CHOLESTEROL DIET INCREASES PLASMA AND LIVER CONCENTRATIONS OF CHOLESTEROL EPOXIDES AND CHOLESTANETRIOL

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Cholesterol α -epoxide (5,6 α -epoxy-5 α -cholestan-3 β -ol), cholesterol β -epoxide (5,6 β -epoxy-5 β -cholestan-3 β -ol), and cholestanetriol (5 α -cholestane-3 β ,5,6 β -triol) were isolated from plasma and liver of rabbits by high performance liquid chromatography and identified by gas-chromatography-mass spectrometry. The 5,6-oxygenated cholestanols in the plasma and liver of rabbits fed for two months on a diet supplemented with cholesterol were elevated to 2—5 times and 5—8 times the normal level, respectively. Among the 5,6-oxygenated steroids, the β -epoxide existed at the highest level in tissues of both control and cholesterol-fed rabbits. The ratios of the β -epoxide to the α -epoxide were 2—3 in all the examined biological specimens just as the previously demonstrated ones in the *in vitro* lipid peroxidation-mediated reaction of cholesterol. These results strongly suggest that the epoxidation of the cholesterol double bond in the animal may be mediated by lipid peroxidation. The elevated 5,6-oxygenated cholestanol levels on long term cholesterol feeding will be discussed in relation to a possible physiological role of cholestanetriol in regulation of tissue cholesterol levels.

Keywords—5,6 α -epoxy-5 α -cholestan-3 β -ol; 5,6 β -epoxy-5 β -cholestan-3 β -ol; cholesterol epoxide; 5 α -cholestane-3 β ,5,6 β -triol; lipid peroxidation; cholesterol metabolism

Cholesterol α -epoxide (5,6 α -epoxy-5 α -cholestan-3 β -ol, α -EC) has recently been suspected as a systemic carcinogen. As early as 1969, Bischoff¹⁾ demonstrated that α -EC given subcutaneously to rats and mice caused sarcomas at injection sites. Furthermore, Black and Douglas²⁾ reported a suggested role of α -EC as a chemical mediator in the induction of skin tumor in hairless mice irradiated by ultraviolet ray. More recently, α -EC has been demonstrated to induce malignant transformation of cells in culture³⁾ and form a covalent bonding to the bases of isolated DNA.⁴⁾ The work of Reddy and Wynder⁵⁾ has shown that the hydration product of α -EC, cholestanetriol (5 α -cholestane-3 β ,5,6 β -triol, CT) is excreted in significantly higher quantity in the feces of patients with ulcerative colitis and colon cancer. In connection with this, Hwang and Kelesy⁶⁾ have demonstrated the presence of α -EC hydratase activity in normal adult human gut flora. Apart

from its carcinogenic activity, α -EC has been reported to be found at significantly high levels in sera of patients with high blood pressure, peptic ulcers, and hypercholesterolemia⁷⁾ as well as in the organs of patients with Wolman's disease.⁸⁾ The present paper provides the first direct evidence for the occurrence of both α -EC and cholesterol β -epoxide (5,6 β -epoxy-5 β -cholestan-3 β -ol, β -EC) in the animal body. Evidence will also be shown that tissue concentrations of both epoxides and their hydrolytic product, CT, increase remarkably in the animal fed on a cholesterol diet.

The epoxide, α -EC, is most likely to arise *in vivo* from lipid peroxidation-mediated oxidation of cholesterol. Nitrogen dioxide, an inducer of lipid peroxidation in the lung when inhaled, has been reported to increase cholesterol epoxide contents in rat lung lipid with concomitant increase in the amounts of epoxides of unsaturated fatty acids as components of tissue triglycerides and

phospholipids.⁹⁾ An *in vitro* investigation carried out by Aringer and Eneroth¹⁰⁾ indicated that hepatic microsomal lipid peroxidation, induced with a ferrous-ADP complex in the presence of an NADPH-generating system, followed the oxidation of cholesterol to α -EC and β -EC in the ratio 1:3, both of which were hydrolysed in part by microsomal cholesterol epoxide hydratase to CT as the sole product. More direct evidence for the lipid peroxidation-mediated epoxidation has been obtained by using a system consisting of linolate-lipoxygenase and cholesterol.¹⁰⁾ In this system as well as in other chemical oxidation systems such as a ferric-acetylacetone complex and either hydrogen peroxide¹¹⁾ or *t*-butyl hydroperoxide,¹²⁾ the preferential formation of β -EC to α -EC also occurred. The hepatic microsomal epoxidation of the cholesterol double bond, however, is not catalyzed by P-450,¹³⁾ whereas the 16,17-double bond of 1,3,5(10), 16-estratetraen-3-ol is epoxidized by the hepatic monooxygenase,¹⁴⁾ like many other exogenous ethylenic compounds.¹⁵⁻¹⁷⁾

Though cholesterol β -epoxide is the major product in the lipid peroxidation-mediated cholesterol epoxidation, nothing has been known of its existence in tissue specimens as well as its physiological or toxic role in the animal body. In addition, all the previous studies on the isolation, identification, and quantification of cholesterol epoxides from the animal body failed to distinguish the α -isomer from the β -isomer since they were inseparable by conventional chromatographic systems, including GLC. Namely, α -EC and β -EC have been identified and quantified merely as their derivatives such as diols after reduction with lithium aluminium hydride^{7,8)} and methoxydiols after solvolysis with methanol in the presence of boron trifluoride.⁹⁾

For the isolation and identification of the 5,6-oxygenated steroids, male albino rabbits weighing 2–2.2 kg were sacrificed. Their livers immediately isolated were homogenized in a cold isotonic KCl solution containing 5 mM EDTA for preventing endogenous cholesterol from autooxidation. Rabbit plasma specimens were also diluted with

the same KCl solution. The biological specimens were extracted with chloroform-methanol (2:1). The extract obtained on the evaporation of the solvent under reduced pressure was refluxed in 0.5N KOH methanolic solution for 30 min. After the dilution with water, the reaction mixture was extracted with ethyl acetate. The organic phase separated was concentrated to dryness, dissolved in a small volume of benzene, and poured onto a silica gel column packed with benzene. After the elution of the column with benzene-acetone (40:1) for the elimination of cholesterol, polar steroids were collected in a fraction by the elution with benzene-acetone (1:1). To the polar steroid fraction was added 20-methyl-5-pregnen-3 β -ol acetate as an internal reference for the chromatographic isolation of 5,6-oxygenated cholestanols. High performance liquid chromatography (HPLC) was carried out on a reverse partition octadecylsilicone column, Lichrosorb RP-18 (5 μ in particle size, 4 mm $\times$ 15 cm) in methanol (1 ml/min) as the eluant. Under the chromatographic conditions, CT, α -EC, and β -EC had relative retention times of 0.27, 0.60, and 0.50, respectively, to the retention time of the internal reference (9.6 min). The effluent was collected into CT, α -EC, and β -EC fractions according to their relative retention times to the internal standard. The residue obtained on the evaporation of the solvent from the chromatographic fraction was heated at 80° for 1 hr in a mixture of a 20% aqueous potassium hydroxide solution and pyridine (1:5) containing 10% *p*-nitrobenzoyl chloride. The reaction mixture was diluted with water and extracted with benzene. The organic phase separated was concentrated to dryness, and the residue subjected to HPLC under the same conditions as mentioned above with monitoring the absorbance at 260 nm. Peaks with retention times of 10.0, 8.7, and 5.7 min in the respective chromatograms corresponded to those of 3-*p*-nitrobenzoates of α -EC, β -EC, and 3,6-di-*p*-nitrobenzoate of CT, respectively, but were not well separated from back ground peaks surrounding them. Each peak was isolated and rechromatographed on a silica gel column

(μ Porasil, 3.9 mm $\times$ 30 cm) in hexane-tetrahydrofuran (2 ml/min) by HPLC. In the chromatograms, they appeared as well separated single peaks at 8.2 and 9.6 min for the monoesters of α -EC and β -EC in hexane-THF (50:1) and 12.7 min for the diester of CT in hexane-THF (15:1). The *p*-nitrobenzoates isolated by adsorption chromatography were analyzed by mass spectrometry. Their mass spectra were superimposable on those of respective authentic specimens. Identity of the isolated steroids was also confirmed by mass spectrometry after hydrolysis of the *p*-nitrobenzoates in a methanolic potassium hydroxide solution.

For the quantitative determination of α -EC, β -EC, and CT in plasma and liver of rabbits, the isotope dilution method was used. Fifteen ml of a 33% liver homogenate in the KCl solution or plasma diluted three times with the KCl solution was extracted with chloroform-methanol containing [4- 14 C] α -EC, β -EC, and CT (2000 dpm each). The extract was worked up by the same procedures as mentioned above. Specific radioactivities of the 5,6-oxygenated cholestanols were obtained by measuring peak areas of the *p*-nitrobenzoates in the HPLC chromatograms and counting radioactivities of eluates on a liquid scintillation counter. β -EC showed 2–3 times as high concentrations as the α -EC in both plasma and liver specimens from three control rabbits (Table I).

In rabbits fed on a diet containing 1% pure cholesterol for 2 months, all the 5,6-oxygenated cholestanols increased remarkably, *i.e.* the concentration of α -EC, β -EC, and CT in the plasma was elevated to 2.6, 2.3, and 4.6 times, and that in the liver to 7.5, 6.0, and 5.0 times the normal levels, respectively (Table I). In the cholesterol-fed animal, the plasma and liver total cholesterol levels determined by gas-chromatography were 17.5 mg/ml and 34.6 mg/g wet weight, respectively, whereas in the normal animals they were 0.28 mg/ml and 1.61 mg/g, respectively. That the 5,6-oxygenated cholestanols isolated and determined by the present procedure were not artifacts due to autoxidation of endogenous cholesterol but true tissue components was confirmed by the addition of 14 C-cholesterol instead of 14 C-5,6-oxygenated cholestanols to the biological specimens, followed by the determination of radioactivities in the polar steroid fractions isolated under the same conditions.

The ratios of α -EC to β -EC in plasma and liver from cholesterol-fed rabbits were similar to those in the ones from control rabbits. This implies that epoxidation of the cholesterol double bond is most likely to be mediated by lipid peroxidation both in the normal and in the cholesterol-fed rabbits. CT would arise from the epoxides by the catalytic action of epoxide hydratase as has already been demonstrated *in vivo*¹⁸⁾ as well as *in*

TABLE I. Contents of Cholesterol Epoxides and Cholestanetriol in Plasma and Liver of Rabbits

Steroid	Tissue content			
	Plasma (μ g/ml)		Liver (μ g/g)	
	Control ^{a)}	Cholesterol fed ^{b)}	Control ^{a)}	Cholesterol fed ^{b)}
Cholesterol				
α -epoxide	0.09	0.24	0.08	0.60
Cholesterol				
β -epoxide	0.22	0.50	0.27	1.63
Cholestanetriol	0.07	0.32	0.16	0.80

Data are expressed as the arithmetic mean values from 3 animals.

a) Rabbits fed on a commercial diet for 2 months were used.

b) Rabbits fed on the diet supplemented with cholesterol (1%) for 2 months were used.

vitro.^{10,19)} Rabbit is one of the representative animals which cause significant hypercholesterolemia when fed on a cholesterol diet, and its plasma cholesterol level has been demonstrated to decrease to the normal level on co-feeding with CT.²⁰⁾ The present data, therefore, might suggest that in the hypercholesterolemic animal CT synthesis is required to be accelerated for lowering tissue cholesterol levels. In connection with this, an interesting investigation carried out by the British research group, Gray *et al.*,⁷⁾ should be recalled. They pointed out that patients with hypercholesterolemic high blood pressure showed extremely high plasma concentrations of α -EC. They, however, showed no data of β -EC and CT with these patients. A comparative study on plasma concentrations of α -EC, β -EC, and CT between patients and normal subjects is now in progress in our laboratory.

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