Identification and characterization of dehydrocholic acid reductase system in the cytosol of human red blood cells

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Abstract: We conducted in vivo and in vitro studies of the reductive metabolism of the cholagogue, dehydrocholic acid (DHCA). Immediately after the intravenous administration of 1 g of DHCA in normal subjects (n = 6), the concentration of the reductive metabolite, 3α-hydroxy-7,12-dioxo-cholanoic acid (unconjugated form), increased sharply in the systemic circulation, rising to 95.8 μM 10 min after administration. The results of in vitro experiments with DHCA and whole blood showed that 3α-hydroxy-7,12-dioxo-cholanoic acid and 3β-hydroxy-7,12-dioxo-cholanoic acid were produced from DHCA. In vitro experiments using DHCA and the red blood cell fraction, and DHCA and the red blood cell cytoplasmic fraction gave similar results to those described above with whole blood. However, a reductive metabolite was not formed by the incubation of DHCA and the red blood cell membrane fraction. These findings indicated that, contrary to the conventional theory that intravenously administered DHCA is subjected to reduction only in the liver, reduction also occurs in the systemic circulation, and the mechanism for this reductive metabolism is present in the cytoplasmic fraction of red blood cells. Further investigation to characterize this reductive metabolic system revealed an optimum temperature of 37°C, an optimum pH of 7.4, a Km value of $2.0 \times 10^{-3}$ M, and inactivation by heat treatment (70°C for 2 min).

Key words: dehydrocholic acid, human red blood cell cytoplasmic fraction, 3α-hydroxy-7,12-dioxo-cholanoic acid, 3β-hydroxy-7,12-dioxo-cholanoic acid

Introduction

The bile acid, dehydrocholic acid (DHCA), 3,7,12-trioxo-5β-cholanoic acid, has been used widely for some time, both as a cholagogue and for the measurement of blood circulation time.1,2 Considerable research on DHCA metabolism has been conducted in the past, leading to the widespread belief that DHCA administered intravenously to humans is taken up by the liver, where taurine and glycine are conjugated and the oxo groups are reduced by reductase at the C-3, C-7, and C-12 positions in that sequence, and that most DHCA is excreted in the bile and some in the urine.3-5 However, in 1990, Yoneda et al.6 demonstrated that intravenous administration of DHCA in two cholangiocarcinoma patients who underwent percutaneous transhepatic cholangial drainage (PTCD) led to elevated blood levels of unconjugated 3α-hydroxy-7,12-dioxo-cholanoic acid, a DHCA metabolite, thereby indicating that a mechanism for reducing the oxo groups of DHCA exists in the blood of the systemic circulation.

In our present study, consisting of an in vivo experiment, in which we intravenously administered DHCA to healthy human subjects, and in vitro experiments with red blood cells, we found a DHCA reductase system to be present in the cell cytoplasmic fraction. We conducted an investigation to characterize this enzyme system, and we describe our findings in this report.

Materials and methods

Reagents

Hydrochol (10 ml of 10% Na-DHCA solution in one ampoule), obtained from Kyorin Pharmaceutical Co., Ltd. (Tokyo, Japan), was used for the sodium dehy-
drocholate injection in the in vivo experiment. The purity of this material was shown to be 98% or higher by gas liquid chromatography (GLC) and the level of contaminating 3α-hydroxy bile acid was shown by an enzymatic method to be extremely low, 0.38% or lower. Na-DHCA used in the in vitro experiments was obtained from Tokyo Tanabe Co., Ltd. (Tokyo, Japan) and was confirmed by GLC analysis to have a purity of 99% or higher. The reference substances, 3α-hydroxy-7,12-dioxo-cholanoic acid (3α-OH-7,12-dioxo), 7α-hydroxy-3,12-dioxo-cholanoic acid, 3α, 7α-dihydroxy-12-oxo-cholanoic acid, and 3α,12α-dihydroxy-7-oxo-cholanoic acid were generously supplied by Professor Ikawa of the Institute of Steroid Research of the Tottori University School of Medicine. In addition, 3β-hydroxy-7,12-dioxo-cholanoic acid (3β-OH-7,12-dioxo) was synthesized by one of the authors (J.G.). All other reagents used in the experiment were of special grade.

**Intravenous Na-DHCA loading**

The contents of one hydrochol ampoule (1g of Na-DHCA) were administered to each of six healthy subjects via the cubital vein early in the morning after they had fasted overnight. Blood was sampled from the cubital vein on the contralateral side before administration and 3, 5, 10, 20, 30, 60, 90, and 120 min after administration. The blood samples were freeze-stored at −20°C after centrifugation until use. Tests of peripheral blood in these six healthy subjects showed a red blood cell count of (mean ± SD; 480 ± 20) × 10⁶/μl, and a hematocrit (Ht) of 42 ± 2%; none of them showed any anemia.

**Serum bile acid analysis**

Total bile acid concentration in the serum (serum 3α-hydroxy bile acid) was measured by the enzymatic method of Mashigie et al., using 3α-hydroxysteroid dehydrogenase (3α-HSD), and serum DHCA concentration was determined by the method of Yoneda et al., using 3-oxo-3β-A⁴-dehydrogenase. The bile acid fraction of the blood was analyzed by GLC. Briefly, this method consisted of diluting 0.5 ml of the serum sample with 2 ml of 0.1 N NaOH and applying this to a Bond elut C18 column (Analytical International, Harbor City, Calif.). The pH of the reaction solution was adjusted to 1.0 with 6N HCl and the bile acid was extracted with ethyl acetate. After methylation with diazomethan, a trifluoroacetate derivative was prepared. Samples prepared in this way were dissolved in acetone and subjected to GLC analysis. A 1.5% QF-1 column (250°C) was used for the GLC column. The value of each bile acid in serum was then corrected with the peak area/μg of standard bile acid.

**Identification of 3α- and 3β-OH-7,12-dioxo**

We identified 3α-and 3β-OH-7,12-dioxo by GLC analysis and gas chromatography-mass spectrometry (GC-MS). The relative retention times of the sample and reference compounds were compared by GLC analysis, and the mass spectra of the unknown sample and reference compound by GC-MS analysis. Prior to analysis by GC-MS, a trimethylsilyl-ether derivative of bile acid pentafuorobenzyl (PFB) ester was prepared and GC-MS was carried out with VG analytical MM-12030 quadruple mass spectrometer (VG Analytical, Manchester, England).

**In vitro tests of DHCA reduction by whole blood and blood components**

Heparinized blood samples from the healthy subjects were preheated for 5 min at 37°C. A 0.12-ml aliquot of 12.5 mM Na-DHCA solution and 0.08 ml of physiological saline solution were added to 1.0 ml of preheated blood, and the mixture was shaken for 1 h at 37°C. Hence, the concentration of the DHCA substrate was 1.25 mM. The tube containing this substrate was immediately cooled in ice, and then subjected to cooling centrifugation at 1500 g at 4°C for 10 min. After separation of the plasma, the samples were freeze-stored at −20°C. Similarly to the measurement of serum bile acids, the plasma bile acids were analyzed by GLC and identification was done by GC-MS. The same experiment was repeated using plasma, red blood cells, and white blood cell/platelet suspensions. After the red blood cells were separated from the whole blood, they were washed with physiological saline solution, and then subjected to centrifugation at 1000 g for 20 min. Similar washing was repeated three times, and a 42% Ht physiological saline solution was then prepared. A white blood cell/platelet suspension was prepared (concentration, 1 × 10⁷ white blood cells/ml physiological saline solution) by the discontinuous concentration gradient method, using Ficoll-Hypaque (Histopaque; Sigma) in accordance with the method of English and Anderson. Subsequently, 0.12 ml of 12.5 mM Na-DHCA and 0.08 ml of physiological saline solution were added to 1.0 ml of the plasma, to 1.0 ml of the red blood cell suspension, or to 1.0 ml of the white blood cell/platelet suspension, respectively (giving a final DHCA con-