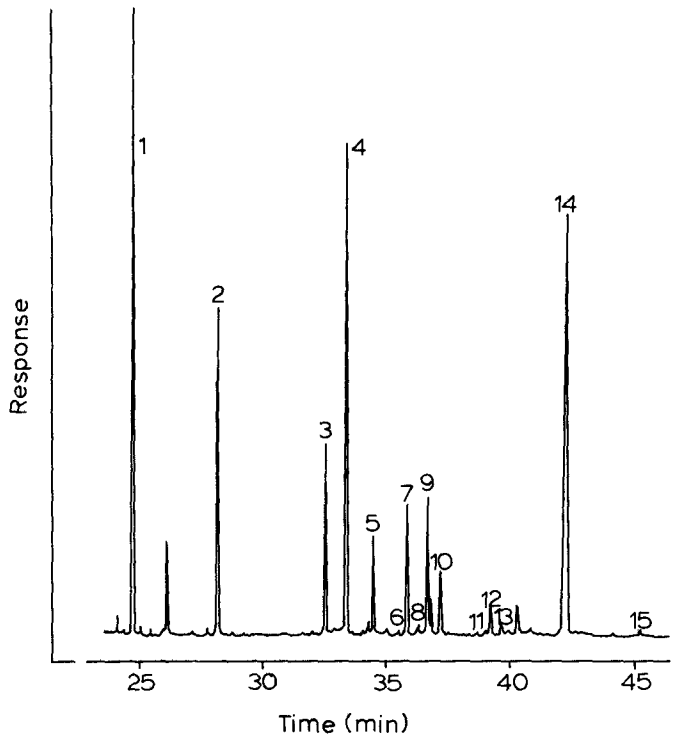

FIG. 1. Gas chromatographic separation of cholesterol and its oxides. Peak identification: (1) 5α-Androstan-3β-ol-17-one acetate (internal standard); (2) 3,5-cholestadiene; (3) 7α-hydroxycholesterol; (4) cholesterol; (5) 3,5-cholestadien-7-one; (6) cholest-4-ene-3-one; (7) 7β-hydroxycholesterol; (8) cholesta-4,6-dien-3-one; (9) 5,6β-epoxy-5β-cholestan-3β-ol; (10) 5,6α-epoxy-5α-cholestan-3β-ol; (11) 20α-hydroxycholesterol; (12) 25-hydroxycholesterol; (13) 5α-cholestan-3,6-dione; (14) 7-ketocholesterol; and (15) 5α-cholestan-3β,5,6β-triol.

tion factors based on the response of each compound to the analytical technique, including column chromatography, silylation and GC detector response were established. These were 0.74, 0.91, 0.86 and 0.85 for cholesterol, 7-ketocholesterol, 7-hydroxycholesterols and 5,6-epoxides, respectively.

Heat treatment. Dry mixtures of 1 mg cholesterol plus 1 mg of various amino acids, triacylglycerols and dipalmitoylphosphatidylethanolamine were heated in open vials at 180°C for 1 hr. The temperature chosen for the present experiments is that commonly used for frying. The effects of temperature as well as other parameters on cholesterol oxidation are considered in a subsequent report.

RESULTS AND DISCUSSION

Quantitative comparison of the cholesterol remaining after heating indicates that triacylglycerols accelerated cholesterol oxidation, with oleate being the most effective (Table 1).

When cholesterol was heated alone at 180°C for 1 hr, the main oxidation products were 7-ketocholesterol, the 7-hydroxycholesterols and the 5,6-epoxides. When triacylglycerols were added to cholesterol, the oxide profile in the heated mixture was qualitatively similar to that of cholesterol alone, but the quantitative pattern was different. The ratio of total epoxides to 7-derivatives was higher when triacylglycerols were present during heating.

In both the presence and absence of triacylglycerols, the total oxides detected, *i.e.*, those listed in Table 1 plus other minor compounds identified but not listed, accounted for only 30% of the total decomposition. A major portion of the cholesterol decomposition may have occurred *via* a dif-

TABLE 1

Effect of Phosphatidylethanolamine and Triacylglycerols on Cholesterol Oxidation at 180°C for 1 hr (mg/100 mg Initial Cholesterol)[a]

	Cholesterol only	Cholesterol + phospholipid	Cholesterol + linoleate	Cholesterol + stearate	Cholesterol + oleate
Unaltered cholesterol	28.8 ± 2.4*	46.7 ± 0.4	23.1 ± 2.6	20.2 ± 0.7	16.9 ± 5.9
Altered cholesterol	71.2	53.3	76.9	79.8	83.1
7-Derivatives	11.9	1.5	11.7	12.2	12.5
7-Ketocholesterol	9.2 ± 1.5		9.4 ± 0.9	10.3 ± 0.1	10.7 ± 0.1
7α-Hydroxycholesterol	0.6 ± 0.1				
7β-Hydroxycholesterol	1.4 ± 0.7		1.6 ± 0.3	1.3 ± 0.2	1.2 ± 0.1
3,5-Cholestadien-7-one	0.6 ± 0.3	1.5 ± 0.0	0.8 ± 0.1	0.7 ± 0.3	0.7 ± 0.1
Epoxides	6.5		9.5	8.0	10.5
5,6α-Epoxide	3.6 ± 1.1		4.3 ± 0.2	4.0 ± 0.7	4.6 ± 0.1
5,6β-Epoxide	2.9 ± 1.6		5.2 ± 1.1	4.1 ± 2.2	5.9 ± 1.4
Epoxides/7-deriv.	0.6		0.8	0.7	0.8
Total oxides detected	21.9	1.5	23.4	22.2	24.0
(% of altered chol.)	(30.7)	(2.8)	(30.5)	(27.9)	(28.9)

[a]All mixtures were at a ratio of 1:1 (w/w).
*Mean ± standard deviation, five determinations.

TABLE 2

Effect of Amino Acids on Cholesterol Oxidation at 180°C for 1 hr (mg/100 mg Initial Cholesterol)[a]

	Cholesterol only	Cholesterol + cysteine	Cholesterol + alanine	Cholesterol + serine	Cholesterol + aspartic acid	Cholesterol + lysine	Cholesterol + histidine
Unaltered cholesterol	28.8 ± 2.4*	95.3 ± 15.2	92.3 ± 3.1	68.8 ± 5.4	65.8 ± 7.0	57.1 ± 5.2	51.7 ± 0.8
Altered cholesterol	71.2	4.7	7.7	31.2	34.2	42.9	48.4
7-Ketocholesterol	9.2 ± 1.5	2.0 ± 0.1	0.9 ± 0.1	0.3 ± 0.0	0.4 ± 0.0	0.6 ± 0.0	2.2 ± 0.8
7α-Hydroxycholesterol	0.6 ± 0.1			0.2 ± 0.1			0.1 ± 0.0
7β-Hydroxycholesterol	1.4 ± 0.7	1.2 ± 0.4	1.4 ± 0.3	0.8 ± 0.1	2.7 ± 0.2		0.3 ± 0.1
3,5-Cholestadien-7-one	0.6 ± 0.3	0.5 ± 0.1	0.5 ± 0.2	0.6 ± 0.7	1.3 ± 0.1	4.2 ± 0.0	1.5 ± 0.1
5,6α-Epoxide	3.6 ± 1.1		0.3 ± 0.1	1.4 ± 0.0	1.5 ± 0.1	0.6 ± 0.0	0.5 ± 0.4
5,6β-Epoxide	2.9 ± 1.6		0.7 ± 0.1	2.2 ± 0.0	1.4 ± 0.1		1.0 ± 0.0
Total oxides detected	21.9	4.2	4.1	6.6	8.1	8.0	7.1
(% of altered chol.)	(30.7)	(89.3)	(53.5)	(21.1)	(23.7)	(18.7)	(14.7)

[a]All mixtures were at a ratio of 1:1 (w/w).
*Mean ± standard deviation, five determinations.

ferent pathway, *e.g.*, polymerization. It is also possible that a considerable amount of oxides produced may have undergone further breakdown at the high temperature used.

In contrast to triacylglycerols, the phospholipid dipalmitoylphoshatidylethanolamine (PE) protected cholesterol against oxidation. Fifty-three percent of the substrate was altered, as compared to 71% when cholesterol was heated alone. Table 1 also shows that in the presence of PE, only 3,5-cholestadien-7-one was detected. In this case, the portion of cholesterol decomposition unaccounted for is even greater than in the case of triacylglycerols.

All the amino acids tested exhibited strong protection when mixed with cholesterol, especially cysteine and alanine (Table 2). However, in contrast to PE and the triacylglycerols, the amino acids did not exert a significant effect on the oxide profiles. It is interesting to note that cysteine and alanine, which exhibitd very strong protection, also gave rise to a higher proportion of oxides relative to total decomposition. It is possible that these amino acids also protect the oxides against further oxidation. Alternatively, they may inhibit other pathways, *e.g.*, polymerization.

This study shows that the cholesterol oxide patterns obtained from the heated mixtures are significantly different from each other, indicating that the various added compounds not only influenced the rate of cholesterol oxidation but also exerted different influences on its oxidative pathway.

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