3β, 5α-DIHYDROXYCHESTAN-6-ONE EXISTS IN HUMAN BLOOD

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3β,5α-Dihydroxycholestan-6-one was detected in fresh human plasma at the concentration of 14-44 ng/mL. The oxysterol binds specifically to phorbol ester specific binding protein in vitro, and may be an endogenous ligand of the protein.

KEY WORDS cholesterol; oxysterol; phorbol ester; 3β,5α-dihydroxycholestan-6-one; HPLC/MS; GC/MS.

Cholesterol undergoes oxidation to produce a number of oxidized products generally called oxysterols, many of which possess biological activities such as cytotoxicity,1 atherogenicity,2 mutagenicity,3 etc. Oxysterols are as intermediates in several biosynthetic pathways, and autoregulate their metabolic fate by end-product repression and activation of downstream catabolism.5 One of them, 3β,5α-dihydroxycholestan-6-one (YS-64), is cytotoxic.5 We have prepared YS-64 as a mimic of 12-O-tetradecanoylphorbol-13-acetae (TPA).6 Though this oxysterol did not bind protein kinase C, it showed significant binding to the TPA specific nuclear binding protein,7 the binding constant being 104 M-1.9 this suggests that the oxysterol may have an important physiological role. Recent report on the oxysterols as nuclear receptor ligands9 also suggest the idea that YS-64 is a putative nuclear receptor ligand. This compound was also identified in feces of rats fed cholestan-3β,5α,6β-triol.10 Here, we report that 3β,5α-dihydroxycholestan-6-one is present in fresh human blood.

Method Bovine adult serum (500 mL) from Sigma (B2771) was added to 1600 mL of chloroform-methanol (1:2). The mixture was vigorously shaken and centrifuged at 4000 rpm. The supernatant was extracted with chloroform (2x 500 mL), and the organic layer was evaporated in vacuo. The residue was dissolved in toluene (5 mL). For solid-phase extraction, a silica cartridge column (Sep-Pak Vak) was conditioned by washing with 30%
isopropanol-hexane and then hexane. The above toluene solution (1 mL) was applied to the conditioned column and eluted with 100 mL of 1% isopropanol-hexane (essentially all the cholesterol was eluted), followed by 100 mL of 30% isopropanol-hexane. The later fraction, which contains YS-64, was concentrated \textit{in vacuo}. The fraction was then subjected to HPLC on a Hibar LiChrosorb Si60 column (Merck, 10 mm/25 cm), and eluted with 2% methanol-dichoromethane (5 mL/min). The YS-64 fraction was again chromatographed by on the same column with 5% isopropanol-hexane (5 mL/min). The YS-64 fraction was analyzed by HPLC/MS and GC/MS. Human plasma was prepared by centrifugation (3000 rpm, 10 min) from fresh human blood (100 mL, 100 mg of EDTA was added), and butylhydroxytoluene (BHT) was added to it (50 mg/mL plasma). Blood cells were washed five times with phosphate-buffered saline and BHT was added. The extraction and purification procedure was the same as in the case of bovine serum. The HPLC/MS and GC/MS conditions are described in the figure legends.

Fig 1. Mass chromatograms on HPLC/MS: (a) m/z 401, (b) m/z 419 and mass spectra (c) of 3β,5α-dihydroxycholestan-6-one. left: authentic, right: typical sample of bovine adult serum. All separations were carried out on a 2.0-mm x 150-mm Develosil packed column (Nomura Chemical) with 0.5% glycerol in acetonitrile. Detection were performed by FAB.

Results and Discussion First, we found the presence of YS-64 in bovine serum and established the analytical procedures. Recovery of YS-64 on the extraction and purification process was estimated to be roughly 50-60% by using 22,23-di-\textsuperscript{3}H]-YS-64. The detection limit was 1-5 ng per one HPLC/MS or GC/MS shot, or at the level of ng/mL plasma. The concentrated YS-64 fractions were analyzed by HPLC/MS: at the retention time (13.3 min when 2.5% water-methanol as eluent) of authentic YS-64: Peaks corresponding to the molecular ion of YS-64 (M+H\textsuperscript{+} = 419), a dehydrated peak (M-H\textsubscript{2}O+H\textsuperscript{+} = 401 and M-2H\textsubscript{2}O+H\textsuperscript{+} = 383) were observed. The mass spectrum of the peak was essentially identical with that of the authentic sample. This identity was complete when the eluate was 100% acetonitrile. GC/MS analysis also confirmed the presence of YS-64 in plasma. The extract gave the same three peaks at the retention time of 21.8 min when monitored at these mass numbers. The amount of YS-64 in the bovine serum was 9 ng/mL. The amounts found in the two human fresh samples were 14 and 44 ng/mL in plasma and 4 and 10 ng/mL in blood cells, respectively. During identification, care was taken to avoid artificial oxidation. Cholesterol, which exists in a large amount, was first removed to minimize artificial production of oxysterols.\textsuperscript{10} Also, we did not use any alkali treatment for the hydrolysis of cholesterol esters in order to avoid oxidation under the alkaline condition:
only free YS-64 was analyzed. Though we could not absolutely eliminate the possibility of artificial formation during the procedure, the results indicate the presence of 3β,5α-dihydroxycholestan-6-one in fresh human blood. The discovery of this novel metabolite of cholesterol should be helpful in establishing the physiological roles of oxysterols.

Fig 2. Selected ion chromatograms on GC/MS: (a) m/z 318, (b) m/z 418 and mass spectra (c) of 3β,5α-dihydroxycholestan-6-one. Left: authentic, right: typical sample of human plasma. All separations were carried out on a Shimadzu CBP1-M25-025 column. The injector temperature was 250°C. The oven temperature was set to an initial value of 50°C during 2 min following sample injection and then increased at a rate of 40°C/min up to 300°C, where the temperature was held at 300°C. Detection was performed by EI.

REFERENCES

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