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Extra-hepatic metabolism of 7-ketocholesterol occurs by esterification to fatty acids via cPLA2α and SOAT1 followed by selective efflux to HDL

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ABSTRACT

Accumulation of 7-ketocholesterol (7KCh) in tissues has been previously associated with various chronic aging diseases. Orally ingested 7KCh is readily metabolized by the liver and does not pose a toxicity threat. However, 7KCh formed in situ, usually associated with lipoprotein deposits, can adversely affect surrounding tissues by causing inflammation and cytotoxicity. In this study we have investigated various mechanisms for extra-hepatic metabolism of 7KCh (e.g. hydroxylation, sulfation) and found only esterification to fatty acids. The esterification of 7KCh to fatty acids involves the combined action of cytosolic phospholipase A2 alpha (cPLA2α) and sterol O-acyltransferase (SOAT1). Inhibition of either one of these enzymes ablates 7KCh-fatty acid ester (7KFAE) formation. The 7KFAEs are not toxic and do not induce inflammatory responses. However, they can be unstable and re-release 7KCh. The higher the degree of unsaturation, the more unstable the 7KFAE (e.g. 18:0 > 18:1 > 18:2 > 18:3 ≫ 20:4). Biochemical inhibition and siRNA knockdown of SOAT1 and cPLA2α ablated the 7KFAE synthesis in cultured ARPE19 cells, but had little effect on the 7KCh-induced inflammatory response. Overexpression of SOAT1 reduced the 7KCh-induced inflammatory response and provided some protection from cell death. This effect is likely due to the increased conversion of 7KCh to 7KFAEs, which reduced the intracellular 7KCh levels. Addition of HDL selectively increased the efflux of 7KFAEs and enhanced the effect of SOAT1 overexpression. Our data suggests an additional function for HDL in aiding extra-hepatic tissues to eliminate 7KCh by returning 7KFAEs to the liver for bile acid formation.

1. Introduction

7-Ketocholesterol (7KCh) is a well-studied oxysterol known for its inflammatory and cytotoxic properties [1]. This oxysterol has been implicated in the pathogenesis of most human age-related chronic diseases [1,2]. It forms by the autooxidation of cholesterol and cholesterol-fatty acid esters (CEs) which are abundantly found in lipoprotein deposits [3]. In the retina, 7KCh has been found associated with lipoprotein deposit in Bruch's membrane and the choriocapillaris [4]. In monkey ocular tissues it was recently reported to accumulate as a process of aging [5]. The levels are particularly pronounced in the retinal pigment epithelium and choriocapillaris (RPE/CH) [5]. In some elderly human RPE/CH tissues and drusen deposits the levels can be comparable to those found in atheromatous plaques [5].

7-KCh is a potent inhibitor of 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMG-CoA reductase), the rate limiting enzyme in cholesterol synthesis, and was briefly investigated as a potential cholesterol lowering drug in rats [6]. This proved short-lived, because the rat liver adapted very quickly and metabolized the orally administered 7KCh into more polar derivatives and bile acids, which were quickly excreted [7]. 7KCh is known to activate hepatic cholesterol 7α-hydroxylase (CYP7A1) [8]. CYP7A1 is the rate-limiting enzyme in bile acid synthesis [9], and its deficiency is known to cause premature atherosclerosis in humans [10]. Other studies by a different group have also demonstrated that dietary 7KCh is very quickly metabolized into bile acids and excreted [8,11]. These investigators concluded that dietary 7KCh is not the source of the 7KCh found in the atherosclerotic plaques [11].

Another enzyme known to metabolize 7KCh is sterol 27-hydroxylase (CYP27A1) [12]. However, *Cyp27A1* −/− null mice had very little difficulty in metabolizing 7KCh into bile acids [13]. Thus, it seems that CYP27A1 is unlikely to be involved in metabolizing dietary 7KCh. Another enzyme that may be involved in the hydroxylation of 7KCh is oxysterol and steroid 7-alpha-hydroxylase (CYP7B1), which is broadly expressed in various tissues [14]. However, disruption of this gene in mice causes no defects in bile acid synthesis [15]. This enzyme seems to be involved in the metabolism of steroids such as androgens and estrogens [16]. This was further confirmed when the CYP7B1 knockout mouse demonstrated sexual development abnormalities [17]. Yet another enzyme reported to be able to metabolize 7KCh is cholesterol sulfotransferase (SULT2B1b) [18]. Another study demonstrated

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that by overexpressing SULT2B1b in 293T cells 7KCh-sulfate was formed [19]. However, SULT2B1b is expressed in very low levels in most tissues, and its main function seems to be in regulating adrenal androgens [20]. Thus it seems that the only enzyme that has been clearly demonstrated to metabolize 7KCh is CYP7A1. Unfortunately, this enzyme is only expressed in the liver [21].

In this study we examined the levels of various enzymes that have either been previously reported and/or could potentially metabolize 7KCh in extra-hepatic tissues. We also analyzed by LCMS various metabolites generated from 7KCh in cultured ARPE19 cells as well as tissues with high 7KCh content, such as the retinal pigment epithelium and choriocapillaris [4]. Based on our results we conclude that the main extra-hepatic metabolic pathway for 7KCh is via esterification to 7KCh-fatty acid esters (7KFAEs), by the combined action of cytosolic phospholipase A2 alpha (cPLA2α, to release membrane fatty acids) and sterol O-acyltransferase (SOAT1, esterification to fatty acids). This is followed by efflux to HDL and presumably returning to the liver for bile acid formation and excretion.

2. Materials and methods

2.1. Materials

Cholesterol (Ch) and 7-ketocholesterol (7KCh) were purchased from Steraloids Inc. (Newport, RI). Hydroxypropyl β-cyclodextrin (HPBCD), cholesteryl-fatty acid esters (CEs) and high density lipoprotein (HDL) were purchased from Sigma-Aldrich (St. Louis, MO). Fatty acids, stearic, oleic, linoleic and linolenic were purchased from Thermo Fisher Scientific Inc. (Waltham, MA). Acetonitrile and methanol were purchased from Fisher Scientific (Fair Lawn, NJ). The SOAT1 selective inhibitor (K-604) was a kind gift from Kowa Company Ltd. (Tokyo, Japan). The cPLA2α inhibitor (Cat# 525143) was purchased from EMD Millipore (Billerica, MA). An affinity-purified rabbit anti-SOAT1 polyclonal antibody was purchased from Cayman Chemicals Co. (Ann Arbor, MI) (Cat# 100028). A polyclonal rabbit anti-GAPDH human antibody was purchased from Invitrogen Corp. (Carlsbad, CA). Total RNA from adult human tissues (retina, lung, placenta, brain, liver, kidney, heart, testis, stomach, spleen, small intestines, prostate, and skeletal muscles) was purchased from BD Biosciences (Mountain View, CA). RNA from human skin was purchased from BioChain (Hayward, CA). Total cellular RNA from cultured human RPE cell lines ARPE19 and D407 cells was isolated using TRIzol reagent (Invitrogen Corp., Carlsbad, CA) and purified with RNeasy mini kit (Qiagen, Valencia, CA).

2.2. cDNA synthesis

Concentrations of total RNA were determined by spectrophotometry (Nanodrop ND-100 Spectrophotometer; Biolab, Melbourne, Vic, Australia). Complementary DNA was synthesized from 2 μg of total RNA previously treated with DNase in a 20 μl reaction, using SuperScript III First-strand Synthesis System kit (Invitrogen Corp., Carlsbad, CA). The cDNA from each preparation was diluted 1:5, and 2 μl of each dilution was used for real-time PCR.

2.3. Copy number determination

Expression of mRNAs from CYP7A1, CYP7B1, CYP27A1, CYP46A1, CYP11A1, CH25H and SULT2B1b was quantified by qRT-PCR using SYBR green in an ABI 7500 instrument (Applied Biosystems Inc., Foster City, CA). To measure copy number, plasmid DNA was used to prepare standards. Each gene was amplified from tissue cDNAs with full-length ORF primers and cloned into pcDNA3.1/CT-GFP expression vector. The DNA concentration is measured by A260 and converted to the number of copies using the molecular weight of the DNA. Copy numbers were determined for each test and genes assayed based upon linear regression equations from standard curve assays. Melting curve analysis was performed to confirm production of a single product in each reaction. PCR reactions were performed two independent times in triplicate each time and validated by analysis of template titration and dissociation curves. Table 1 lists the primer pairs for each of the mRNAs quantified in this study. The changes in gene expression were normalized to the 18S ribosomal RNA.

2.4. Relative quantification for qPCR

Quantitative RT-PCR was performed using Taqman ready-to-use primer sets using an AB instrument (ABI 7500; Applied Biosystems, Foster City, CA) according to the manufacturer's specifications. SOAT1 (Hs00162077_m1), SOAT2 (Hs00186048_m1) and cPLA2 (Hs00233352_ml) were measured and normalized to the housekeeping gene GAPD (ABI4352934E). PCR reactions were performed three independent times in triplicate each time.

2.5. Monkey tissue collection

The monkey eyes and tissues were provided by the Pathology Department of the Division of Veterinary Resources after the completion of various NIH-wide protocols. These protocols were unrelated to this study and the tissues were provided as a courtesy to various NIH researchers. Fresh monkey eyes from 6 year old male Rhesus macaques (*Macacca mulatta*) were enucleated within minutes after euthanasia and the eyes were placed on ice.

2.6. Immunoblotting analyses

Monkey tissues and the cultured human RPE-derived cells, ARPE19 and D407, were homogenized in M-PER solution (Pierce, Rockford, IL) containing the Complete® protease inhibitor cocktail (Roche Diagnostics, Mannheim, Germany). Total protein content was determined by the BCA method (Pierce, Rockford, IL). Protein in the cell extract was precipitated using 10% trichloroacetic acid then collected by centrifugation at 15,000 rpm for 20 min at 4 °C. Protein samples (50 μg protein/lane) were separated in 4–12% NuPAGE Novex Bis–Tris Gels running in 1× NuPAGE MOPS SDS Running Buffer at room temperature for 50 min at 200 V (Invitrogen Corp., Carlsbad, CA). The proteins were transferred using Invitrogen iBlot transfer system. Blots were blocked in 1× TBS, pH 7.4, 5% nonfat milk, and 0.05% Tween-20, for 1 h at room temperature and incubated with rabbit anti-SOAT1 human antibody at 1:1000 dilution or rabbit anti-GAPDH human antibody at 1:2000 dilution overnight at 4 °C. The blot was developed using anti-rabbit IgG HRP conjugated secondary antibody at a dilution of 1:20,000 (KPL, Gaithersburg, MD) and imaged on X-ray film using SuperSignal West Pico Chemiluminescent Substrate (Pierce, Rockford, IL).

2.7. Preparation of water soluble 7KCh solution

A 10 mM stock solution was prepared by dissolving the appropriate amount of 7KCh in 1× PBS containing 45% w/v hydroxypropyl-β-cyclodextrin (HPBCD). Briefly, an appropriate amount of 7KCh was dissolved in ethanol and placed in a volumetric flask. The solution of 45% HPBCD is added to 60–70% of the total volume of the flask and sonicated at 45 °C for 1 h. The remainder of the 45% HPBCD is added to the volume of the flask. A 1 mM working solution was prepared by diluting the stock solution ten-fold in 1× PBS.

2.8. Cell culture

ARPE19 cells were obtained from the American Type Culture Collection (Manassas, VA) at passage 22 and grown in DMEM/F12 (1:1, by vol., Mediatech Inc., Herndon, VA) medium containing 10% fetal bovine serum (FBS), 2 mM glutamine, 100 IU/ml penicillin, and 100 μg/ml