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## The ABC of cholesterol transport

Plösch, Torsten

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## Summary

Cholesterol fulfills an indispensable role in mammalian physiology. It is an important constituent of all cell membranes. Furthermore, it is the precursor of steroid hormones, which regulate a variety of physiological functions, and of bile salts, which are necessary for the generation of bile flow and the intestinal absorption of lipids and lipophilic vitamins. Cholesterol can be derived from the diet as well as from endogenous synthesis, the latter being the major source in humans.

Apart from its vital role in the body, cholesterol also poses a potential threat on human well-being. High plasma cholesterol concentrations, especially in the Low-Density-Lipoprotein (LDL) fraction, are associated with an increased risk for development of atherosclerosis. This is due to its accumulation in macrophages present in blood vessels, representing a key step in the formation of an atherosclerotic plaque. Therefore, it is not surprising that various, tightly regulated pathways exist to eliminate excess cholesterol from the body. The route from peripheral tissues via the liver to the feces has been defined as *reverse cholesterol transport*. Shortly, at the level of the peripheral cell, cholesterol is excreted via the action of the ATP-binding cassette transporter ABCA1 towards High-Density-Lipoprotein particles (HDL), the so-called “good cholesterol”. The parenchymal cells of the liver (hepatocytes) take up cholesterol from HDL and excrete it into the bile either as free cholesterol or after its conversion into bile salts. Subsequently, cholesterol (or its bacterial metabolites) and bile salts end up in the feces.

Different mechanisms exist to maintain cellular cholesterol homeostasis. On the one hand, the expression of enzymes which are involved in cholesterol and bile salt synthesis (liver cells only) is regulated at various levels, controlling both cholesterol synthesis and catabolism. On the other hand, the expression of transport proteins, which promote uptake, trafficking and excretion of cholesterol in the cell, is also tightly regulated. As a consequence cholesterol fluxes within the body are also tightly controlled. Gene expression of the major transporters studied in this thesis is regulated by a transcription factor called *Liver-X-Receptor* (LXR). This receptor senses the level of oxidized cholesterol derivatives, oxysterols, and thereby indirectly the concentration of cholesterol in the cell. This thesis aims to elucidate the mechanisms involved in cholesterol transport and their molecular regulation by LXR, especially at the level of the enterocyte and the hepatocyte. **Chapter 1** provides a summary of the processes involved in maintenance of cholesterol homeostasis.

It has previously been postulated that the beneficial effect of HDL is its function as a cholesterol carrier involved in reverse cholesterol transport from the periphery to the liver. In other words, cholesterol found in HDL was considered to be on its way to elimination. The ABC-transporter ABCA1, which is ubiquitously present in different cell types in the body, including hepatocytes, enterocytes, and macrophages, is of crucial importance for the

formation of HDL. **Chapter 2** characterizes reverse cholesterol transport in mice lacking *Abca1* (*Abca1*<sup>-/-</sup>). As expected, these mice completely lack HDL. However, both hepatobiliary cholesterol excretion and fecal cholesterol disposal are entirely normal in *Abca1*<sup>-/-</sup> mice compared to wild-type mice. Moreover, also as on activation of LXR with its synthetic ligand T091317, *Abca1*<sup>-/-</sup> mice are indistinguishable from their wild-type littermates. Both strains show a marked increase in hepatobiliary and fecal cholesterol excretion. This clearly demonstrates that *Abca1* – and also HDL – does not play a critical role in mass reverse cholesterol transport. Also a second finding of this study is remarkable: when summing up dietary cholesterol intake and hepatobiliary cholesterol excretion, and subtracting fecal cholesterol disposal, one can calculate the amount of cholesterol which is absorbed in the intestine. Interestingly, this amount is *negative* under conditions of LXR activation, which means that the intestine actively excretes cholesterol. This issue is further addressed in Chapter 5 (below).

Cholesterol differs only in the side chain from sterols which are common in plants, *e.g.*, sitosterol and campesterol. These plant sterols contain additional methyl- or ethyl-groups at their side chain. In a typical "Western style" diet, the intake of plant sterols approximately equals that of cholesterol. These plant sterols are, however, only found in trace amounts in the body. The ABC half-transporters *Abcg5* and *Abcg8* have been demonstrated to limit absorption of plant sterols by efficiently pumping them back from the enterocyte into the intestinal lumen. The rare inherited disease *sitosterolemia* has been demonstrated to be caused by mutations in the gene of either *Abcg5* or *Abcg8*. These so-called half-transporters putatively act as a heterodimer. In **Chapter 3** we describe a mouse model of sitosterolemia, the *Abcg5* knockout mouse. These mice show symptoms very similar to human sitosterolemia patients, *i.e.*, increased plasma plant sterol levels. This shows that absence of *Abcg5* alone is sufficient to abolish the protective function of the complex. Treatment with the LXR agonist T091317, which results in increased expression of, *e.g.*, *Abcg5*, *Abcg8*, and *Abca1*, did not influence fractional cholesterol absorption in *Abcg5* knockout mice. In wild-type mice, however, it reduced fractional cholesterol absorption, which means that *Abcg5/Abcg8* may also influence cholesterol uptake. Very surprisingly, LXR activation increased plasma plant sterol levels and therefore aggravated sitosterolemia in the *Abcg5* knockout mice, possibly by an intestinal *Abca1*-dependent mechanism.

**Chapter 4** characterizes the hepatic function of *Abcg5/Abcg8*. There were differing data available on cholesterol concentrations in gallbladder bile of *Abcg5*- and *Abcg8*-knockout mice and *Abcg5/Abcg8* double-knockout mice, the latter having the lowest concentrations of all. To determine the role of *Abcg5* in hepatobiliary cholesterol excretion, the cholesterol excretion rate rather than gallbladder concentrations were measured in *Abcg5*<sup>-/-</sup>, *Abcg5*<sup>+/-</sup> and wild-type mice. While bile flow and bile salt output were normal in *Abcg5*<sup>-/-</sup> mice, cholesterol and phospholipid output were reduced by 85 and 31%, respectively. To get insight not only in the steady-state situation, but also in the maximal cholesterol excretion rate, mice were

infused with an increasing concentration of the hydrophilic bile salt tauroursodeoxycholate to stimulate bile flow. Similar *relative* increases in cholesterol- and phospholipid excretion rates were observed. Upon LXR activation, the maximal cholesterol- and phospholipid output rates were dramatically increased in wild-type mice, whereas there was no effect in *Abcg5*<sup>-/-</sup> mice. These data suggest that *Abcg5* is indeed strongly involved in the control of hepatobiliary cholesterol excretion. However, LXR-independent routes may exist that may contribute to overall hepatobiliary cholesterol output. This is supported by findings in *LXRα*<sup>-/-</sup> mice: upon feeding a high-cholesterol diet, these mice showed an increased hepatobiliary cholesterol excretion rate, similar to that of wild type or *LXRα*<sup>+/-</sup> mice on the same diet. However, *Abcg5*- and *Abcg8* expression levels were not increased in *LXRα*<sup>-/-</sup> mice in contrast to heterozygote or wild type mice. Hence, in this particular model *Abcg5* and *Abcg8* appear to be not rate-controlling for hepatobiliary cholesterol excretion.

The liver has been thought to be the main organ for cholesterol disposal. However, in Chapter 3 we describe that, upon LXR activation, the intestine must be a source of fecal cholesterol in mice. In **Chapter 5**, *Mdr2* knockout mice were used to further evaluate the role of the intestine in fecal cholesterol excretion. *Mdr2* transports phospholipids from the hepatocyte to bile, a process which is indispensable for cholesterol excretion. Consequently, *Mdr2*<sup>-/-</sup> mice do not excrete cholesterol into bile. Upon treatment with the synthetic LXR agonist GW3965, fecal cholesterol excretion increased in both wild-type and *Mdr2* knockout mice to the same extent, independent of biliary cholesterol excretion. In addition, LXR activation stimulated the excretion of [<sup>3</sup>H]-cholesterol from plasma to the feces also in *Mdr2*<sup>-/-</sup> mice. The data presented in Chapter 5 therefore give a strong indication that the intestine may play an important and novel role in reverse cholesterol transport.

Under pathophysiological conditions, hepatobiliary cholesterol excretion may be disturbed in the absence of functional cholestasis. This is the case in an animal model of *erythropoietic protoporphyria*, the *fech/fech* mouse, which is discussed in **Chapter 6**. *Fech/fech* mice have a mutation in the gene encoding the enzyme ferrochelatase, which catalyzes the ultimate step in heme synthesis. The biochemical hallmark of the disease is the accumulation of the hydrophobic heme precursor protoporphyrin in the body. This leads to bile duct proliferation and biliary fibrosis, but normal bile flow, in the *fech/fech* mice. We found that, in addition, *fech/fech* mice have elevated liver and plasma cholesterol levels. This increase is predominantly caused by free cholesterol which, together with phospholipids, is present in *lipoprotein-X* (Lp-X). Lp-X is an unusual particle commonly found under cholestatic conditions and in the rare metabolic disorder *familial lecithin:cholesterol acyltransferase (LCAT) deficiency*. It is thought to be derived from cholesterol and phospholipids which are en route to bile, but ultimately cannot be excreted due to cholestasis. The *fech/fech* mouse is, besides the *Lcat*<sup>-/-</sup> mouse, the first model in which Lp-X is found in the absence of cholestasis. However, *fech/fech* mice show an uncoupling of biliary bile salt from lipid secretion, *i.e.*, relatively more bile salts are needed for the excretion of cholesterol

and phospholipids. This indicates that the process of hepatobiliary cholesterol excretion is impaired in these mice, which may contribute to the accumulation of bile-destined lipids in plasma.

Finally, **Chapter 7** reviews the complex network of transport proteins and regulating factors involved in the maintenance of cholesterol homeostasis in the body. It summarizes the regulation of intestinal cholesterol absorption and hepatobiliary cholesterol excretion. Putative mechanisms are proposed which separate the handling of cholesterol from that of plant sterols in the enterocyte. Furthermore, the mode of action of new drugs developed for the treatment of hypercholesterolemia is described.

In conclusion, cholesterol homeostasis in mammals is achieved by regulating endogenous synthesis and intestinal uptake of cholesterol on the one hand and cholesterol catabolism (*i.e.*, to bile salts and steroid hormones) and excretion to the feces (either via the hepatocyte or the enterocyte) on the other hand. Some of the regulatory mechanisms involved are already targets for drugs to lower plasma cholesterol and consequently the risk for coronary vascular diseases. Detailed knowledge of all of these processes from the molecular to the systemic level is a crucial landmark on the way to an efficient cholesterol-lowering treatment in humans.