Insulin sensitivity of hepatic glucose and lipid metabolism in animal models of hepatic steatosis
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Chapter 9

Summary and general discussion
World-wide prevalences of nonalcoholic fatty liver diseases, such as nonalcoholic steatohepatitis (NASH), and diabetes mellitus type 2 have and will increase markedly during the coming years (1-4). The hallmark of the fatty liver is an excessive accumulation of triglycerides (TGs) and various human studies showed that this fat accumulation is associated with insulin resistance and diabetes mellitus type 2 (5-10). The increasing prevalence of these conditions are a result of the prevailing sedentary life-style, characterized by high food consumption and low physical activity (11). The metabolic syndrome, often referred to as a readout of the sedentary life-style in Western societies, comprises a cluster of metabolic abnormalities, among which obesity and insulin resistance are key features (5,12,13).

Although the condense summary of human data suggest a strong association between increased hepatic TG concentrations and insulin resistance, only a few basal studies on metabolic and cellular processes involved have been published. The primary research question addressed in this thesis was therefore whether increased hepatic TG concentrations induced by any cause, by definition, lead to (hepatic) insulin resistance.

Metabolism of the energy-bearing molecules glucose, fatty acids and TGs is closely linked in the liver and insulin plays a pivotal role in this interaction. In the liver, glucose is stored as glycogen, but this organ is also capable to produce glucose. Energy needed for de novo glucose production (gluconeogenesis) can be generated by hepatic oxidation of fatty acids (β-oxidation). On the other hand, the liver can convert glucose into fatty acids and TGs (de novo lipogenesis) and subsequently secrete TGs in very low density lipoprotein (VLDL) particles. In fed conditions, when blood glucose levels rise, the blood glucose lowering hormone insulin is secreted by the pancreas. Insulin inhibits hepatic glucose production (HGP), enhances conversion of glucose into glycogen, stimulates de novo lipogenesis, and inhibits gluconeogenesis and VLDL secretion.

Defects in the actions of insulin on lipid and glucose metabolism are thought to result in various hallmarks of insulin resistance, the metabolic syndrome and diabetes mellitus type 2. The work described in this thesis addresses the (patho)physiological effects of hepatic steatosis on these metabolic pathways in several animal models. The models used were the leptin-deficient \textit{ob/ob} mouse, pharmacological activation of the liver X receptor, pharmacological inhibition of β-oxidation, and pharmacological inhibition of glucose-6-phosphatase.

**The \textit{ob/ob} mouse**

Leptin-deficient \textit{ob/ob} mice are obese, hyperinsulinemic, hyperglycemic, have hepatic steatosis, and are a commonly employed animal model of diabetes mellitus type 2. In the studies described in chapter 2, we used novel stable isotope techniques to quantify the hepatic glucose fluxes and metabolic clearance rate (MCR) of glucose in \textit{ob/ob} mice compared to wild-type, lean littermates. These studies showed that HGP did not differ between \textit{ob/ob} and lean mice, despite higher plasma insulin levels in the \textit{ob/ob} mice. Because the elevated plasma insulin levels did not suppress HGP, the hepatic steatosis of \textit{ob/ob} mice is by definition associated with decreased insulin sensitivity of hepatic glucose metabolism. Previous experiments of our laboratory showed that hyperinsulinemia did not suppress the VLDL production rate in \textit{ob/ob} mice (14). Thus, the \textit{ob/ob} mouse is an useful animal model of hepatic steatosis associated with insulin resistance of both hepatic glucose and lipid metabolism. In the studies described in chapter 4, insulin sensitivity of glucose metabolism was studied in more detail in \textit{ob/ob} mice. For this, we used the ‘golden standard’ to determine insulin sensitivity: the hyperinsulinemic euglycemic clamp. In this technique, mice receive an infusion with insulin to achieve a stable, relatively high plasma concentration of the hormone.
Upon the hyperinsulinemia thus created, blood glucose will start to decrease. To maintain euglycemia, a glucose solution is also infused. The infusion rate of the latter solution can be adjusted so that euglycemic conditions are met. The glucose infusion rate is a measure of the insulin sensitivity of, in this case, the mouse. Addition of $[^{13}\text{C}]$-glucose to the solutions allowed us to calculate HGP and MCR. Hyperinsulinemia suppressed HGP by 94% in lean mice but by only 48% in $ob/ob$ mice. This underscores insulin resistance of hepatic glucose metabolism in $ob/ob$ mice. Moreover, hyperinsulinemia increased MCR 5.5-fold in lean mice, whereas no increase was seen in $ob/ob$ mice, indicating insulin resistance of peripheral glucose metabolism.

In conclusion, hepatic steatosis in $ob/ob$ mice is associated with decreased insulin sensitivity of hepatic glucose and lipid metabolism. Concerning glucose metabolism, $ob/ob$ mice are also insulin resistant in the periphery. Because plasma FFA levels are elevated in $ob/ob$ mice, peripheral lipid metabolism (e.g., insulin-mediated suppression of lipolysis) is probably also affected. Although $ob/ob$ mice are commonly used in diabetic and obesity research, one should carefully interpret results gathered with these animals because leptin deficiency is not a normal cause of obesity, hepatic steatosis and insulin resistance in humans. Only few families are known that are homozygous for a mutation in the leptin gene (15,16). In general, monogenetic obesity in humans is scarce (17). A monogenic animal model of obesity and/or hepatic steatosis is therefore probably not the best model to study hepatic steatosis. On the other hand, because $ob/ob$ mice are commonly used, detailed and physiologically-relevant information about this model is available.

For the latter reason, we used $ob/ob$ mice in the studies described in chapter 5 to assess the metabolic consequences of the iminosugar derivative N-$(5'\text{-adamantane-1'-yl-methoxy})$-pentyl-1-deoxynojirimycin (AMP-DNM), a novel inhibitor of glucosylceramide transferase. When administered to $ob/ob$ mice, AMP-DNM normalised elevated tissue glucosylceramide levels and improved insulin sensitivity of both peripheral tissues and the liver. Because ceramide levels were not affected, these data strongly suggest that ceramide metabolites may be involve din the link between increased fatty acids and insulin resistance.

**Pharmacological activation of the liver X receptor**

The liver X receptor (LXR) is a member of the 48-member superfamily of nuclear receptors that can activate or inhibit transcription of genes upon their activation by, in most cases, a small-molecular ligand. Oxysterols are oxygenated metabolites of cholesterol and are considered the endogenous ligands of LXR. The synthetic LXR ligands T0901317 and GW3965 are useful tools in LXR-related research. Upon activation, LXR stimulates transcription of various genes, *i.e.*, those encoding enzymes involved in the transport of cholesterol from the peripheral tissues to the liver and in secretion of cholesterol into bile and subsequently the feces (18). Clinical application of synthetic LXR ligands is hampered by the fact that LXR also stimulates transcription of genes encoding enzymes involved in de novo lipogenesis. Administration of synthetic LXR ligands to rodents results in severe hepatic steatosis (19). In the studies described in chapter 3, T0901317-induced hepatic steatosis was accompanied by a more than 2-fold induction of VLDL production. The secretion of large, TG-rich VLDL particles completely accounted for this increase. It is known that large VLDL particles will finally be converted in the very atherosclerotic small, dense low density lipoprotein (LDL) particles. In the studies described in chapter 4, hyperinsulinemic euglycemic clamp techniques and stable isotopes were used to study the effects of LXR activation on glucose metabolism and insulin sensitivity. GW3965-induced hepatic steatosis was not associated with reduced insulin sensitivity of hepatic glucose metabolism: in LXR-
ligand treated mice, HGP was suppressed by 86% and hepatic glucose fluxes were not affected. In ob/ob mice, in contrast, LXR activation resulted in slightly improved insulin sensitivity of peripheral glucose metabolism. This improvement was mainly due to effects of the LXR ligand on adipose tissue.

In conclusion, LXR-induced hepatic steatosis is not associated with decreased insulin sensitivity of hepatic glucose metabolism. So far, no studies have been performed to investigate the effects of LXR activation on insulin sensitivity of lipid metabolism, e.g., peripheral lipolysis and hepatic VLDL production. The studies described in chapter 4 were among the first to show that hepatic steatosis per se does not lead to (hepatic) insulin sensitivity. Moreover, the studies from chapters 3 and 4 and those of others (19) were among the first to show that clinical use of pharmacological broad-acting LXR ligands as anti-atherosclerotic or anti-diabetic drugs, is hampered by their undesirable side effects. Potential application of LXR modulators in diabetes treatment will require the development of gene- and/or organ-specific compounds.

**Pharmacological inhibition of β-oxidation**

In fasted conditions, dietary glucose supply is absent. In this condition, the body largely depends on the liver for maintaining euglycemia because only the liver (and the kidney to a small extent) is capable to produce glucose. For this, glycogen is broken down into glucose, but gluconeogenesis is also facilitated. Fatty acid β-oxidation, according the textbook biochemistry, yields the energy needed for gluconeogenesis. With low insulin levels, lipolysis of peripheral (adipose) TGs is enhanced and the free fatty acids (FFAs) thus generated are transported to the liver. Fatty acids are broken down in the β-oxidation process localised to hepatic mitochondria. Transport of fatty acids over the mitochondrial membranes, controlled by carnitine palmitoyl transferase (CPT) -1 and -2, is considered rate-limiting in the β-oxidation processes. In the studies described in chapter 6 we showed that pharmacological CPT1 inhibition with tetradecylglycidic acid (TDGA) resulted in severe microvesicular hepatic steatosis and hypoglycemia upon fasting. VLDL production rates did not differ between the TDGA-treated and control mice and hyperinsulinemic euglycemic clamps equally suppressed VLDL production in control and treated mice. Moreover, hepatic intracellular insulin signaling was not affected upon TDGA treatment. In conclusion, hepatic steatosis due to pharmacological inhibition of β-oxidation is not associated with reduced insulin sensitivity of hepatic lipid metabolism.

In humans, most fatty livers show large lipid droplets, referred to as macrovesicular steatosis. Only few clinical fatty liver diseases are microvesicular, i.e., fatty livers due to disturbances in β-oxidation. For instance, medium chain acyl-CoA dehydrogenase (MCAD) deficiency, assumed to be the most common inherited disorder of fatty acid metabolism (20) is associated with microvesicular fat accumulation. Upon fasting, MCAD deficient patients develop lethargy which may proceed into coma or sudden death (20). It is not known whether this syndrome is associated with insulin resistance.

**Pharmacological inhibition of glucose-6-phosphatase**

In the past, our laboratory has performed detailed studies with S4048, a pharmacological inhibitor of glucose-6-phosphate translocase (G6PT). G6PT is an enzyme of the glucose-6-phosphatase (G6Pase) complex and its inhibition leads to a condition similar to glycogen storage disease type I (GSDI). In rats, inhibition of G6Pase resulted in increased de novo
lipogenesis and hepatic steatosis (21,22). Remarkably, the VLDL production rate was not affected in S4048-treated animals (22). The studies described in chapter 7 showed that S4048 treatment also resulted in hepatic steatosis in mice. This condition was not associated with decreased intracellular hepatic insulin signaling, i.e., phosphorylation of protein kinase B (PKB) upon insulin injection. Although it was expected that the transcription factor carbohydrate responsive element binding protein (ChREBP) was involved in the development of hepatic steatosis, the translocation of this transcription factor to the nucleus was not enhanced. The increased phosphorylation and activity of AMP-activated protein kinase (AMPK) upon S4048 treatment might, in part, be responsible for this phenomenon. However, expression of genes encoding proteins involved in de novo lipogenesis was clearly increased upon S4048-treatment, in a LXR-independent fashion.

It is known that liver enlargement in GSDI patients is not solely the result of glycogen accumulation, but also of increased TG levels due to enhanced de novo lipogenesis (23). To study the role of ChREBP in S4048-induced hepatic steatosis and its role in the livers of GSDI patients, more experiments should be performed, especially with ChREBP knockout mice (24).

**Overall conclusion**

From the studies described in this thesis, it is clear that hepatic TG accumulation per se does not necessarily negatively interfere with insulin sensitivity of hepatic glucose and lipid metabolism. However, one should be aware that all studies described, except those with the ob/ob mice, were conducted in mice with “short-term” hepatic steatosis. Moreover, all fatty livers were the result of manipulation of de novo lipogenesis and/or β-oxidation. Various research groups have performed studies with other mouse models of hepatic. For instance, hepatic steatosis in CD36 knockout mice is due to increased FFA flux to the liver and associated with decreased insulin sensitivity (25,26). In addition, decreased hepatic TG levels in hormone sensitive lipase (HSL) knockout mice resulted in improved insulin sensitivity (27). Comparison of these latter studies with the ones described in this thesis strengthens the overall conclusion that hepatic steatosis in mice is not always associated with insulin resistance. Upon prolonged fasting, for instance during the night, humans also develop hepatic steatosis as a result of increased FFA flux from peripheral tissue to the liver. So far, it has not been reported that this “daily fatty liver” is associated with insulin resistance. Also in mice, 16-h fasting did not show an effect on insulin sensitivity of HGP, despite increased hepatic TG levels (28).

**Properties of the fat droplets**

In the future, more studies should be done to precisely determine the effects lipid droplet-associated factors. First, the size of the fat droplets within the cells, i.e., the metabolic differences between macro- and microvesicular hepatic steatosis, might play a role. At first sight, one could argue that because larger fat droplets use more cytosolic space, they can harm more of the intracytosolic processes, for instance insulin signaling. Smaller droplets, as seen in livers of patients with β-oxidation disorders, use less cytosolic space and leave the intracellular organelles more intact. However, microvesicular hepatic steatosis implies a more severe disease than macrovesicular steatosis (reviewed by Fromenty and Pessayre (29)). The reason for this is probably the fact that the main cause of microvesicular hepatic steatosis, impairment of mitochondrial β-oxidation, is a severe condition itself, with clinical features such as hypoglycemia, hyperammonememia, brain disorders and eventually death.
Secondly, different sources of fatty acids within the droplets might have different effects. As described above, there are differences in (hepatic) insulin sensitivity in animals with hepatic steatosis due to high fat diets and hepatic steatosis due to enhanced de novo lipogenesis and/or reduced β-oxidation. The localisation of the lipids within the liver might play a role herein. In the periportal zone dietary fatty acid accumulation co-localises with processes involved in gluconeogenesis and β-oxidation. Glycolysis and lipogenesis are presumably located predominantly in the perivenous zone (30). It is of importance to notice that diabetes-associated steatosis is predominantly present in the perivenous zones of the liver.

Thirdly, as already discussed, the duration of the hepatic steatosis and duration of adverse effects might be related. Ob/ob mice, for instance, have a life-long hepatic steatosis and also suffer from the adverse effects of this condition. Considering this, the effects of hepatic TG concentrations seen in the CD36 and HSL knockout mice (25-27) might also be the result of life-long fat accumulation. As mentioned in the introduction of this thesis, inflammation is the major factor in the transition of simple hepatic TG accumulation towards fibrosis and cirrhosis (31). Adipose tissue and tissues with excessive TG concentrations are intrinsically inflammatory active, and thus longer existing hepatic steatosis might, via inflammatory molecules, disturb intra- and extrahepatic insulin sensitivity.

The structure of the fatty acids (saturated, monounsaturated, and polyunsaturated) could differentially affect hepatic metabolism and insulin sensitivity. This is, of course, closely related to the source of the fatty acids. Various studies have shown that polyunsaturated fatty acids (PUFAs) are healthier: they suppress activity of the major lipogenesis regulatory factor sterol-regulatory element-binding protein-1c (SREBP-1c), antagonize LXR and improve plasma TG profiles (32). Moreover, PUFAs reduce de novo lipogenesis via inhibition translocation and activation of ChREBP (33). Studies with monounsaturated fatty acids also showed less negative effects compared to saturated fatty acids (34).

**Extrahepatic regulation**

Next to the intrahepatic aspects mentioned, other organs might also play roles in the (lack of) association between hepatic steatosis and insulin resistance, for instance, via the adipocyte-derived hormones leptin and adiponectin. Deficiency of leptin is associated with both obesity and insulin resistance (14-16;35). Low plasma adiponectin levels are associated with the development of insulin resistance (36). Administration of adiponectin to mice resulted in decreased liver TG concentrations and increased insulin sensitivity (37). Next to these hormones, the brain can also influence hepatic insulin-mediated effects (38-40). In conclusion, complex interactions between endocrine, metabolic, and transcriptional pathways are involved in TG-induced hepatic insulin resistance, as recently discussed by Den Boer et al. (41).

**What does lead to what?**

The question that remains unsolved is whether hepatic steatosis is caused by insulin resistance, or that hepatic steatosis predisposes for insulin resistance. The majority of studies described in this thesis started with the assumption that insulin resistance is a result of hepatic steatosis, or, in other words, that fatty acids, TGs and/or their metabolites and derivatives interfere with insulin signaling. However, this seemed be true in ob/ob mice only. In other studies, in which endogenous fatty acid fluxes were manipulated, hepatic TG concentration showed a negative correlation with the insulin sensitivity (25-27,42). More studies focussing on the role of fatty acid derivatives ceramide and glycosphingolipid will be necessary to gain insight about their role in mediating peripheral and hepatic insulin signaling and insulin sensitivity.
The two “hits” hypothesis (43) suggests that hepatic steatosis is due to reduced (peripheral) insulin sensitivity. Upon decreased insulin sensitivity, insulin-mediated suppression of lipolysis is hampered and, as a result, more fatty acids are directed to the liver. As a result, these fatty acids are stored upon esterification as TGs but will also enhance \( \beta \)-oxidation via activation of peroxisome proliferator activated receptor \( \alpha \) (PPAR\( \alpha \)). Finally, this will result in enhanced gluconeogenesis and HGP. In the future, experiments need to be performed with rodents upon a high fat diet to study the time-course of development of insulin resistant. Everybody agrees that obesity and hepatic steatosis upon enhanced caloric intake and reduced physical activity is truly associated with insulin resistance and type 2 diabetes mellitus in humans (2-4,11,44,45). In the experiments with mice upon a high fat diet, the questions that need answers are (i) whether different diets, i.e., different fatty acid compositions, cause more or less insulin resistant, and (ii) whether hepatic steatosis firstly affects peripheral or hepatic insulin sensitivity resistance In studies with non-diabetic patients with NAFLD, it became clear that NAFLD is associated with reduced insulin sensitivity of the periphery, not of the liver. For instance, hyperinsulinemia suppressed HGP in both NAFLD and control subjects, but insulin-enhanced glucose disposal was severely impaired in the NAFLD subjects compared to controls (46) Using the hyperinsulinemic clamp technique, Marchesini \textit{et al.} (9) also demonstrated a reduction of insulin-mediated glucose disposal in NAFLD.

In conclusion, fatty liver per se is not associated with reduced insulin sensitivity of hepatic glucose and lipid metabolism. Factors related with duration, inflammatory grade, fatty acid composition, and size of the fat droplets might influence severity of the fatty liver and its association with other clinical symptoms, for instance insulin resistance and diabetes mellitus type 2.

References

Summary and general discussion
