CHAPTER 8

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The two main processes involved in fat absorption are lipolysis and solubilization. The first part of this thesis deals with the process of lipolysis. Lipolysis is an important step in the overall process of fat absorption and a shortage of lipase due to pancreatic insufficiency may lead to severely reduced levels of fat absorption. A rat model in which fat malabsorption is caused by impaired lipolysis of dietary triacylglycerols has been developed and characterized (chapter 2). Impaired lipolysis was induced by feeding rats different doses of orlistat, an inhibitor of gastric and pancreatic lipase. It has been demonstrated that orlistat inactivates lipase enzymes by reacting covalently with serine (Ser-152) in the active site of the catalytic subunit. Orlistat has been shown to reproducibly induce fat malabsorption in a dose-dependent fashion [1,2]. The percentage of total dietary fat absorption was examined upon feeding the rats 4 different doses of orlistat, i.e. 0, 50, 200, and 800 mg orlistat per kg rat chow. Fat absorption decreased in a dose-dependent way from 80.2 ± 2.2% in control rats (mean ± SEM) to 32.8 ± 3.7% when 800 mg orlistat per kg rat chow was added (P<0.001).
A potential test for the diagnosis of impaired lipolysis would be the $^{13}$C-MTG breath test, which was previously described by Vantrappen et al. [3]. The advantage of the $^{13}$C-MTG is its sensitivity to pancreatic lipase activity, the most important enzyme with respect to hydrolysis of triacylglycerols. The principle of the $^{13}$C-MTG breath test is based on lipolysis-dependent $^{13}$CO$_2$ excretion via the breath. The relationship between the extent of fat malabsorption and the recovery of $^{13}$CO$_2$ in breath after oral ingestion of $^{13}$C-MTG was investigated in control and orlistat-fed rats. Percentage of $^{13}$CO$_2$ in breath was examined upon feeding the rats 0, 50, 200, and 800 mg orlistat per kg rat chow. A significant correlation ($r=0.88, P<0.001)$ was observed between percentage of total fat absorption and 6-h recovery of $^{13}$CO$_2$ in breath. The correlation was especially strong in rats having major fat malabsorption, indicating that the $^{13}$C-MTG breath test can be used as a tool to detect impaired lipolysis when fat malabsorption is severe. However, in rats with fat absorption higher than 75% the coefficient of variation of cumulative breath $^{13}$CO$_2$ excretion was large (15%) compared to that of fat absorption (5%). Thus, even under controlled circumstances in a homogeneous group of rats with similar genetic background and standard diet, a considerable variation in $^{13}$CO$_2$ expiration was observed. Potential causes of this large variation may be differences in gastric emptying, hepatic clearance and metabolism, β-oxidation, endogenous CO$_2$ production and pulmonary excretion, since all these factors may influence the recovery of $^{13}$CO$_2$ in the breath. In summary, when fat malabsorption is severe, impaired lipolysis is evidently the rate-limiting step, which can be identified by the $^{13}$C-MTG breath test. However, upon rather mild fat malabsorption, the rate-limiting step of $^{13}$CO$_2$ expiration is shifted from impaired lipolysis to one of the other mentioned factors, resulting in large variations upon application of the $^{13}$C-MTG breath test. Although no direct extrapolation from rats to humans can be performed, these results indicate the diagnostic limitations of the $^{13}$C-MTG breath test in subjects with mild to moderate fat malabsorption.

The mechanistic studies were extended by characterization of the $^{13}$C-MTG breath test in healthy adults (chapter 3). The effects of various test conditions on the $^{13}$CO$_2$ response have only partially been elucidated. Therefore, it was determined which factors, apart from pancreatic insufficiency, may influence the quantitative recovery of $^{13}$CO$_2$ in breath. The physiological variation of the $^{13}$CO$_2$ response in healthy human adults was examined by performing the test twice under exactly the same test circumstances within 4 weeks. The repeatability was calculated according to Bland and Altman [4] and it was found that when the $^{13}$C-MTG breath test is repeated in the same individual, the results of both tests are considerably different. In order to explore whether the application of the $^{13}$C-MTG breath test could be simplified or the sensitivity improved, the following factors were studied: the effect of two different test meals on the $^{13}$CO$_2$ response, the effect of an additional meal during the test, and the effect of physical exercise during the test. A variety of test meals has been described for breath tests with a diversity of substrates [3,5-7]. So far, no standardized test meal for clinical purpose of these breath tests has been proposed. A disadvantage of a test meal containing bread and butter could be the extended time it would take for children to consume it, and the risk of not consuming it quantitatively. Also, such a test meal is not applicable to small infants. It was examined whether the $^{13}$CO$_2$ response of a liquid test meal (75 mL cream) is similar to the $^{13}$CO$_2$ response of a solid test meal (2 slices of bread and 25 g
butter). The results suggest that these two distinct test meals give a similar cumulative $^{13}$CO$_2$ response. Since it may be cumbersome to keep patients, in particular infants, fasted for several hours, the effect of consuming an extra meal during the test on the $^{13}$CO$_2$ response was examined. The extra meal was ingested 3 h after the start of the experiment and consisted of 2 slices of bread and 30 g of strawberry jam. It appeared that stringency on continuous fasting during the test is unnecessary, which is in favor of the applicability of the test in pediatric patients. It is known that physical activity considerably affects the production rate of CO$_2$ and nutrient oxidation [8-10], however, it is not established to what extent it influences the results of the $^{13}$C-MTG test. Subjects were asked to perform moderate exercise (50 Watt) during the first 5 h of the test on a bicycle ergometer. Most subjects showed a large increase in their $^{13}$CO$_2$ recovery in the breath, and thus standardization of resting conditions still seems preferable.

A second important step in the overall process of fat absorption is solubilization of the lipolytic products by bile components. A rat model has been developed and characterized in which fat malabsorption is induced by long-term bile diversion (chapter 4). Bile diversion was achieved by providing rats with a permanent catheter in the bile duct in order to interrupt the enterohepatic circulation. This experimental model allows for physiological studies in unanesthetized rats with long-term bile diversion without the interference of stress or restraint. After 6 days of bile diversion, no bile is available in the intestine for the formation of mixed micelles, and theoretically, lipid absorption is expected to be decreased. When rats were fed a standard diet (14 en% fat), however, percentage of dietary fat absorption still appeared to be efficient and decreased only from 96.7 ± 0.2% in control rats to 87.2 ± 0.9% ($P<0.001$) in bile-diverted rats. In order to challenge the absorptive system of the rat, fat absorption was also studied when rats were fed a high-fat diet (35 en% fat). Percentage of total dietary fat absorption again was highly efficient in control rats (93.2 ± 0.4%), but was considerably decreased in bile-diverted rats (53.9 ± 3.9%, $P<0.001$). The presently characterized rat model allows the systematic evaluation of the quantitative role of the various bile components on intestinal lipid absorption, in particular by reconstitution experiments involving the continuous infusion of model bile solutions. In addition, this rat model will allow to evaluate the potency of novel therapeutic approaches/drugs to improve lipid absorption under conditions of impaired bile formation.

A potential substrate for the diagnosis of impaired solubilization would be a $^{13}$C-labeled long-chain fatty acid. A $^{13}$C-labeled long-chain fatty acid test measures the whole process of uptake of long-chain fatty acids: solubilization of lipolytic products by bile components and translocation of the fatty acids over the intestinal mucosa. In this thesis, the substrate [1-$^{13}$C]palmitic acid was selected, because palmitic acid is the most predominant saturated fatty acid in the Western diet. The potency of the [1-$^{13}$C]palmitic acid test to quantify fat malabsorption due to impaired intestinal uptake of long-chain fatty acids was investigated in chronically bile-diverted rats (chapter 4). So far, the use of $^{13}$C-labeled long-chain fatty acids for quantitative studies on defective fat absorption has been limited to breath and feces analysis [5,11,12]. The excretion rate of $^{13}$C in the form of exhaled $^{13}$CO$_2$, however, does not necessarily reflect quantitative differences in the absorption of the $^{13}$C-labeled parent compound, e.g., due to variations in the post-absorptive metabolism [7,13]. The determination
of plasma concentrations of absorbed $^{13}$C-labeled fats as a measure of their absorption offers a theoretical advantage over breath $^{13}$CO$_2$ analysis, since numerous steps are involved in the post-absorptive metabolism of the tracer prior to exhalation of $^{13}$CO$_2$ [14]. After intraduodenal administration of [1-$^{13}$C]palmitic acid, plasma $^{13}$C-palmitic acid concentrations clearly differentiated between control rats and chronically bile-diverted rats. When 10% dose L$^{-1}$ plasma was used as the lower limit of normal plasma values and 91% as the lower limit of normal dietary fat absorption, the test had a sensitivity and specificity of 100% for detecting fat malabsorption under the test conditions used. The results were essentially similar on the standard and high-fat diet, emphasizing the potency of the [1-$^{13}$C]palmitic acid absorption test to detect impaired intestinal uptake of long-chain fatty acids.

In order to investigate the sensitivity of the [1-$^{13}$C]palmitic acid test in humans, the test was applied to a group of healthy volunteers in which fat absorption was slightly reduced by dietary supplementation of calcium (chapter 5). It has been shown that calcium can form insoluble precipitates, consisting of calcium, phosphate, bile acids and long-chain fatty acids [15,16]. Oral calcium supplementation to healthy subjects has been reported to increase fat excretion via the feces in a dose-dependent fashion [15,17-20]. Upon calcium administration fat absorption significantly decreased from 96.6 ± 0.6% to 94.9 ± 0.9% ($P<0.05$). Thus, oral calcium supplementation in humans seems to be a method to reduce fat absorption to a minor extent. It was examined whether this effect could be quantified by means of orally administered [1-$^{13}$C]palmitic acid. Upon calcium administration, plasma $^{13}$C-palmitic acid concentrations after 8 h were significantly increased when compared to control values, yet, cumulative expiration of $^{13}$CO$_2$ was significantly decreased. This discrepancy between the results of the [1-$^{13}$C]palmitic acid test in plasma and breath indicates that post-absorptive metabolism is changed upon calcium supplementation. Percentage of dietary fat absorption did not correlate to either breath $^{13}$CO$_2$ recovery or plasma $^{13}$C-palmitic acid concentrations. Although calcium supplementation clearly affects the outcomes of the [1-$^{13}$C]palmitic acid test, present data do not indicate that the test is sensitive enough to reliably quantify this small degree of fat malabsorption in human adults.

A frequently encountered disorder in Caucasian populations associated with fat malabsorption is cystic fibrosis. The pathophysiology of fat malabsorption in human cystic fibrosis patients may involve both pancreatic insufficiency and bile acid deficiency. However, so far it has not been possible to determine in the individual patient which of the processes is rate-limiting, especially not when patients are supplemented with pancreatic enzymes. In order to obtain more insight into the impaired processes of fat malabsorption in cystic fibrosis we performed a study in pediatric cystic fibrosis patients treated with their usual pancreatic enzyme replacement therapy (chapter 6). The substrates $^{13}$C-MTG and uniformly labeled $^{13}$C-linoleic acid were both applied to determine whether the rate-limiting step behind their fat malabsorption was either impaired lipolysis or impaired intestinal uptake of long-chain fatty acids, respectively. $^{13}$C-linoleic acid was selected as the substrate because, theoretically, it could provide information on the essential fatty acid status and metabolism of the patients. The $^{13}$C-linoleic acid test and the $^{13}$C-MTG breath test were both applied to 10 pediatric cystic fibrosis patients receiving their habitual pancreatic enzymes. During the test days, a fat balance was performed for 3 days to determine dietary fat absorption. Fecal fat excretion ranged from
5.1 to 27.8 g day\(^{-1}\) and fat absorption ranged from 79 to 93\%. After ingestion of \(^{13}\)C-MTG no relationship was observed between breath \(^{13}\)CO\(_2\) recovery and dietary fat absorption (\(r=0.04\)). In contrast, a strong relationship was observed between 8-h plasma \(^{13}\)C-linoleic acid concentrations and dietary fat absorption after ingestion of \(^{13}\)C-linoleic acid (\(r=0.88\), \(P<0.001\)). Our results suggest that fat malabsorption in cystic fibrosis patients on enzyme replacement therapy is not likely due to insufficient lipolytic enzyme activity, but rather due to defective intestinal uptake of long-chain fatty acids. Therefore, therapeutic attempts to normalize fat absorption in cystic fibrosis patients need to include a strategy to improve intestinal uptake of long-chain fatty acids.

Impaired intestinal uptake of long-chain fatty acids may be caused by (combinations of) processes such as altered bile composition, bile salt precipitation, decreased intestinal bile salt concentration, small bowel mucosal dysfunction and/or alterations in the mucus layer [21-25]. In order to obtain more mechanistic insight into the involved processes, we studied two cystic fibrosis mouse models (chapter 7). The two cystic fibrosis mouse models are: 1. mice with the \(\Delta F508\) mutation in the \(cftr\) gene, \(\Delta F508/\Delta F508\) (129/FVB genetic background), and 2. mice with complete inactivation of the \(cftr\) gene, \(cftr\ -/-\) (129/C57/Bl6 genetic background) [26,27]. The basic defect in cystic fibrosis lies in the cystic fibrosis transmembrane regulator (CFTR), a protein responsible for chloride ion transport. In \(\Delta F508/\Delta F508\) mice, the biosynthetic processing of the \(cftr\) gene product to its mature glycosylated form is disrupted [28], so that the protein is retained in the endoplasmic reticulum and is then degraded [29]. However, it has been observed that \(\Delta F508/\Delta F508\) mice exhibit residual \(cftr\) activity in the gallbladder and ileum [26]. In \(cftr\ -/-\) mice, \(cftr\) function is completely abolished, and epithelia lack \(cftr\) in the apical plasma membrane and, therefore, lack cAMP-stimulated Cl\(^-\) permeability [30]. Fat absorption was studied after feeding the mice a standard (14 en\% fat) or a high-fat (35 en\% fat) diet for 2 weeks. In \(\Delta F508/\Delta F508\) mice, dietary fat absorption was similar compared with controls on both diets (standard diet: 94.8 ± 0.7\% compared to 95.5 ± 0.6\%, respectively; high-fat diet: 93.8 ± 2.2\% compared to 95.3 ± 2.4 \%, respectively). Absorption of dietary fats by \(cftr\ -/-\) mice, however, was significantly less efficient when compared with their control counterparts (standard diet: 82.8 ± 3.0\% compared to 93.9 ± 1.3\%, respectively, \(P<0.01\); high-fat diet: 88.8 ± 1.6\% compared to 95.0 ± 1.4\%, respectively, \(P<0.01\)). These data indicate that the complete disruption of the \(cftr\) gene leads to moderate fat malabsorption, in contrast to introduction of the \(\Delta F508\) mutation. However, it can not be completely excluded that the observed difference in fat malabsorption is also influenced by the different genetic background of the mice. In order to study the processes behind defective uptake of long-chain fatty acids in more detail, biliary secretion of bile salts was determined after cannulation of the gallbladder for 80 min and fecal bile salt excretion was determined. Biliary bile salt pool sizes and biliary bile salt secretion rates were similar for the CF mouse models and their respective controls on either diet, indicating that this is not the reason for fat malabsorption in \(cftr\ -/-\) mice. Fecal bile salt excretion was increased in \(\Delta F508/\Delta F508\) and in \(cftr\ -/-\) mice when compared with their respective controls (10 versus 5 \(\mu\)mol g\(^{-1}\) feces, respectively, \(P<0.01\)). No significant correlation was observed between fecal bile salt excretion and fecal fat, indicating that the increased excretion of fecal bile salts is not secondary to fat malabsorption. Biliary bile salts in \(\Delta F508/\Delta F508\), \(cftr\ -/-\) and control mice were predominantly composed of cholate and
ß-muricholate, with minor contributions of deoxycholate, ursodeoxycholate and hyodeoxycholate. Significantly increased cholate and decreased deoxycholate concentrations were observed in bile of ΔF508/ΔF508 and cftr -/- mice when compared with their controls. This result is consistent with the finding of increased fecal bile salt loss, which upregulates neosynthesis of bile salts thereby resulting in higher amounts of primary bile salts and decreased amounts of secondary bile salts. In conclusion, in this study we have shown that cftr -/- mice, but not ΔF508/ΔF508 mice, have an impaired dietary fat absorption, which is not likely due to either decreased bile salt pool size or altered bile composition. In both CF mouse models, fecal bile salt excretion was increased, which was not secondary to increased fecal fat excretion. Bile composition data indicate that the increased fecal loss of bile salts is compensated for by an increased bile salt neosynthesis. Further investigations are needed to establish whether impaired pancreatic function, intestinal mucosal dysfunction or alterations in the intestinal mucosa are responsible for the observed fat malabsorption in cftr -/- mice.

In summary, in this thesis more information is obtained with respect to the various pathophysiological processes involved in fat malabsorption. Various animal models for lipid malabsorption were described and characterized: orlistat-fed rats to study impaired lipolysis, bile-diverted rats to study bile deficiency, and transgenic and knock-out mice for the study of cystic fibrosis. These animal models are expected to be of significant importance to investigate the potency of novel diagnostic and therapeutic strategies in the near future. In addition, the potency of diagnostic tests such as the 13C-MTG breath test and the 13C-palmitic acid test was investigated to characterize the etiology behind fat malabsorption in animal models and in humans. The present data in cystic fibrosis patients and mice do not only open the possibility to determine pathophysiological mechanisms of lipid malabsorption in individual patients, but they also give way for titration of therapy to individual patients.

References


