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EPOXIDATION OF CHOLESTEROL BY HEPATIC MICROSOMAL LIPID HYDROPEROXIDES

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SUMMARY: [1,2-^{3}H]Cholesterol was epoxidized to radioactive cholesterol α- and β-epoxides (5,6α-epoxy-5α- and 5,6β-epoxy-5β-cholestan-3β-ols) in the ratio 1:4 by hepatic microsomal lipid hydroperoxides (MsOOH, 1 mM as active oxygen) in the presence of ferrous ion. MsOOH could be replaced by methyl linoleate hydroperoxides (MOOH) under the same conditions although the latter was less effective than the former. None of cumene hydroperoxide, *t*-butyl hydroperoxide, and hydrogen peroxide was an effective oxidant even at 10 mM. Neither ADP nor EDTA had an effect on the epoxidation of cholesterol by MsOOH as well as by MOOH. Ferrous ion could not be replaced by ferric ion in the hydroperoxide-mediated epoxidation. Cyanide anion potentially inhibited the reaction.

INTRODUCTION

Cholesterol α-epoxide (5,6α-epoxy-5α-cholestan-3β-ol), a toxic steroid, has been suggested to be closely related to skin tumorigenesis in hairless mice irradiated by ultraviolet ray (1) as well as to hypercholesterolemic hyperpressure (2) and Wolman's disease (3). In addition, patients with colon cancer have been demonstrated to excrete larger amounts of its hydrolytic product, 5α-cholestane-3β,5,6β-triol, in their feces (4). The steroid epoxide not only covalently binds to isolated DNA (5), but also induces chromosome aberrations (6) and malignant transformations (7) of cells in culture.

Cholesterol α-epoxide is found together with the isomeric β-epoxide (5,6β-epoxy-5β-cholestan-3β-ol) in the ratio 1 to 2-4 as normal constituents in various tissues of mammals (8, 9), including human (3). The previous workers have demonstrated that epoxidation of cholesterol is mediated by hepatic microsomes in the presence of an NADPH-generating system, accelerated by the addition of a ferrous ion-ADP complex with concomitant formation of increasing amounts of malondialdehyde, and potentially inhibited by the metal-chelating agent, EDTA, with suppressed formation of the dialdehyde (10-12). The hepatic microsomal epoxidation of cholesterol is not inhibited by carbon monoxide (12), strongly suggesting that it occurs by reaction with microsomal lipid hydroperoxides *in vivo* as well as *in vitro*. Actually, the ratio of

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cholesterol α- and β-epoxides present in rat liver has been demonstrated to be very close to that observed with hepatic microsomal epoxidation of cholesterol *in vitro* (13). However, it should be noticed that no direct evidence has been available for the participation of microsomal lipid hydroperoxides as true oxidants in the microsomal cholesterol epoxidation. The present investigation provides the first direct evidence for the epoxidation of cholesterol with microsomal lipid hydroperoxides (MsOOH) and also for the requirement of ferrous ion as an essential factor.

MATERIALS AND METHODS

Materials —— [1,2-^{3}H]Cholesterol (43 Ci/mmol) was purchased from Radiochemical Centre, Amersham, diluted with unlabeled cholesterol, and purified before use by HPLC on a Lichrosorb RP-18 ODS column in methanol. Adenosine 5'-diphosphate (ADP), EDTA, and methylene blue were obtained from Wako Pure Chemical Industries, Ltd., Osaka, and cumene hydroperoxide (98% pure as active oxygen) from Nakarai Chemical Co., Kyoto. Cholesterol α-epoxide (14), β-epoxide (15), *t*-butyl hydroperoxide (99% pure as active oxygen) (16), and methyl linoleate hydroperoxides (MOOH, 95% pure as active oxygen) (17) were synthesized by the previous methods.

Microsomal lipid hydroperoxides (MsOOH) were prepared as follows: a suspension of microsomes (protein content: 540 mg by the Lowry method (18)) from male young adult Wistar rat liver (30 g) in 0.1 M phosphate buffer, pH 7.4 (90 ml), was shaken with a mixture of chloroform and methanol (2:1 v/v, 500 ml). The residue obtained on the evaporation of the solvent from the organic phase separated by centrifugation was suspended in ethanol (200 ml). To the ethanolic solution was added methylene blue (0.11 mM) after the removal of undissolved materials by centrifugation. The mixture was vigorously aerated at 15° for 6 hr in light of a tungsten lamp (300 W) and then condensed *in vacuo* at 15° to 50 ml. The condensate was shaken with ether and water (100 ml each). The organic layer separated was washed twice with water (0.5 vol. each), dried over anhydrous sodium sulfate, and condensed to 10 ml. The final condensate had 35 μmol/ml of active oxygen and was able to be stored without any appreciable loss of active oxygen at -20° in dark at least for 2 months.

Active oxygen of the hydroperoxides was determined by iodometry as previously reported (19).

Incubation of Radioactive Cholesterol —— A typical incubation system was made as follows: to a solution of [^{3}H]cholesterol (1 μCi, 0.05 μmol) and a hydroperoxide (1 μmol as active oxygen) in methanol (0.1 ml) containing 0.5% Tween 80 was added a solution of ADP (1 μmol) in 0.1 M phosphate buffer, pH 7.4 (0.5 ml). The mixture was preincubated for 5 min at 37°, and the reaction was started by the addition of an aqueous solution (0.4 ml) of ferrous sulfate (1 μmol). Other ingredients when used were dissolved in water and added to the incubation mixture. Incubations were carried out at 37° for 60 min.

High Pressure Liquid Chromatography (HPLC) —— HPLC was carried out for the separation of cholesterol epoxides on a LDC model Constametric II high pressure liquid chromatograph equipped with a RI monitor and a Lichrosorb RP-18 ODS column (Merck Co., Darmstadt, 5 μ in particle size, 4 x 150 mm). A silica column (Lichrosorb SI-60, 5 μ in particle size, 4 x 300 mm) was used for the determination of radiochemical purity of the epoxides isolated on the ODS column. The ODS and silica columns were eluted at 20° with 1 and 2 ml/min developing solvents, respectively.

Determination of Epoxides —— Radioactive cholesterol α- and β-epoxides formed from tritirated cholesterol by reactions with hydroperoxides were separated by HPLC and determined by the reverse isotope dilution method, including the scintillation counting of their radioactivities and the

determination of carrier steroids by GLC as follows: from the reaction mixture were extracted radioactive epoxides into peroxide-free ether (2 ml) containing unlabeled cholesterol α- and β-epoxides (0.5 mg each) in the presence of a saturating amount of sodium chloride. The solvent was evaporated in a nitrogen stream from the organic phase separated. The residue obtained was dissolved in a mixture of methanol and ethanol (10:3, 0.2 ml) and immediately subjected to reverse partition HPLC carried out in the same solvent mixture to collect the epoxides appearing at 4.7-5.6 min; under these conditions, cholesterol was eluted at 9.8 min. The eluate corresponding to the epoxide fraction was redissolved in methanol (0.2 ml) after the evaporation of the solvent in nitrogen. The methanolic solution was subjected to HPLC carried out on the same column in methanol; under these conditions cholesterol α- and β-epoxides were eluted at 8.0 and 6.4 min, respectively. The epoxide fractions were separately collected, and their radioactivity to carrier ratios were measured by the previously reported method (12).

RESULTS AND DISCUSSION

Epoxidation of [^{3}H]Cholesterol by Hepatic Microsomal Lipid Hydroperoxides (MsOOH)

[1,2-^{3}H]Cholesterol was incubated with rat liver MsOOH in the presence of ferrous ion and ADP in an aqueous medium, pH 7.4 (complete system). Radioactive cholesterol epoxides formed were extracted together with unlabeled cholesterol α- and β-epoxides as carriers for the reverse isotope dilution method. The epoxides were immediately separated from the radioactive substrate by HPLC on an ODS column in methanol containing ethanol into a single fraction eluted at 4.7-5.6 min. The α- and β-epoxides were then well separated at 8.0 and 6.4 min, respectively, on an ODS column by the elution with methanol. Radiochemical homogeneity of the separated epoxides was confirmed by HPLC on a silica column; retention times of the α- and β-epoxides were 11.3 and 13.5 min, respectively. The radioactivity to carrier ratios of the α- and β-epoxides, determined by a combination of the scintillation counting method with GLC (12), were unchanged before and after HPLC on the adsorbent column with the specimens separated by the 2nd reverse partition HPLC.

Per cent conversion of [^{3}H]cholesterol to the radioactive α- and β-epoxides by MsOOH (1 mM as active oxygen) was 1.6% in the presence of ferrous ion and ADP (Table I). The β-epoxide was yielded preferentially to the α-epoxide in the ratio very similar to that of the previously demonstrated hepatic microsomal epoxidation (1.3%) of cholesterol (0.34 μM) in the presence of ferrous ion (0.2 mM), ADP (1 mM), and an NADPH-generating system (12). Methyl linoleate hydroperoxides (MOOH) were one half as effective as MsOOH for epoxidation of cholesterol. The α- to β-ratio of the epoxides formed by MOOH was very similar to the case of MsOOH. However, none of cumene hydroperoxide, *t*-butyl hydroperoxide, and hydrogen peroxide was an effective oxidant even at 10 mM as active oxygen under the same incubation conditions.

Factors Influencing Cholesterol Epoxidation by Lipid Hydroperoxides

Lipid hydroperoxidation in hepatic microsomes has been well recognized to be remarkably accelerated either by ferric ion or by ferrous ion in the

Table I. Epoxidation of Cholesterol by Various Hydroperoxides

Hydroperoxide	Concentration of active oxygen (mM)	Radioactive products formed (%) α-Epoxide	β-Epoxide	β-/α-Epoxides
Microsomal lipid hydroperoxide (MsOOH)	1	0.31	1.33	4.3
Methyl linoleate hydroperoxide (MOOH)	1	0.19	0.76	4.0
Cumene hydroperoxide	1	0.00	0.03	-
	10	0.03	0.16	-
t-Butyl hydroperoxide	1	0.01	0.04	-
	10	0.02	0.08	-
Hydrogen peroxide	1	0.00	0.01	-
	10	0.02	0.02	-

The reaction mixture contained ADP (1 mM), $FeSO_4$ (1 mM), [1,2-^{3}H]cholesterol (1 μCi, 50 μM), Tween 80 (0.05%), and the hydroperoxide (1 mM as active oxygen) in 0.05 M phosphate buffer, pH 7.4.

presence of NADPH and ADP and potentially inhibited by 0.5 mM or higher concentrations of EDTA (20). In this hydroperoxidation system, ferric ion has been suggested to be reduced to ferrous ion, a possibly effective form for the activation of molecular oxygen (21).

Surprisingly, epoxidation of cholesterol by MsOOH in the presence of ferrous ion was little influenced by omitting ADP or by the addition of EDTA up to 10 mM to the complete incubation mixture. Omitting ferrous ion from the complete system or replacing ferrous ion by ferric ion, the epoxidation did not occur to any appreciable extent (Table II). Potassium cyanide completely inhibited the epoxidation at a seven times higher concentration than that of

Table II. Epoxidation of Cholesterol by Microsomal Lipid Hydroperoxides

Incubation system	Radioactive products formed (%) α-Epoxide	β-Epoxide	β-/α-Epoxides
Complete [a]	0.31	1.33	4.3
- ADP	0.31	1.47	4.7
+ EDTA (1 mM)	0.31	1.20	3.9
(10 mM)	0.33	1.33	4.0
- Fe^{2+}	0.03	0.14	-
- Fe^{2+} + Fe^{3+} (1 mM)	0.02	0.10	-
+ KCN (7 mM)	0.00	0.00	-

[a] The reaction mixture contained microsomal lipid hydroperoxides (1 mM as active oxygen) and other ingredients as described in Table I.

Table III. Epoxidation of Cholesterol by Methyl Linoleate Hydroperoxides

Incubation system	Radioactive products formed (%)		β- /α-Epoxides
	α-Epoxide	β-Epoxide	
Complete [a]	0.19	0.76	4.0
- ADP	0.18	0.68	3.8
+ EDTA (1 mM)	0.17	0.60	3.5
(10 mM)	0.18	0.77	4.3
- Fe^{2+}	0.00	0.01	-
- Fe^{2+} + Fe^{3+} (1 mM)	0.00	0.02	-
+ KCN (7 mM)	0.00	0.00	-

[a] The reaction mixture contained methyl linoleate hydroperoxides (1 mM as active oxygen) and other ingredients as described in Table I.

ferrous ion. Factors influencing epoxidation of cholesterol by MOOH were very similar to those by MsOOH (Table III).

No appreciable inhibitory effect of EDTA on the epoxidation of cholesterol by hydroperoxides in the presence of ferrous ion strongly suggests that the chelating agent would inhibit the initial step for the lipid hydroperoxide formation in hepatic microsomes, but not influence the epoxidation. That may be the reason for the potent inhibition with EDTA of the microsomal malondialdehyde formation as well as of microsomal epoxidation of cholesterol in the presence of ferrous ion and NADPH. ADP was not necessarily an essential factor for the cholesterol epoxidation whereas it has been demonstrated to be an accelerating factor for the formation of malondialdehyde (20) and cholesterol epoxides (12) in hepatic microsomes. This strongly suggests that the nucleotide may serve as a chelating agent for the formation of an iron complex to catalyze the formation of lipid hydroperoxides from polyunsaturated fatty acyl lipids, but not for the formation of active oxygen from MsOOH and ferrous ion which catalyzes the epoxidation of cholesterol. Potassium cyanide is likely to block the formation of the active oxygen-iron complex to catalyze the epoxidation. Thus, a proposed mechanism for microsomal epoxidation of cholesterol which involves MsOOH as a direct oxidant may be depicted as illustrated in Fig. 1.

The α- to β-ratio of the epoxides formed by oxidation of the cholesterol double bond varies with oxidizing agents, *e. g.*, the β-epoxide is yielded 8-10 times higher than the α-epoxide by air oxidation (22) or by a high concentration of hydrogen peroxide (23). On the contrary, the α-epoxide is a major product when cholesterol is treated with peracids in organic solvents (24) or by OH radical in an aqueous solution (25). A ferric ion-acetylacetone complex and *t*-butyl hydroperoxide have been shown to convert cholesterol in chloroform

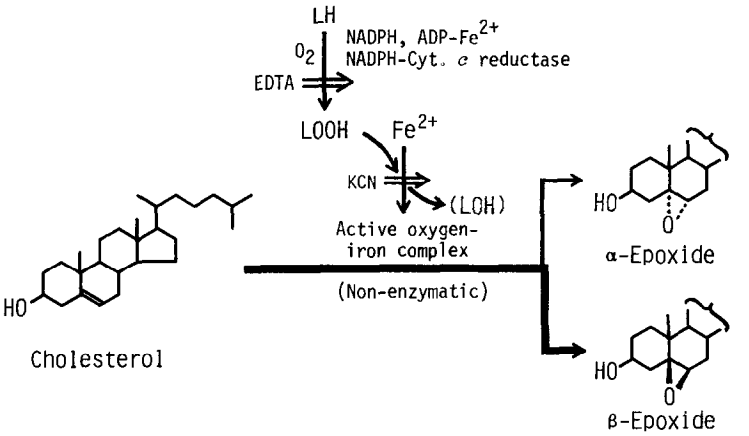

Fig. 1 A proposed mechanism for microsomal epoxidation of cholesterol. LH and LOOH represent polyunsaturated fatty acid residues of microsomal lipids and their hydroperoxides, respectively. Hydroperoxidation of LH with molecular oxygen is mediated by NADPH-cytochrome *c* reductase and accelerated by an ADP-Fe^{2+} complex as has previously been demonstrated (21).

to the α- and β-epoxides in the ratio very similar to the microsomal epoxidation (26, 27). However, the present investigation demonstrated that neither ferric ion nor *t*-butyl hydroperoxide was an effective agent for the epoxidation of cholesterol in an aqueous medium. A study on the participation of hydroperoxides of other microsomal polyunsaturated fatty acids in cholesterol epoxidation is now in progress in our laboratory.

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