Hepatobiliary transport of trace metals, functional characterization of novel hepatic copper transporter (cCOP)
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SUMMARY

Essential trace elements are chemical elements required for functioning of the organism in only minute amounts. By definition, the term essential implies that it is required in the diet, and an element is considered to be a trace element when it constitutes less than 0.01% of total body weight. The body requirements of trace elements (e.g., iron, copper, zinc, manganese, molybdenum, chromium, cobalt, iodine) range from a fraction of a milligram to some milligrams daily. Nevertheless, they fulfill several major physiological functions. It is important that each trace element is maintained at an optimal level and that too low as well as too high concentrations are prevented. As dietary intake usually exceeds the trace amounts required for optimal functioning of the organism, well-regulated mechanism(s) are needed for maintenance of body homeostasis. Control may be exerted at the level of absorption from the gastrointestinal tract, distribution over/in tissues and organs, and/or elimination from the body. Absorption can be influenced by dietary components by formation of soluble or insoluble complexes with trace elements. Ascorbic acid can influence the solubility and hence the absorption of iron and copper, whereas phytate, fiber, phosphate and tannins form insoluble complexes with zinc, copper, manganese and iron. Interaction and competition of trace metals for their absorption can be found as well. Once absorbed trace metals reach the liver via the portal circulation. The liver functions as site of storage, as the place where incorporation of trace metals into metalloenzymes takes place and as secretory organ. The liver secretes trace metals into bile or back into the blood stream after incorporation into proteins, thereby functioning in their redistribution over the various organs and tissues of the body followed by their elimination via the intestinal wall, pancreas, urine or feces.

This thesis deals with the mechanism(s) involved in hepatobiliary transport of trace metals, with emphasis on biliary secretion of copper. The experiments were performed in vivo in the rat and by an in vitro approach, making use of bile canalicular membranes isolated from rat and human liver.

Bile secretion of naturally occurring metals (endogenous) and intravenously (i.v.) administered metals (exogenous) were studied in rats equipped with a silastic catheter in the common bile duct. In rat bile the trace elements iron, copper, zinc, manganese, molybdenum and bromine could always be detected by PIXE (Proton Induced X-ray Emission). This methodology allows simultaneous determination of all elements with a molecular mass >19 and an abundance exceeding 0.5 μg/ml in a single small sample. Biliary secretion rates of iron, molybdenum and bromine closely followed changes in bile flow. When bile flow increased or decreased their concentration in bile did not alter. The secretory process of iron, molybdenum and bromine may therefore involve paracellular transport from plasma to bile,
i.e., diffusion through tight-junctions, and seems not to be regulated at the cellular level. This suggests that biliary secretion of these trace elements is of minor importance for maintenance of their body homeostasis.

Elimination of endogenous and i.v. administered zinc via bile may be connected to glutathione (GSH) secretion. GSH is a tripeptide present in liver and bile in millimolar concentrations and partly responsible for formation of bile. However, GSH does not play a regulatory role in endogenous Zn secretion but somehow is required for efficient removal of Zn via the biliary pathway.

Biliary secretion of endogenous manganese and copper seemed to occur independently of bile flow and GSH output, changes in bile flow were compensated for by adaptations in their concentration. Their secretion therefore seems to be carefully regulated (Appendices 1, 2 and 3).

Because of the clear relevance of Cu metabolism in several forms of human liver disease (primary biliary cirrhosis, Indian childhood cirrhosis, cholestasis) and in Wilson disease, an autosomal recessive disorder characterized by an inability of the liver to secrete copper into bile, together with the fact that regulatory control mechanisms must exist for the maintenance of copper homeostasis, the hepatic processing of copper became subject of further studies as described in this thesis. Knowledge concerning mechanisms involved in biliary copper secretion was minimal. The point of view up to about 1990 comprised two potential routes for copper transfer across the canalicular membrane; transport via GSH transporting system(s) and via lysosomal exocytosis, a process for elimination of lysosomal content, including copper under certain physiological and pathological conditions, into the bile canaliculus. However, our results did not support the concept that lysosomal exocytosis plays a quantitative important role in biliary copper secretion (Appendix 6).

The role of GSH in biliary secretion of copper was investigated in more detail in control Wistar and mutant GY (Groningen Yellow) Wistar rats. These mutant GY rats are deficient in activity of the ATP-dependent canalicular Multi Organic Anion Transporter (cMOAT), the transport system responsible for hepatobiliary transport of organic anions and GSH-conjugates. GSH is virtually absent in bile of these animals, hepatic GSH levels on the other hand are twice as high as in their control counterparts.

Biliary secretion of endogenous copper was unaffected in GY rats. On the other hand, secretion of copper after an i.v. copper load was markedly impaired in these mutants when compared to control rats. A maximal secretion rate of about 30-35 nmol/h/100g body wt was observed in GY rats. The capacity of the copper secretion rate in control rats depended on the administered dose of copper. Treatment of rats with diethylmaleate (DEM) to deplete hepatic GSH and biliary GSH secretion, followed by i.v. copper injection reduced copper secretion in control rats to a value comparable to that observed in GY rats with and without DEM pretreatment. Biliary secretion of endogenous copper can proceed independently of biliary GSH. In contrast, secretion of i.v. administered copper into bile can be observed when GSH is present in bile. This suggests a role of copper-GSH
complex formation followed by secretion via a GSH-conjugate transporting system (probably cMOAT) in the case of excess Cu overload, and partly separate pathways for biliary secretion of endogenous and exogenously administered copper may exist.

We have elaborated this concept further by comparing biliary copper removal after manipulation of the endogenous pathway by supplementation of copper to diets of control and GY rats (8 mM CuSO\textsubscript{4} in drinking water). Biliary copper secretion rates were 6-fold increased both in control and GY rats within 2 weeks to a level of about 30-35 nmol/h/100g body wt and remained stable during the subsequent experimental period of dietary copper supplementation. This biliary secretion rate might reflect a limited capacity for copper transfer across the canalicular membrane and indicates the existence of a saturable GSH-independent copper-transporting system in rat liver. Apparently, biliary GSH nor the pathway dependent on GSH (cMOAT) are involved in removal of excess dietary Cu (Appendix 5).

During the course of our experiments, the gene for Wilson disease was identified as encoding for a putative copper-binding P-type ATPase. The gene is predominantly expressed in the liver. It is conceivable that the gene product, the copper-transporting ATPase (ATP7B), plays a role in biliary secretion of endogenous copper. Based on our in vivo results, indicating the existence of a saturable copper transporting system, the protein may be functionally localized at the canalicular membrane. In isolated rat and human liver plasma membrane vesicles, enriched in the canalicular domain of the liver cell, a clear ATP-dependent uptake of copper was measured (Appendices 7 and 8). By further characterization, biochemical evidence was obtained for a saturable ATP-dependent copper transport system. We have proposed to name this putative canalicular copper-transporting system cCOP, in analogy to other ATP-dependent transport systems at the canalicular membrane, i.e. cBAT for bile acids and cMOAT for glutathione-conjugates and organic anions. The potential involvement of cMOAT in the measured copper transport activity was excluded by the observation of a comparable ATP-dependent copper uptake in canalicular membrane vesicles isolated from control and GY rat livers. Concerning the functional localization of the ATP-dependent copper transporter at the canalicular membrane it may also reside in lysosomal membranes and/or the membrane of the endoplasmic reticulum (ER), the compartment where ceruloplasmin synthesis takes place. A clear correlation could be demonstrated between the activity of leucine aminopeptidase (marker for canalicular membrane) and ATP-dependent copper transport, but not with activities of acid-phosphatase and glucose-6-phosphatase, markers for lysosomes and ER, respectively, indicating that cCOP is mainly functionally located at the canalicular pole of the liver cell.

Because heavy metals like cadmium and silver are predominantly secreted into bile, rather than into urine, it is likely that these metals are transported by pathways that exist for elimination of biologically essential trace metals. For copper transfer across the canalicular membrane, sofar at least two transport systems can be distinguished; transport...
via cCOP functioning in the elimination of endogenous copper and transport via a pathway requiring cMOAT activity, in the case of excessive i.v. copper load. The involvement of cMOAT is also apparent for biliary elimination of zinc. Indications were obtained that cadmium does not compete with endogenous copper for its biliary elimination. In GY rats biliary cadmium concentration after i.v. administration remained below detection limits. These data strongly suggest that cadmium might be secreted as a complex with GSH probably via cMOAT. Biliary elimination of i.v. administered silver was impaired in GY rats and did not compete with endogenous copper for its biliary secretion. When silver and copper were administered simultaneously, recovery of silver and copper were both strongly reduced in control and GY rats when compared to single administration of the metals. Copper and silver probably share common transport systems for hepatobiliary removal, being in part dependent on the presence of GSH in bile and/or cMOAT activity. GSH-independent secretion may be mediated by cCOP (Appendix 9).