Communication

24(S),25-Epoxycholesterol

EVIDENCE CONSISTENT WITH A ROLE IN THE REGULATION OF HEPATIC CHOLESTEROSTERGENESIS*

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Previously we showed that 24(S),25-epoxyccholesterol is formed from acetate, via squalene 2,3(S), 22(S),25-dioxido and 24(S),25-oxidolanosterol, during the normal course of cholesterol biosynthesis in S0 rat liver homogenate (Nelson, J. A., Steckbeck, S. R., and Spencer, T. A. (1981) J. Biol. Chem. 256, 1067-1068; Nelson, J. A., Steckbeck, S. R., and Spencer, T. A. (1981) J. Am. Chem. Soc. 103, 6974-6975). Herein we demonstrate that the nonapoifiable extract from human liver tissue contains 24(S),25-epoxycholesterol in an amount approximately 10^-3 relative to cholesterol. We show that 24(S),25-epoxyccholesterol, like many other oxygenated sterols, represses hydroxyoemethylglutaryl-CoA reductase activity in cultured cells and binds to the cytosolic oxysterol-binding protein. Furthermore, we show that this epoxide is not rapidly metabolized in cultured cells. These results suggest that 24(S),25-epoxyccholesterol may participate in the regulation of hepatic cholesterol metabolism in vivo.

It was found by us (1, 2) and by others (3, 4) that inhibitors of oxidosqualene cyclase cause an accumulation of squalene 2,3(S), 22(S),25-dioxido and as squalene 2,3(S)-oxide during sterol biosynthesis. We then demonstrated that squalene 2,3(S), 22(S),25-dioxido is converted by S0 rat liver homogenate to 24(S),25-epoxyccholesterol via 24(S),25-oxidolanosterol, approximately as efficiently as squalene 2,3(S)-oxide is converted to cholesterol (5). We found further that incubation of acetate with rat liver homogenate in the absence of any cyclohexane inhibitor results in the formation of approximately 5% of 24(S),25-epoxyccholesterol relative to cholesterol (6). These and other (7) observations suggest that sterol epoxides formed from squalene 2,3(S), 22(S),25-dioxido may have biochemical significance, particularly since numerous cholesterol derivatives with oxygenated side chains are effective represors of HMG-CoA reductase, and, consequently, of cholesterol biosynthesis (8). Accordingly, it is important to determine whether production of such epoxy sterols occurs under physiological conditions, and whether these sterols play a role in cholesterol regulation.

In this study, we demonstrate that 24(S),25-epoxyccholesterol is present in human liver. We also show that this epoxide, like sterols with side chain hydroxyl or keto groups, represses HMG-CoA reductase in cultured cells and binds to the cytosolic oxysterol-binding protein (9). Incubation of tritiated 24(S),25-epoxyccholesterol with cultured fibroblasts leaves the epoxide largely unchanged, with no detectable conversion into 25-hydroxycholesterol, 24-cholehydroxycholesterol, desmosterol, or cholesterol. These results support a hypothesis that aetabolic generation of 24(S),25-epoxyccholesterol may be a mechanism for the regulation of cholesterol biosynthesis.

EXPERIMENTAL PROCEDURES

Sterols—Unlabeled 24(S),25-epoxyccholesterol, m.p. 158-160 °C (m.p. (literature) 190-192 °C (6)), and 24(R),25-epoxyccholesterol, m.p. 164-165.5 °C (m.p. (literature) 165-165.5 °C (6)), were prepared from desmosterol (Steraloids, Inc., Wilton, NH) as previously described (5, 6). Both were homogeneous by TLC and HPLC. The 24(S),25-epoxyccholesterol was further characterized by 1) 'H NMR (determined in CDCl3 on a Varian XL-300 spectrometer): δ 6.09 (s), 0.84 (d, J = 6.6 Hz), 1.01 (s), 1.26 (s), 1.31 (s), 2.68 (m), 3.52 (m), 5.36 ppm (m); and 2) mass spectroscopy (performed by Philip Thorne of Dartmouth College on a Finnegan 4000 instrument at 35 eV using a solid probe): m/z (relative intensity) 400 (28), 382 (29), 367 (20), 315 (22), 271 (65), 255 (27), 213 (52), 170 (50), 43 (100). Radio labeled 24(S),25-epoxyccholesterol was synthesized as follows. According to the method of Ikemawa and co-workers (10), cholic acid (Steraloids, Inc.) was converted to its tetrahydropropanoyl ether, which was reduced with lithium aluminum hydride in tetrahydrofuran and then oxidized with pyridinium chlorochromate to afford the corresponding C-24 aldehyde as its tetrahydropropanoyl ether. This material was labeled with 'H by triethylamine-catalyzed proton exchange according to the method of Barton et al. (11), to afford 23-[3H]aldehyde which was treated with isopropylidene triphenylphosphorane, by a modification of a procedure used previously to prepare 25-[3H]desmosterol (12), to afford 23-[3H]desmosterol with specific activity = 94,000 dpm/μg. By the same procedure used with the unlabeled desmosterol, this material was converted to 23-[3H]24(S),25-epoxyccholesterol. This labeled epoxide was purified before use by reverse-phase HPLC on a Resolve 5-μm C8 column (Waters) using 88:12 CH3OH:H2O as solvent to give a single symmetrical radioactive peak with specific activity = 93,000 dpm/μs, 25-Hydroxycholesterol was purchased from Steraloids, Inc. 24-Hydroxycholesterol was prepared as a mixture of R and S epimers by NaBH4 reduction of 24-ketocholesterol (Analsabs, Inc., North Haven, CT); this epimeric mixture, which was not resolved by the reverse-phase HPLC conditions employed, was used to test whether either 24(S)-hydroxycholesterol, the chemically plausible product of reduction of 24(S),25-epoxyccholesterol, or 24(R)-hydroxycholesterol was being formed. 4-[14C]Cholesterol was purchased from Amersham Corp.

Analysis of Human Liver for 24(S),25-Epoxycholesterol—Samples from two human livers were obtained from Dr. James Trudell of the Department of Anaesthesia, Stanford University School of Medicine. The samples were from donors in the Stanford Heart Transplant Program and were obtained as rapidly as possibly following removal of the heart for cardiac transplantation. Donors were on cardiac bypass up to the time of excision of the heart. The liver samples were frozen at liquid nitrogen temperature immediately after excision and were kept frozen until they were saponified as described next. The

* The abbreviations used were: HMG, 3-hydroxy-3-methylglutaryl; HPLC, high performance liquid chromatography.

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protocol of Dr. Trudell was approved by the Stanford Human Subjects Experimentation Committee.

To a sample of liver (approximately 75 g, wet weight) which had been chopped into small pieces was added approximately 700 ml of ethanol and approximately 100 ml of 30% KOH in H₂O, and the mixture was heated at reflux for 4 h. The cooled extract was exhaustively extracted with petroleum ether. The extracts were washed with H₂O and evaporated to afford approximately 1 g of nonsaponifiable extract. A 30-40 mg sample of this extract was dissolved in 3 ml of ethyl acetate and put through a Sep-Pak C₁₈ cartridge. The eluted sample was then subjected to preparative TLC on Silica Gel 60 F₂₅₄ (EM Reagents, Cincinnati, OH) using three developments with 4:1 hexane:ether. A broad band corresponding to cholesterol, which would contain any 24(S),25-epoxycholesterol present, was scraped, eluted with ethyl acetate, and passed through a Sep-Pak C₁₈ cartridge. Evaporation afforded material which was used for mass spectroscopy and in the following experiments designed to establish the identity of the epoxide by demonstration: 1) its liability upon mild acid treatment, and 2) its conversion to cholesterol by treatment with lithium aluminum hydride.

1) A sample sufficient to give a readily detectable HPLC signal of the material thought to be 24(S),25-epoxycholesterol (approximately 1 μg) was dissolved in 500 μl of tetrahydrofuran, and 2 drops of water and 100 μl of 60% perchloric acid were added. The mixture was stirred at room temperature for 1 h. Ethyl acetate (10 ml) was added, and the mixture was washed with 1 ml of aqueous sodium bicarbonate and 1 ml of brine. Evaporation of the organic layer afforded a residue which was subjected to preparative TLC and HPLC analysis as described above. A control experiment was performed identically except that no perchloric acid was added.

2) Another sample of the putative epoxide from liver was dissolved in 3 ml of freshly distilled dimethoxyethane at 0 °C under N₂, and 20 mg of lithium aluminum hydride was added. The resulting suspension was heated at reflux for 72 h under N₂, with care being taken to maintain the solvent level. The mixture was cooled and then treated with 3 ml of ethyl acetate, followed by 0.5 ml of H₂O. The solution was extracted with ethyl acetate, and the organic extracts were evaporated to afford a residue which was subjected to preparative TLC and HPLC analysis as described above.

Normal-phase HPLC, used only to separate 24(S),25-epoxycholesterol and 24(R),25-epoxycholesterol, was performed under the same instrumental conditions used for reverse-phase analysis of liver samples on an Ultrasphere-Si 5-μm, 4.6 × 250-mm column (Beckman Instruments) with 98:2:1 hexane/isopropanol alcohol as eluent at a flow rate of 1 ml/min.

In order to determine the amount of material with the retention time of 24(S),25-epoxycholesterol relative to the amount of cholesterol in the sample, HPLC response curves for authentic samples of 24(S),25-epoxycholesterol and cholesterol were determined. The intensity of the HPLC response at 205 nm was identical for the two compounds.

Analysis of Human Liver for 24(S),25-Epoxysterol—Nonsaponifiable extracts from samples of two human livers were purified chromatographically as described under "Experimental Procedures" and were analyzed by reverse-phase HPLC. A peak was found which had a retention time identical to that of authentic 24(S),25-epoxycholesterol when eluting with 95:5 or 90:10 methanol/water was used as eluent. When 95:5 methanol/water was used, typical retention times were for 25-hydroxycholesterol, 10.5 min; 24(S),25-epoxycholesterol, 13.5 min; desmosterol, 30.0 min; and cholesterol, 39.3 min. A representative HPLC trace of the "cholesterol fraction" from one liver is shown in Fig. 1.

Experiments were run as follows to establish that the ratio of 24(S),25-epoxycholesterol to cholesterol thus determined was an accurate reflection of the ratio in intact liver. To the saponification mixture for a sample of liver were added known amounts of 23,24-[3H] 24(S),25-epoxycholesterol and 4,4'-[3H]cholesterol. The nonsaponifiable extract was prepared exactly as usual, and an aliquot of the extract was analyzed for 3H and 14C. The per cent of the 3H and 14C having the TLC mobility of 24(S),25-epoxycholesterol and cholesterol was determined with another aliquot. The above analyses were repeated after the nonsaponifiable extract was passed through a Sep-Pak C₁₈ cartridge and after preparative TLC as described above.

Repression of HMG-CoA Reductase Activity in Culture L cells (mouse fibroblasts) by 24(S),25-epoxycholesterol was assayed as described (9). The relative binding affinity of the sterol for the oxysterol-binding protein was determined by measuring its affinity to trap the 25-[3H]hydroxycholesterol from specific sites. Conditions for preparation of the oxysterol-binding protein from L cells, for binding of the ligand at pH 5.5 or 7.4, and for assay of the complex by velocity sedimentation in a sucrose gradient were as described (9).

Metabolism of 24(S),25-Epoxysterol—Approximately 2 × 10⁷ cells from 4 L cell spinner culture (9) were pipetted into a 150-ml culture flask (Corning) and incubated for 2 h at 37 °C in a 5% CO₂ incubator. The cells attached to the flask's surface during this time, and the medium was then decanted and replaced with 10 ml of fresh medium containing 1 μg/ml vitamin E. The sterol solution was prepared by mixing 0.1 ml of an ethanol solution of 25-[3H]24(S),25-epoxycholesterol with 1 ml of medium containing 5% bovine serum albumin (Sigma, essentially fatty acid-free). This mixture was then added to the culture medium to give a final concentration of epoxide of 0.37 μg (30,000 dpm)/ml. After 5 h of incubation at 37 °C, the cells were scraped into the medium, collected by centrifugation, and washed twice with 0.1 M NaCl. The sterols were extracted by adding chloroform/methanol 2:1, and the phases were separated by centrifugation. The lower chloroform phase was dried under a stream of nitrogen, and the residue was dissolved in 1 ml of hexane/1 M NaCl, and the phases were separated by centrifugation. The intensity of the HPLC response at 205 nm was identical for the two compounds.
Hepatic 24(S),25-Epoxycholesterol

FIG. 1. HPLC analysis of human liver for 24(S),25-epoxycholesterol. Reverse-phase HPLC analysis as described under "Experimental Procedures" of the portion of the nonsaponifiable extract from human liver which has the chromatographic mobility of cholesterol on normal-phase preparative TLC is shown. The arrow indicates the peak which was shown to be 24(S),25-epoxycholesterol (retention time = 13.5 min). Cholesterol has retention time of 39.3 min.

Figure 1 shows the HPLC analysis of human liver for 24(S),25-epoxycholesterol. The arrow indicates the peak which was shown to be 24(S),25-epoxycholesterol (retention time = 13.5 min). Cholesterol has a retention time of 39.3 min.

FIG. 2. 24(S),25-Epoxycholesterol repression of HMG-CoA reductase in L cell cultures (A) and displacement of 25-[3H]hydroxycholesterol from the oxyysterol-binding protein (B). Points in A represent results from two identical assays. In B, points represent results from three assays, in one of which (O) binding of the sterols to the binding protein occurred over 24 h in 0.05 M phosphate buffer, pH 7.4. In the other two (●), the buffer was 0.05 M citric acid phosphate buffer, pH 5.5, and the incubation time was 2 h.

Figure 2 shows the 24(S),25-Epoxycholesterol repression of HMG-CoA reductase in L cell cultures (A) and displacement of 25-[3H]hydroxycholesterol from the oxyysterol-binding protein (B). Points in A represent results from two identical assays. In B, points represent results from three assays, in one of which (O) binding of the sterols to the binding protein occurred over 24 h in 0.05 M phosphate buffer, pH 7.4. In the other two (●), the buffer was 0.05 M citric acid phosphate buffer, pH 5.5, and the incubation time was 2 h.
function at pH 5.5. The similar results obtained under both conditions suggest that this was not an important factor in the assay. The concentrations of the epoxide required to suppress HMG-CoA reductase by 50% (0.89 \mu M) and to displace 50% of the 25-[\text{H}]hydroxycholesterol from the binding protein (0.55 \mu M) can be compared with published values for a wide range of oxysterols (9). The activity of 24(S),25-epoxycholesterol is intermediate between the least and most active sterols tested; it is approximately one-seventh that of 25-hydroxycholesterol.

To try to make certain that the repression of HMG-CoA reductase in L cells was caused by 24(S),25-epoxycholesterol itself and not by a metabolite, [\text{H}]24(S),25-epoxycholesterol was incubated for 5 h with L cells, and the sterol fraction was extracted and analyzed by HPLC. The distribution of recovered radioactivity is shown in Fig. 3. About 88% of the counts were found as unaltered epoxide. The remaining radioactivity was scattered in the highly polar fractions; no significant amounts with the retention times of 25-hydroxycholesterol, 24-hydroxycholesterol, desmosterol, or cholesterol were detected.

These results suggest that the repression of HMG-CoA reductase is indeed caused by 24(S),25-epoxycholesterol and not by a product formed from it intracellularly. The fact that the activity measured in the binding assay is in good agreement with that determined by the reductase repression assay further supports the conclusion that the epoxide itself represses HMG-CoA reductase.

By use of literature values indicating that the concentration of cholesterol in liver is approximately 2.5 mg/g, wet weight (15), and that water constitutes about 75% of the wet weight (16), the concentration of the epoxide can be estimated to be in the range of 10–30 \mu M. This range is well above the concentration of 0.89 \mu M required in a fibroblast cell culture to suppress HMG-CoA reductase activity by 50%. However, an unknown proportion of the epoxide present in liver may have been esterified and unavailable for a regulatory role. Furthermore, cultures of liver cells appear to be about 10 times more resistant than other cell cultures to the inhibitory effects of oxysterols (8). Nonetheless, despite the uncertainties involved in these estimates, the concentration of the epoxide in liver seems to be high enough to function in the regulation of HMG-CoA reductase. We have found (17) that similar levels of 24(S),25-epoxycholesterol, apparently adequate for regulation of the reductase, are synthesized from mevalonic acid in cultured fibroblasts, indicating that the epoxide could also play a regulatory role in non-hepatic cells.

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