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Inhibition of soluble epoxide hydrolase in mice promotes reverse cholesterol transport and regression of atherosclerosis

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ABSTRACT

Adipose tissue is the body largest free cholesterol reservoir and abundantly expresses ATP binding cassette transporter A1 (ABCA1), which maintains plasma high-density lipoprotein (HDL) levels. HDLs have a protective role in atherosclerosis by mediating reverse cholesterol transport (RCT). Soluble epoxide hydrolase (sEH) is a cytosolic enzyme whose inhibition has various beneficial effects on cardiovascular disease. The sEH is highly expressed in adipocytes, and it converts epoxyeicosatrienoic acids (EETs) into less bioactive dihydroxyeicosatrienoic acids. We previously showed that increasing EETs levels with a sEH inhibitor (sEHI) (*t*-AUCB) resulted in elevated ABCA1 expression and promoted ABCA1-mediated cholesterol efflux from 3T3-L1 adipocytes. The present study investigates the impacts of *t*-AUCB in mice deficient for the low density lipoprotein (LDL) receptor ($Ldlr^{-/-}$ mice) with established atherosclerotic plaques. The sEH inhibitor delivered *in vivo* for 4 weeks decreased the activity of sEH in adipose tissue, enhanced ABCA1 expression and cholesterol efflux from adipose depots, and consequently increased HDL levels. Furthermore, *t*-AUCB enhanced RCT to the plasma, liver, bile and feces. It also showed the reduction of plasma LDL-C levels. Consistently, *t*-AUCB–treated mice showed reductions in the size of atherosclerotic plaques. These studies establish that raising adipose ABCA1 expression, cholesterol efflux, and plasma HDL levels with *t*-AUCB treatment promotes RCT, decreasing LDL-C and atherosclerosis regression, suggesting that sEH inhibition may be a promising strategy to treat atherosclerotic vascular disease.

1. Introduction

Plasma high density lipoprotein cholesterol (HDL-C) levels bear a strong reverse relationship with atherosclerotic cardiovascular disease risk [1]. HDL plays a key role in reverse cholesterol transport (RCT) by promoting cholesterol efflux from peripheral cells, and delivering the cholesterol to the liver for excretion, a process that is believed to be atheroprotective [2]. Many studies strongly suggest that HDL-raising strategies may be effective therapy for the treatment of atherosclerosis [3–5]. However, our understanding of the mechanisms that contribute to HDL regulation remain largely incomplete. ATP-binding cassette transporter A1 (ABCA1) is an essential membrane protein for the initial step of HDL biogenesis by facilitating the efflux of cellular free cholesterol to extracellular lipid-free apolipoprotein A-I (apoA-I), forming nascent HDL particles [6]. The critical role of ABCA1 in HDL metabolism was first identified as the gene mutated in Tangier disease [7], which is characterized by markedly low levels of circulating HDL-C [6]. ABCA1 is highly expressed in several tissues [8]. The expression of ABCA1 in liver, adipose tissue and intestine are reported to contribute plasma HDL-C pool [9,10]. The liver is considered the

Abbreviations: ABCA1, ATP binding cassette transporter A1; HDL, high-density lipoprotein; RCT, reverse cholesterol transport; sEH, soluble epoxide hydrolase; EETs, epoxyeicosatrienoic acids; DHETs, dihydroxyeicosatrienoic acids; sEHI, sEH inhibitors; ATD, atherogenic diet; *t*-AUCB, *trans*-4-[4-(3-adamantan-1-yl-ureido)-cyclohexyloxy]-benzoic acid; SCD, standard chow diet; acLDL, acetylated LDL; LSC, liquid scintillation counting.

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principal organ for cholesterol biosynthesis and catabolism through bile acid production. However, in obese individuals, adipose tissues are the largest cholesterol reservoir [11]. Moreover, the obesity state is associated with lower plasma HDL-C, which may be attributable to the impaired ability of adipocytes to efflux cholesterol to HDL [1]. Thus, in obese persons, ABCA1 role in HDL regulation is more important in adipose tissue than in liver and intestine. In 2010, Zhang et al. [12] presented the first evidence that adipocytes transfer of cholesterol to HDL *in vitro* is mediated by ABCA1. Furthermore, Chung et al. [13] findings suggested a role for adipose ABCA1 in contributing to plasma HDL-C, particularly in the obese state. Lack of adipose ABCA1 impaired cholesterol efflux to apoA-I, and reduced circulating HDL-C. Therefore, promoting adipose ABCA1-mediated cholesterol efflux should raise HDL-C levels, and thus have positive effects on reducing atherosclerosis.

The expressions of ABCA1 in adipose tissue is upregulated in states of cholesterol excess by transcriptional and post-transcriptional mechanisms [14]. The enzyme soluble epoxide hydrolase (sEH), which metabolizes endogenous epoxyeicosatrienoic acid (EETs), is abundantly expressed in adipose tissue, and its expression and activity increase with obesity [15]. Recently, our group reported that inhibition of endogenous sEH in adipocytes by a sEH inhibitor (*t*-AUCB) results in an increase in ABCA1 mRNA and protein expression, indicating a possible relevant role for adipose sEH in regulating ABCA1 [16]. Separately, sEHI were found to reduce the progression of atherosclerotic disease in apoE$^{-/-}$ mice [17,18]. These data suggest that inhibition of sEH may be an attractive therapeutic target for the treatment of cardiovascular disease; however, many questions remain to be addressed, in particular regarding the effect of sEH inhibition on the functionality of produced HDL and its ability to promote RCT.

In the current study, we tested the impact of sEH inhibition on RCT and atherosclerosis in the hypercholesterolemic and obese mouse model deficient for the LDL receptor (Ldlr$^{-/-}$ mice). Atherosclerosis was first established in Ldlr$^{-/-}$ mice by feeding them an atherogenic diet (ATD), after which they were treated with an sEHI (*t*-AUCB) for 4 weeks. We demonstrated herein that sEH expression is increased in the adipose tissue of mice fed ATD. Importantly, inhibition of sEH in mice effectively decreases plasma LDL-C levels, increases ABCA1 expression in adipose tissue and effectively raises circulating HDL. Furthermore, inhibition of sEH expression in adipose tissue effectively increases RCT and decreases the size of atherosclerotic plaque. These data establish that inhibition of endogenous sEH therapeutically reduces plasma LDL-C, increases ABCA1 expression in adipose tissue, raises plasma HDL, enhances RCT and induces regression of atherosclerosis.

2. Methods

2.1. Drug delivery

The potent and selective sEHI *trans*-4-[4-(3-adamantan-1-yl-ureido)-cyclohexyloxy]-benzoic acid (*t*-AUCB) was synthesized by Dr. Sung Hee Hwang from the laboratory of Pr. Bruce D Hammock [19]. The structure and physical properties are shown in online Data Supplement S1 and Fig. 1. For mice treatment, 0.5 mg *t*-AUCB was added to 1.0 mL PEG400 and ultrasonicated until the suspension became a completely clear liquid. The stock solution (500 mg/L) was diluted to concentrations of 50, 15 and 5 mg/L. Compared with earlier sEHI, such as AUDA, *t*-AUCB has improved water solubility and high oral availability such that can it be delivered in drinking water. The water solubility of *t*-AUCB is 160 mg/L.

2.2. Mice

Animal experiments were approved by the Institutional Animal Care and Use Committee of the Second Xiangya Hospital of Central South University, China. Forty-two 8-week-old male Ldlr$^{-/-}$ mice (C57BL/6 background) were weaned at 4 weeks of age and placed on an ATD that containing 21% fat and 0.15% cholesterol for 14 weeks, at which point mice were either sacrificed (baseline) (n = 4) or switched to standard chow diet (SCD) for 4 weeks. Coincident with the switch to SCD (n = 6), mice were randomized into 4 groups (n = 8): no treatment (vehicle only) and *t*-AUCB 5, 15 or 50 mg/L. The mice were observed to drink approximately 3–4 mL water per day, which was consistent with published studies [18], indicate this procedure gives a dose of approximately 0.5–5 mg *t*-AUCB per kg per day, clearly with high levels of intake at night. Each mouse was housed in a separate cage in order to monitor the daily water and drug intake.

2.3. Measurement of the concentrations of sEHI in the plasma

Mice were sacrificed after 4 weeks of treatment, and then the blood concentrations of *t*-AUCB were measured as the method, which has previously been described [20]. At the end of experiments, 10 μL of whole blood was placed into 50 μL distilled water containing 10 μL of propane 1, 2-diol and EDTA, and then mixed followed by addition of 200 μL of ethyl acetate. Similarly, added 50 μL internal standard and shook again. The extracted samples were analyzed by liquid chromatography coupled with mass spectrometry [18].

2.4. sEH activity assay

Measurement of sEH activity has previously been described [21,22]. Briefly, epididymal adipose tissue depots were homogenized in PMSF, and cytosolic supernatants were obtained by centrifugation. In brief, 100 μL of supernatants were incubated with or without 20 μM *t*-AUCB at 30 °C for 10 min in 96-well black assay plates, and 80 mM epoxy fluor 7 in 100 μL reaction buffer (50 mM Tris–HCl buffer containing 0.2 mg/mL BSA, pH 7.0) was added to each well. After incubation at 30 °C for 30 min, fluorescence was determined by use of a Spectra Fluor Plus Xuorescent plate reader (Tecan Systems, San Jose, CA, USA) with excitation wavelength 330 nm and 465 nm at 30 °C. sEH activity was the corrected sample fluorescence intensity calculated by sample fluorescence values for the sEHI subtracted from those of the non-sEHI.

2.5. Plasma cholesterol determination and fast protein liquid chromatography

Plasma was collected either by tail or retro-orbital plexus bleeding of mice fasted for 4 h. Plasma total cholesterol (TC), low density lipoprotein cholesterol (LDL-C) and HDL-C levels were measured by enzymatic method (bioMerieux, Lyon, France) using an automated analyzer (Type 7170A, Hitachi). For fast protein liquid chromatography analysis, 300 μL pooled plasma (n = 4 mice total) was separated on a Superose-6 TM column (Amersham Biosciences) at a flow rate of 0.4 mL/min as described previously [23].

2.6. Adipose cholesterol determination

Lipid extracts of liver tissue were assayed for cholesterol according to the manufacturer's protocol using the Cholesterol Quantification Kit (BioVision, Mountain View, CA). Data were normalized to tissue wet weight or protein, measured by the Lowry assay.