Original Article

MOLECULAR METABOLISM

Intestinal CREBH overexpression prevents high-cholesterol diet-induced hypercholesterolemia by reducing *Npc1l1* expression

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ABSTRACT

Objective: The transcription factor cyclic AMP-responsive element-binding protein H (CREBH, encoded by *Creb3l3*) is highly expressed in the liver and small intestine. Hepatic CREBH contributes to glucose and triglyceride metabolism by regulating fibroblast growth factor 21 (*Fgf21*) expression. However, the intestinal CREBH function remains unknown.

Methods: To investigate the influence of intestinal CREBH on cholesterol metabolism, we compared plasma, bile, fecal, and tissue cholesterol levels between wild-type (WT) mice and mice overexpressing active human CREBH mainly in the small intestine (CREBH Tg mice) under different dietary conditions.

Results: Plasma cholesterol, hepatic lipid, and cholesterol crystal formation in the gallbladder were lower in CREBH Tg mice fed a lithogenic diet (LD) than in LD-fed WTs, while fecal cholesterol output was higher in the former. These results suggest that intestinal CREBH overexpression suppresses cholesterol absorption, leading to reduced plasma cholesterol, limited hepatic supply, and greater excretion. The expression of Niemann—Pick C1-like 1 (*Npc1l1*), a rate-limiting transporter mediating intestinal cholesterol absorption, was reduced in the small intestine of CREBH Tg mice. Adenosine triphosphate-binding cassette transporter A1 (*Abca1*), *Abcg5/8*, and scavenger receptor class B, member 1 (*Srb1*) expression levels were also reduced in CREBH Tg mice. Promoter assays revealed that CREBH directly regulates *Npc1l1* expression. Conversely, CREBH null mice exhibited higher intestinal *Npc1l1* expression, elevated plasma and hepatic cholesterol, and lower fecal output.

Conclusion: Intestinal CREBH regulates dietary cholesterol flow from the small intestine by controlling the expression of multiple intestinal transporters. We propose that intestinal CREBH could be a therapeutic target for hypercholesterolemia.

Keywords CREBH; *Npc1l1*; Cholesterol; Intestine

1. INTRODUCTION

Cholesterol absorption in the gut has been studied extensively because of its significant positive correlation with plasma cholesterol concentration, which, in turn, is a major risk factor for atherosclerosis [1,2]. Cholesterol absorption depends on transport from the intestinal lumen across enterocytes into the plasma [3]. Stimulation of reverse cholesterol transport (RCT), which disposes endogenous cholesterol through feces, can inhibit the development of atherosclerosis. Although the hepato-biliary system is considered the dominant route for RCT, it

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Abbreviations: Abca1, ATP-binding cassette, sub-family A1; ABCG5/8, adenosine triphosphate-binding cassette transporter G5/G8; ALT, alanine aminotransferase; Apoa4, apolipoprotein A-IV; AST, aspartate aminotransferase; CREBH, cyclic AMP-responsive element-binding protein H; Cpt1a, carnitine palmitoyltransferase 1a, liver; Cyp7a1, cytochrome P450, family 7, subfamily a, polypeptide 1; ER, endoplasmic reticulum; FGF21, fibroblast growth factor 21; FXR, Farnesoid X receptor; LD, lithogenic diet; LPL, lipoprotein lipase; LXR, liver X receptor; NEFA, non-esterified fatty acids; NPC1L1, Nieman Pick C1-like 1; PPARα, proliferator activated receptor alpha; RCT, reverse cholesterol transport; Shp, small heterodimer partner; Srb1, scavenger receptor class B, member 1; Srebf, sterol regulatory element-binding factor; SREBP, sterol regulatory element-binding protein; TG, triglyceride; WT, wild type

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has been demonstrated that the small intestine also excretes large amounts of endogenous cholesterol [4,5]. Cholesterol uptake from the lumen by enterocytes is the rate-limiting step in cholesterol absorption [6], and NPC1L1 plays a pivotal role in this process [7,8]. NPC1L1 is exclusively expressed in enterocytes of the proximal small intestine [8]. The clinical anti-hypercholesterolemia drug ezetimibe (Zetia) lowers plasma cholesterol levels by inhibiting NPC1L1 [8,9]. Ezetimibe treatment in mice increased fecal neutral sterol excretion without altering hepato-biliary cholesterol disposal into the bile [10]. ATP binding cassette subfamily G isoforms G5 and G8 are expressed in both the liver and small intestine where they heterodimerize into a functional transporter [11] to promote sterol secretion, with a preference for plant sterols over cholesterol [12]. In the liver, they are expressed on the apical membrane of hepatocytes [13] and secrete both cholesterol and plant sterols into the bile [14,15]. In the small intestine, they are presumed to be expressed on the apical brush border membrane of enterocytes and secrete cholesterol and plant sterols into the intestinal lumen [16].

CREBH is a basic leucine zipper domain transcriptional factor of the CREB/activating transcription factor family [17]. *CrebH* is highly and selectively expressed in gastrointestinal tract tissues, including the liver, pyloric stomach, duodenum, and ileum [18]. Hepatic *CrebH* mRNA expression is regulated by fasting and re-feeding, with nuclear levels of active CREBH increasing in times of starvation [19]. Translated CREBH protein localizes to the endoplasmic reticulum (ER) before transfer to the Golgi apparatus, where the transcriptionally active N-terminal region is cleaved prior to translocation to the nucleus [19]. CREBH and peroxisome proliferator activated receptor alpha (PPARα) synergistically activate hepatic fibroblast growth factor 21 (*Fgf21*) expression and exert effects on energy metabolism through the modulation of plasma FGF21 levels [20,21]. CREBH reduces plasma triglyceride (TG) levels by increasing hepatic gene expression of apolipoproteins such as apolipoprotein A-IV (*Apoa4*), *Apoa5*, and *Apoc2*. These apolipoproteins activate plasma lipoprotein lipase (LPL), resulting in reduced plasma TG levels. However, the function of intestinal CREBH remains unclear.

To investigate the functions of intestinal CREBH, we generated CREBH Tg mice and examined effects of CREBH overexpression on cholesterol metabolism.

2. MATERIALS AND METHODS

2.1. Animals and diets

Wild-type (WT) C57/BL6J mice were obtained from CLEA Japan. To generate CREBH Tg mice, cDNAs encoding the rat *Pck1* promoter, active human CREBH (amino acids 1−320), and the 3′ polyadenylation signal of human growth hormone were microinjected into C57BL6J eggs [21]. *Creb3l3*$^{tm1.1Sad}$*/J* (CREBH null) mice [18] were purchased from The Jackson Laboratory (Bar Harbor, ME, USA). All mice were housed in a pathogen-free barrier facility under a 12-h light/dark cycle and given free access to water. Mice were fed a normal chow diet (MF: Oriental Yeast, Tokyo, Japan) or a lithogenic diet (LD) (16.5% fat, 1.25% cholesterol, 0.5% cholic acid (CA); F2HFD1; Oriental Yeast) (F2HFD1: Oriental Yeast, Tokyo, Japan) [22] for 2 weeks. All animal husbandry and experimental protocols conformed to the University of Tsukuba Regulations of Animal Experiments and were approved by the Animal Experiment Committee of the University of Tsukuba.

2.2. RNA purification and real time-PCR

Total RNA was extracted from frozen mouse tissues using Sepasol (Nakarai Tesque, Kyoto, Japan). For the analysis of jejunal gene expressions, the first 15% length from the pyloric sphincter to the rectum was used. Total RNA was reverse transcribed using the PrimeScript RT Master kit (Takara, Bio Inc., Shiga, Japan). Real-time PCR was performed using the ABI Prism 7300 System with LightCycler-DNA Master SYBR Green I Mix (Roche Diagnostics Ltd, Lewes, UK). mRNA expression was normalized to *cyclophilin* mRNA content and expressed as fold change compared to control mice using the ΔΔCT method.

2.3. Immunoblotting

Immunoblotting of whole cell lysates was performed as described previously [23]. Protein expression levels of NPC1L1 were analyzed in small intestine lysates using a rabbit anti-NPC1L1 antibody (Novus Biologicals cat. NB400-127).

2.4. Histological analysis

The liver was fixed, embedded in paraffin, sectioned, and stained with hematoxylin and eosin (H&E).

2.5. Cell culture, transfection, and luciferase assay

Caco-2 cells were maintained in DMEM supplemented with 10% fetal bovine serum (FBS) and penicillin-streptomycin at 37 °C in a humidified 5% CO_2/95% air environment. Mouse *Npc1l1* promoter-luciferase constructs and expression vectors for CREBH and/or SREBP-2 were co-transfected into Caco-2 cells using lipofectamine 3000 (Invitrogen, Grand Island, NY, USA). A renilla expression construct was co-transfected as an internal control for transfection efficiency. At 48 h after transfection, luciferase and renilla activities were measured using commercial assay systems (Luciferase: PicaGene, Toyo-Inki, Tokyo, Japan; Renilla: Promega, Madison, WI, USA). The promoter activity was expressed as the ratio of luciferase to renilla activities in each sample.

2.6. Electrophoretic mobility shift assay

We generated the HA-tagged active form of CREBH from an expression vector using an *in vitro* reticulocyte transcription−translation system (Promega). We used the following sequences in the electrophoretic mobility shift assays (EMSAs): 5′-ggaagttgacctcagaaggaggagatggaatggca-3′ for −106 to −69 of the *Npc1l1* promoter; 5′-ggcaccatctgatgtaagggaagagaaaataaattattaa-3′ for −75 to −33 of the *Npc1l1* promoter; 5′-gagaaaataaattattaaccagtacgg-3′ for −53 to −23 of the *Npc1l1* promoter; and 5′-gtacggcccagtcctattggccccatgacagacgagg-3′ for −32 to +5 of the *Npc1l1* promoter. We incubated the *in vitro*-translated protein lysate and anti-HA antibodies (12CA5, Santa Cruz) in a reaction mixture as previously described [23] and resolved the DNA−protein complexes on a 4% polyacrylamide gel.

2.7. Plasma, hepatic, and gallbladder bile lipid and gallstone analysis

Plasma and liver parameters [23], hepatic bile acids [22], and gallbladder bile [22] were analyzed as described previously. Gallbladders bile was examined for monohydrate cholesterol crystals under polarizing light microscopy.

2.8. Fecal cholesterol and bile acids output

Fecal cholesterol and bile acid outputs were measured as described [24,25]. Briefly, after 2 weeks of normal or LD feeding, mice were individually housed for fecal collection. The feces were dried, weighed, and crushed into powder. Fecal bile acids were extracted from powdered feces with 90% ethanol [24] and concentrations determined

