CHAPTER 6

Perturbed lysosomal architecture and localization in liver-specific WASH-deficient mice does not affect hepatic cholesterol and bile acid metabolism

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Abstract

The architecture and localization of the endo-lysosomal system are highly dependent on the WASH complex, a pentameric protein complex which promotes the formation of branched actin patches on endosomes. These actin deposits form endosomal subdomains and are requisite for the maintenance of the endo-lysosomal structure. The endo-lysosomal system is indispensable for intracellular cholesterol transport. Cholesterol is transported from the endo-lysosomal systems to other organelles, such as the plasma membrane, the endoplasmic reticulum and the Golgi apparatus. This process is facilitated mainly via membrane contact sites, disruption of which disturbs cellular cholesterol homeostasis. Here, we have studied how perturbation of the endo-lysosomal network by WASH depletion affects hepatic cholesterol homeostasis, by quantifying pathways of cholesterol and bile acid metabolism. We show that WASH depletion results in impaired transactivation of liver X receptor (LXR) target genes. Nevertheless, we found no alterations in cholesterol and bile acid excretion, or in hepatic cholesterol synthesis and lipogenesis. Overall, our data suggest that under the conditions studied the perturbed lysosomal architecture and localization upon WASH ablation do not result in abnormal hepatic cholesterol and bile acid metabolism.

Keywords: endosome, liver, WASH complex, cholesterol metabolism, bile acid metabolism
Introduction

Cholesterol is a crucial component in eukaryotic cell membranes, and is the precursor for the biosynthesis of steroid hormones, bile acids and vitamin D. However, excess cellular cholesterol contributes to the development of multiple diseases, including atherosclerosis and non-alcoholic steatohepatitis (1, 2). In mammals, the liver plays an important role in the maintenance of whole-body cholesterol homeostasis. Essential in this process are hepatocytes, as these cells take up and secrete cholesterol-containing lipoproteins from and into the circulation, contributing massively to whole-body synthesis. Additionally, hepatocytes are also able to remove cholesterol from the body via secretion into bile, either as such or after conversion into bile acids (3). To maintain cellular cholesterol homeostasis, the uptake, biosynthesis and excretion of cholesterol are tightly controlled. These pathways are regulated by the transcription factors liver X receptor (LXR) and the sterol regulatory-element binding proteins (SREBPs). Low intracellular cholesterol activates SREBP-2, which initiates cholesterol uptake and biosynthesis (4-6), whereas high intracellular cholesterol promotes LXR-mediated transcription of genes involved in high-density lipoprotein (HDL) efflux (7, 8), biliary cholesterol excretion (9, 10), and lipogenesis to provide fatty acids for cholesterol esterification (10-12). In mice, LXR also activates Cyp7a1 expression, the gene encoding for the rate-limiting enzyme for cholesterol conversion into bile acids (13, 14). As the cholesterol content of the endoplasmic reticulum (ER) is very low compared to other organelles, it functions as a sensitive cholesterol sensor, allowing the cell to respond to small changes in intracellular cholesterol content (15).

The hepatocyte acquires cholesterol by uptake of low-density lipoproteins (LDL), primarily via the LDL receptor (LDLR). The expression of LDLR is tightly regulated by SREBP-2 (16). Cholesterol esters derived from LDL are hydrolyzed in the late endosomes/lysosomes (LE/LY), and subsequently distributed to other organelles such as the ER, the plasma membrane, the mitochondria and the Golgi apparatus (17-20). Cholesterol transport is dependent on membrane contact sites (MCSs); membrane protein-protein interactions of two organelles in close proximity which enable sterol transfer proteins to shuttle hydrophobic cholesterol between membranes (15, 21-23).

The architecture of LE/LY relies on the pentameric Wiskott-Aldrich syndrome protein and SCAR homolog (WASH) complex (WASHC1-WASHC5) (Chapter 5, 24, 25). WASH activates Arp2/3, a nucleating factor that deposits branched actin on endosomes to form endosomal subdomains. These actin deposits are essential for endosomal structure and fission (26).
Studies in MEFs show that depletion of WASH results in altered morphology of the endo-lysosomal system, including clusters, vacuole-like structures, and short actin-free sorting tubules (26-29). Recently, we showed that WASH is also essential for the endo-lysosomal architecture in mouse hepatocytes; ablation of hepatic WASH led to smaller, highly tubulated lysosomes, which clustered at the perinuclear region (Chapter 5, and data not shown). Furthermore, cell surface levels of the lipoprotein receptors LDLR, LDLR-related proteins 1 (LRP1), and scavenger receptor class B type 1 (SR-BI) were reduced upon WASH ablation, resulting in increased plasma levels of LDL-C and HDL-C (Chapter 5).

Here, we studied the expression of LXR and SREBP-2 target genes and subsequently, quantified the relevant pathways in hepatic cholesterol and bile acid metabolism, to investigate whether the perturbed architecture of the endo-lysosomal network in WASH-deficient livers affects hepatic cholesterol homeostasis.

**Results**

**LXR target gene expression is reduced upon hepatic WASH deficiency**

To assess the effect of perturbed LE/LY architecture in WASH-deficient hepatocytes on cholesterol and bile acid metabolism, we first determined the hepatic expression of LXR and SREBP-2 target genes in liver-specific Washc1 knockout mice (Washc1ΔHep) and wild type littermate controls (hereafter referred to as WT) on a low cholesterol chow diet. Hepatic WASH deficiency resulted in decreased expression of multiple LXR target genes: the cholesterol efflux genes Abcg5 and Abcg8 (Fig. 1A), and the lipogenic regulators Scd1 and Fas1 (Fig. 1B). Washc1 depletion also reduced the mRNA levels of Cyp7a1, but this decrease did not reach statistical significance (p=0.11) (Fig. 1C). Hepatic WASH deficiency did not result in significant alteration in the expression of SREBP-2 target gene Hmgcr (Fig. 1D).

We proceeded to evaluate whether this decrease in LXR target gene expression was due to reduced LXR activity by feeding Washc1ΔHep and WT mice a high-fat high-cholesterol diet (HFC, 45% calories from butterfat, 0.2% cholesterol), as cholesterol feeding activates LXR as a result of oxysterol production, which activates LXR. Diet-induced LXR activation restored the gene expression of Abcg5 and Abcg8 (Fig. 1E), as well as of Scd1 and Fas1 (Fig. 1F). Interestingly, HFC feeding resulted in increased mRNA levels of Cyp7a1 in Washc1ΔHep livers compared to WT livers (Fig. 1G). Hmgcr expression was reduced in the livers of HFC-fed WT mice, but increased in the livers of HFC-fed Washc1ΔHep mice compared with livers of chow-fed WT mice (Fig. 1H).
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Altogether these data imply that hepatic WASH deficiency leads to reduced LXR transactivation, which can be restored by feeding mice a diet supplemented with cholesterol. SREBP-2 activity is not affected by WASH deficiency in chow-fed conditions, but might be increased when exposed to a high influx of dietary cholesterol.

**Reduced hepatic LXR target gene expression in Washc1ΔHep mice affects neither cholesterol catabolism nor cholesterol excretion**

Next, we assessed whether the decreased expression of LXR target genes resulted in altered excretion of bile acids and cholesterol. Cyp7a1 catalyzes the first and rate-limiting step in the conversion of cholesterol into bile acids in mice (30); however, decreased Cyp7a1 expression in Washc1 depleted livers did not translate into changes in bile flow, plasma bile acid concentration, bile acid secretion, or fecal bile acid excretion; factors which represents

Figure 1. Washc1 deficiency decreases LXR target gene expression. Hepatic mRNA expression of genes involved in cholesterol excretion, fatty acid synthesis, cholesterol catabolism and cholesterol synthesis in WT and Washc1ΔHep mice on chow (A-D) and after HFC feeding (E-H). n=6-8. Data presented as mean ± SEM, *p < 0.05, **p < 0.005, ***p < 0.001 as determined by Student’s T-test.
hepatic bile acid synthesis under steady state conditions (Fig. 2A-D).

ABCG5/ABCG8 dimers constitute the main transporter for biliary cholesterol excretion (31), but the reduced Abcg5/g8 expression after hepatic WASH depletion did not result in reduced biliary cholesterol or fecal cholesterol output (Fig. 2E,F). Overall, these data suggest that even though LXR target gene expression in hepatic WASHC1-deficient mice is reduced, bile acid and cholesterol secretion rates are not affected.

**Bile acid secretion is increased after LXR stimulation in Washc1ΔHep mice**

As HFC feeding increased Cyp7a1 expression in WASH-depleted livers, we determined whether this translated into increased hepatic cholesterol catabolism. Bile flow after HFC feeding was similar between WT and Washc1ΔHep mice (Fig. 3A), and we indeed observed an increase in bile acid secretion in Washc1ΔHep mice (Fig. 3B). However, this increase in bile acid secretion did not lead to altered fecal bile acid excretion or plasma bile acid concentrations (Fig. 3C, D). Furthermore, we observed no differences in biliary cholesterol and fecal neutral
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Hepatic WASH deficiency does not affect lipogenesis

To determine whether the decreased expression of LXR target genes Fasn and Scd1 in Washc1ΔHep mice resulted in reduced lipogenesis, we added 13C-acetate to the drinking water of the mice and measured the label distribution pattern of palmitate (C16:0), palmitoleate (C16:1), stearate (C18:0) and oleate (C18:1). Despite reduced mRNA expression of fatty acid synthesis genes in Washc1ΔHep livers, lipogenesis between Washc1ΔHep and WT mice did not change, either in chow- or in HFC-fed mice (Fig. 4A, B).

The WASH complex is not involved in regulation of cholesterol production

Increased hepatic expression of Hmgcr in Washc1ΔHep mice after HFC feeding is expected to translate into an increase in cholesterol biosynthesis. To test this, we added 13C-acetate to

Figure 3. WASH deficiency increases biliary bile acid secretion but not fecal bile acid excretion after HFC feeding. (A) Bile flow in WT and Washc1ΔHep mice. Bile acid excretion (B) and concentration in feces (C) and plasma (D). Biliary cholesterol (E) and fecal neutral sterols (F) in WT and Washc1ΔHep mice on HFC diet. n=5-7. Data presented as mean ± SEM; statistical significance determined by Student’s T-test.

sterol excretion (Fig. 3E, F). Together these data imply that although hepatic WASH deficiency increases bile acid secretion, fecal bile acid and cholesterol excretion remain unaltered after LXR stimulation by HFC feeding.
the drinking water of the mice for three consecutive days. We collected bloodspots twice a day, and determined $^{13}$C-incorporation in cholesterol. Although Hmgcr expression was increased in HFC-fed Washc1ΔHep mice, total cholesterol synthesis did not differ between Washc1ΔHep and WT mice on a chow diet (Fig. 5A). As expected, HFC feeding reduced cholesterol synthesis in both groups, but did not result in statistically significant differences in cholesterol synthesis between Washc1ΔHep and WT mice (Fig. 5B).

**Discussion**

The WASH complex is a multi-protein complex of the endosomal sorting machinery, and facilitates endosomal recycling of integral membrane proteins (32). We recently found that hepatic defects in the WASH complex lead to impaired recycling of lipoprotein receptors LDLR, LRP1 and SR-B1, resulting in decreased hepatic uptake of LDL-C and HDL-C and ultimately in hypercholesterolemia (Chapter 5, (33)). In Chapter 5 we observed that hepatic WASH is also essential for the architecture of the endo-lysosomal network; loss of hepatic WASH resulted in smaller lysosomes, increased lysosomal tubulation and increased perinuclear localization of lysosomes (Chapter 5 and unpublished data). The localization of LE/LY is controlled by the intracellular cholesterol content; high intracellular cholesterol levels lead to perinuclear localization of LE/LY, enabling the formation of LE/LY-ER MCSs for cholesterol transport and hence the ability of the cell to respond to changes in cholesterol content (22, 34). The importance of lysosomal mobility and structure in maintaining cellular cholesterol homeostasis led us to hypothesize that the perturbed lysosomal architecture and localization in WASH-deficient hepatocytes might result in aberrant hepatic cholesterol
WASH-deficiency does not affect hepatic cholesterol and bile acid metabolism. Our hypothesis was supported by reduced LXR target gene expression after hepatic WASH depletion, which could be corrected by LXR stimulation via HFC feeding. However, detailed characterization of hepatic WASH depleted mice did not substantiate disturbed hepato-cellular cholesterol homeostasis, as no differences were observed in lipogenesis, bile acid secretion or fecal bile acid and cholesterol excretion under the tested conditions.

Interestingly, expression of SREBP-2 target gene Hmgcr was increased in Washc1ΔHep mice after HFC feeding. As SREBP-2 is activated by low ER cholesterol levels, this might suggest that upon WASH ablation under HFC feeding conditions cholesterol transport from the lysosomes to the ER is impaired, which might ultimately lead to increased SREBP-2–mediated Hmgcr expression. However, this increased Hmgcr expression did not translate into a significant increase in cholesterol synthesis. Although the liver is the major site of cholesterol production (35) we cannot rule out that other organs may compensate for aberrant hepatic cholesterol production that might mask the phenotype of hepatic WASH-deficient mice.

Intracellular cholesterol transport to the ER can occur via vesicular or non-vesicular trafficking systems, and it has been speculated that in non-vesicular systems MCSs play a major role in this transport (36). LE/LY-ER cholesterol transport can occur via two different routes. LDL-derived cholesterol can be trafficked from LE/LY to the ER via direct LE/LY-ER membrane interactions, or indirectly after cholesterol is first distributed to the plasma membrane (19).

Our data imply that SREBP-2-mediated cholesterol biosynthesis is not affected in WASH-

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**Figure 5. Hepatic Washc1 depletion does not affect cholesterol production.** Cholesterol production on chow (A) and after HFC diet feeding (B) in Washc1ΔHep and WT mice after three days of 13C-acetate supplementation, as measured by MIDA. n=5-7. Data presented as mean ± SEM.
deficient hepatocytes, which suggests either that LE/LY-ER cholesterol trafficking is still intact upon WASH ablation, or that cholesterol transport to the ER is only partially dependent on LE/LY-ER interaction. This is in accordance with previous studies showing that only 30% of LDL-derived cholesterol is transported directly from the LE/LY to the ER (17, 18). A more recent study even suggested that all LDL-derived cholesterol is moved first from LE/LY to the plasma membrane before being transported to the ER (20). These results imply that despite perturbed lysosomal architecture and localization in WASH-deficient hepatocytes, LDL-derived cholesterol is still adequately transferred to the ER, likely via the plasma membrane or vesicular transport.

The WASH subunit WASHC2 colocalizes to ER tubules (37), and a recent study indicated that endosomal-ER contact sites are linked to WASH-dependent endosomal fission events, although direct evidence of a role for the WASH complex in the formation of LE/LY-ER MCSs is still lacking. Yet, this study indicates that post-lysosomal cholesterol transport relies neither on WASH-dependent lysosomal architecture and localization nor on endosomal-ER contact sites.

Altogether, our data suggest that under the conditions studied the atypical lysosomal structure and localization in WASH ablated hepatocytes does not affect hepatic cholesterol homeostasis (Fig. 6). It may well be that more significant conditions, such as overshooting cholesterol synthesis by applying a fasting/refeeding protocol using a high carbohydrate/low fat diet to induce SREBP activities, are required to unmask a phenotype (38).

**Material and Methods**

**Animals**

All animal studies were approved by the Institutional Animal Care and Use Committee,
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University of Groningen (Groningen, the Netherlands). Liver-specific Washc1 knockout mice, were generated as previously described (Chapter 5). All experiments were performed with male mice (n=8-9). Mice were housed individually and fed ad libitum with a standard rodent chow diet (RMH-B, AB Diets, the Netherlands) or a high fat high cholesterol diet (45% calories from butterfat, containing 0.2% cholesterol (SAFE Diets)). Littermates were used as wild type (WT) controls in all experiments. To assess cholesterol synthesis, 2% $^{13}$C-acetate was added ad libitum to the drinking water for 72 h. $^{13}$C-acetate supplementation started at 9 AM, and bloodspots were taken at time points 0, 10, 24, 32, 48, 56, and 72 h. Feces were collected during 24 h prior to termination. Mice were anesthetized by intraperitoneal injection of Hypnorm (1 mL/kg) (fentanyl citrate 0.315 mg/mL and fluanisone 10 mg/mL, VetaPharma, Leeds, UK) and diazepam (10 mg/kg) (Centrafarm, Etten-Leur, the Netherlands) and subjected to gallbladder cannulation for 20 minutes as described previously (39). During bile collection, body temperature was stabilized using an incubator. Bile was stored at −20°C until analysis. Tissues for mRNA expression analysis were snap-frozen in liquid nitrogen and stored at -80°C until further analysis. Blood was drawn by cardiac puncture, and plasma was collected after centrifugation at 3000 rpm at 4°C for 10 min.

Gene expression analysis
Gene expression analysis was performed as described elsewhere (40). Used primers can be found in supplementary table 1. Expression data were analyzed using SDS 2.3 software (Applied Biosystems), using the $\Delta\Delta$CT method of calculation. PPIA expression was used as an internal control.

Biliary excretion of bile acids, cholesterol and phospholipids
Biliary free cholesterol was derivatized using N,O-bis-(trimethylsilyl)trifluoroacetamide and pyridine (1:1) with 1% tri-methylchlorosilane at room temperature. Biliary bile salt, cholesterol, and phospholipid concentrations were determined, and the respective biliary excretion rates were calculated as previously described (41).

Fecal excretion of neutral sterols and bile acids
Fecal samples were dried, weighed, and thoroughly ground. Aliquots were used for determination of BAs and neutral sterols by gas liquid chromatography as described (41).

Hepatic lipid extraction
Liver homogenates prepared as 15% (w/v) solutions in PBS were subjected to lipid extraction according to the Bligh & Dyer method (42). Lipids were resolved in 1 ml of chloroform and
used for further determination of the label distribution patterns of palmitate (C16:0), palmitoate (C16:1), stearate (C18:0) and oleate (C18:1).

Hepatic cholesterol synthesis and lipogenesis
Cholesterol was extracted from blood spots using 1 ml 95% ethanol-acetone (1:1, v/v), and trimethylsilylated using N,O-bis-(trimethyl)trifluoroacetamide (BSTFA) with 1% trimethylchlorosilane (TMCS). Mass isotopomer distribution analysis of cholesterol synthesis was performed using the $^{13}$C-acetate method as previously described (35). Hepatic lipogenesis measurements were performed on Bligh & Dyer extracted liver lipids as previously described (43).
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References

1. Ioannou GN. The Role of Cholesterol in the Pathogenesis of NASH. Trends Endocrinol Metab. 2016 Feb;27(2):84-95.


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Table S1. Primer sequences for qPCR analysis of LXR and SREBP-2 target genes

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<th>Gene</th>
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