Hepatic steatosis : metabolic consequences

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When one has much to put into them,
a day has a hundred pockets.
Friedrich Nietzsche

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Chapter 1

General Introduction

Hepatic steatosis refers to the condition of accumulation of triglycerides (TG) in hepatocytes. From a quantitative perspective this storage capacity of the hepatocytes is much less important than the accumulation of TG in adipocytes. TG accumulation in the liver was thought to be an inert histological epiphenomenon. However, nowadays we know that hepatic steatosis is associated with several metabolic changes in lipid and glucose metabolism not only in the liver, but also throughout the body. In this introduction the emphasis is on TG metabolism since TG are the most important lipids that are involved in hepatic steatosis. The regulation of TG metabolism is described with special focus on the causes and consequences of hepatic steatosis.

Whole body triglyceride and fatty acid metabolism

Dietary triglycerides are absorbed in the intestines and packed into chylomicrons. Chylomicrons are very large particles that contain mainly TG, but also consist of phospholipids, cholesterol and proteins.[1] Upon secretion from the intestines into the lymph and subsequently into the bloodstream, these large TG-containing particles acquire apolipoprotein (apo-)B, E and apoCI, -II and III on their surface. The liver also produces TG-rich lipoproteins, i.e. very-low density lipoproteins (VLDL-TG). These VLDL-TG particles also contain cholesterylesters in the hydrophobic core.[1] The surface monolayer consists of cholesterol, phospholipids and protein. In addition to a single apoB molecule per VLDL-TG particle, the shell of the particles is enriched in apoE and apoCI, -II and -III upon secretion into the circulation.

In the fed state a mixture of VLDL-TG (from the liver) and chylomicrons (from the intestines) enters the circulation, where these TG particles are subject to lipolysis by endothelium-bound lipoprotein lipase (LPL), as shown in Figure 1.[2,3] LPL is synthesized in, and secreted by, parenchymal cells throughout the body. It is most abundant in cardiac and skeletal muscle and adipose tissue. Several apolipoproteins influence the lipolytic conversion by LPL. ApoCII is an activating co-factor for LPL[4,5], whereas apoCI and apoCIII inhibit lipolysis.[6,7] In addition, high amounts of apoE can inhibit LPL-mediated lipolysis.[8,9] The process of local lipolysis by LPL generates fatty acids (FA) that can enter the adipose tissue, muscle or the heart, either for energy provision via β-oxidation or for TG storage, depending on the oxidative requirements of the respective tissues. LPL expression is regulated by tissue-specific mechanisms, that also depend on hormonal and nutritional status.[3,10-12] LPL activity decreases

during fasting and increases after a meal containing fat.[13-15] Postprandially, LPL is abundantly expressed on adipose tissue, whereas during fasting the expression on skeletal muscle increases.[10-12]

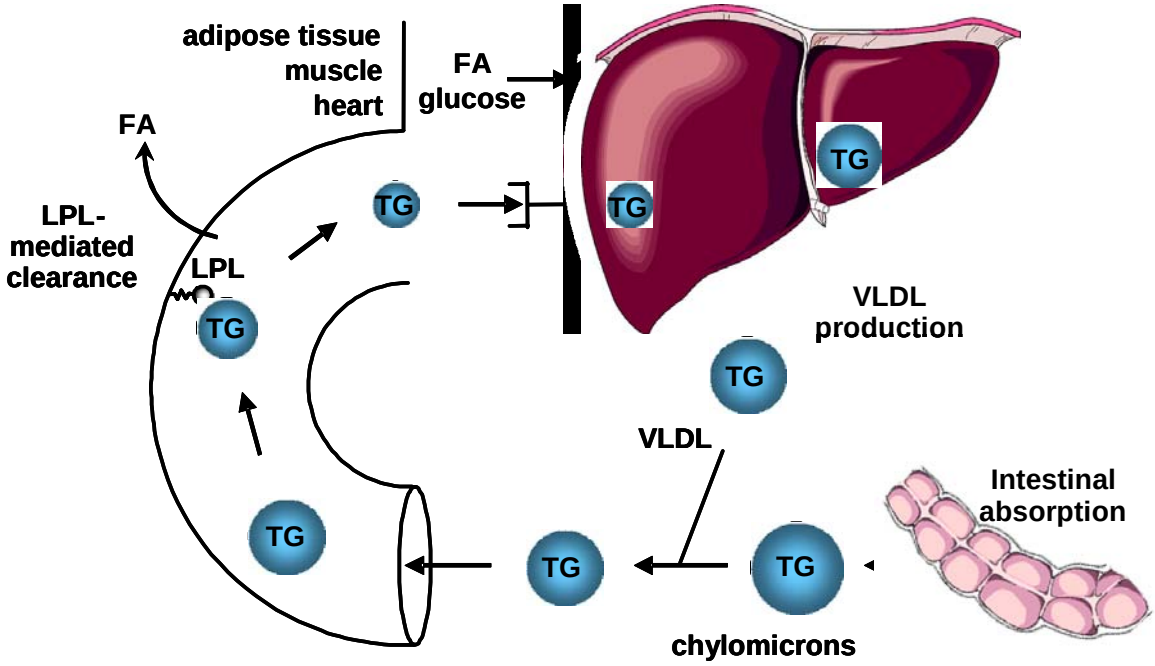

Figure 1. Schematic representation of whole-body TG metabolism. Chylomicrons from the intestines and VLDL-TG from the liver enter the circulation. In the capillaries these particles are lipolyzed by lipoprotein lipase (LPL). The generated FA enter the cardiac and skeletal muscle and the adipose tissue where they can be stored as TG or used for energy provision. After several lipolysis steps the remnant particles are taken up by the liver and further processed.

Hydrolysis of VLDL-TG results in the formation of intermediate density lipoproteins (IDL) and subsequently low density lipoproteins (LDL). In addition to LPL, hepatic lipase (HL) is responsible for further hydrolysis of the particles.[16] After several lipolysis steps, these remnant particles are recognized by the liver by their apoB and apoE, taken up by specific lipoprotein receptors such as LDL-receptor (LDLr) and LDLr-related protein (LRP) and further processed.

Adipose tissue is an important regulator of triglyceride metabolism. It acquires FA from the circulation, either from the free FA pool or from FA derived from plasma TG, through the activity of LPL. Moreover, TG contained within adipose tissue are continuously hydrolyzed by hormone sensitive lipase (HSL) and other lipases[17], which results in the release of FA into the plasma. These FA can subsequently either

be oxidized in other tissues, or be taken up by the liver. Within the liver these FA have two fates: oxidation or re-esterification into TG and subsequent release into the plasma in the form of VLDL-TG. Consequently, there is a futile cycle of FA between adipose tissue and the liver, enabling the body to adapt rapidly to changes in energy requirements. In pathophysiological conditions, in which there are disturbances in this cycle, accumulation of TG may occur in the liver. Since there is a huge fat mass in relation to the very limited maximum storage capacity of TG in the liver, it is likely that only minor changes in fatty acid cycling may result in liver steatosis. This is illustrated by a rapid increase of liver TG after skipping just a few meals during short term starvation.

Hepatic triglyceride and fatty acid metabolism

The liver plays a central role in lipoprotein metabolism. It produces, secretes and takes up lipoproteins. In FA metabolism the liver also plays an important role: it is involved in FA uptake from plasma, FA oxidation, *de novo* FA synthesis, and VLDL-TG secretion. Moreover, FA and their derivatives have major effects on expression levels of many genes, because these FA serve as ligands for several transcription factors which are crucial in the regulation of glucose and fat metabolism.

Hepatic TG metabolism

Hepatic VLDL-TG production (HVP) is mainly substrate-driven, but it is also determined by the hormonal and nutritional status of the individual. VLDL-TG assembly and secretion is a two-step process as described by Alexander et al. in 1976.[18] The first step is the association of one apoB with the core lipids. Microsomal TG transfer protein (MTTP) forms an important step in VLDL-TG-assembly, since it catalyzes the transfer of lipids towards the apoB molecule. This particle fuses with a lipid droplet and generates a mature VLDL-TG particle. The flux of FA to the liver and the amount of TG in the liver are factors influencing HVP. However, this relationship is not always straightforward. The amount of TG accumulated in the liver is not a direct determinant of the production of VLDL-TG. In rats it has been shown that acute stimulation of *de novo* lipogenesis leads to steatosis without affecting VLDL-TG production.[19] Moreover, increased FA flux to the liver does not always lead to increased VLDL-TG production. For instance, CD36 knockout mice have a 60% increase in hepatic TG content[20], but no change in hepatic VLDL-TG production.[21]

Obviously, other factors than merely the availability of FA control hepatic VLDL-TG production. Hormonal effects on HVP will be discussed later on in this introduction.

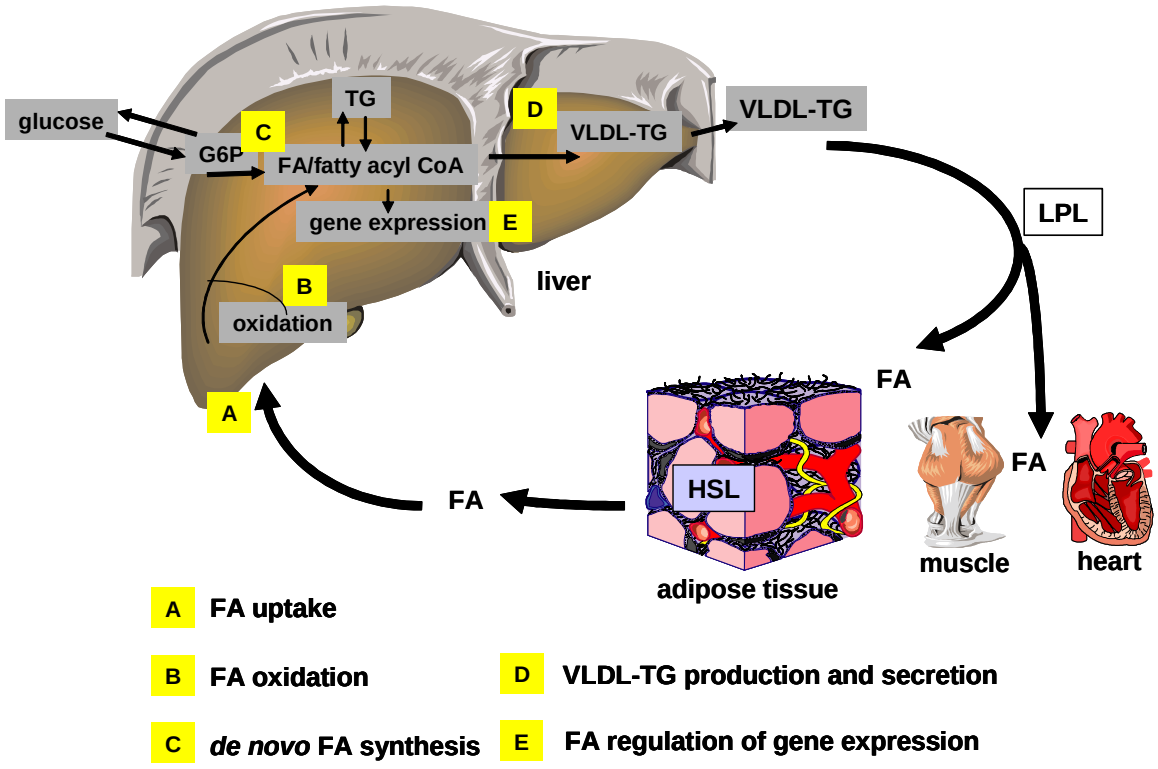

Figure 2. Major pathways of hepatic fatty acid/triglyceride metabolism in the liver. The liver plays a central role in lipid metabolism through **A.** Uptake of fatty acids, **B.** Fatty acid oxidation, **C.** *De novo* fatty acid synthesis, **D.** Assembly and secretion of VLDL-TG, **E.** Effects of fatty acids on gene expression. FA = fatty acids, HSL = hormone sensitive lipase, LPL = lipoprotein lipase, G6P = glucose-6-phosphate.

Hepatic FA metabolism

The liver is important in FA metabolism, because it takes up FA from the circulation (Figure 2). Most medium- and long-chain FA that enter the liver are oxidized in the mitochondrial β-oxidation system. Very-long-chain FA are mainly oxidized in the peroxisome.[22-24] During fasting FA that enter the liver can be metabolized via acetyl coenzyme A (acetyl CoA) to form ketone bodies that can serve as fuel for other tissues such as the brain.[25,26] When the flux of FA towards the liver exceeds β-oxidation capacity, this can lead to accumulation of TG in the liver. FA in the liver are continuously being re-esterified into TG. TG from this pool can be used for hepatic VLDL-TG synthesis and secretion. Actually, most of the plasma VLDL-TG pool is derived from plasma FA, re-esterified by the liver and secreted into the plasma,

whereas only a very small fraction of plasma VLDL-TG is derived from *de novo* fatty acid synthesis in the liver.[27]

Hepatic glucose metabolism

Glucose is the most important energy source in the mammalian body. Especially the brain is depending primarily on readily available glucose. Therefore, it is very important for the body to control plasma glucose concentrations tightly. The plasma glucose level is determined by the balance between dietary uptake in the intestines, glucose uptake by peripheral tissues and the production of glucose by the liver. The liver plays a very important role in the glucose homeostasis by controlling the balance of appearance and disappearance of glucose.

Storage of glucose

Excess glucose is stored as glycogen (glycogenesis) which is a very efficient storage form of glucose.[28] In the liver hexokinase, also known as glucokinase, mediates the first step of hepatic glucose metabolism, which involves the conversion of glucose into glucose-6-phosphate (G6P). G6P is an important metabolic intermediate in the glucose metabolism.[29] In the second step glycogen is produced via uridine diphosphate (UDP-) glucose. In this step glycogen synthase is the rate-controlling enzyme. Not all glucose can be stored as glycogen since the liver's storage capacity is limited. The excess glucose is broken down to pyruvate and lactate so that it can be used in *de novo* lipogenesis to produce FA.

Production of glucose

During fasting the mammalian body depends on the liver and (to a much lesser extent) the kidneys for production of glucose.[30] In the early phase of fasting the liver produces glucose by hydrolysing glycogen via 3 steps (glycolysis). First, a single glucose-1-phosphate is cleaved from glycogen mediated by glycogen phosphatase and then a debranching enzyme converts glucose-1-phosphate to G6P. Finally, G6P is dephosphorylated to glucose and this process is under control of glucose-6-phosphatase (G6Pase).[31] The liver can also form glucose from alternative substrates such as amino acids, glycerol, pyruvate and lactate to maintain stable blood glucose levels (gluconeogenesis).[32] Phosphoenolpyruvate carboxykinase (PEPCK) controls the regulation of the latter process. To protect against complete breakdown of the

glycogen stores, the liver progressively increases gluconeogenesis during prolonged periods of fasting.[33]

Insulin action

Insulin is a key regulator in both glucose and lipid metabolism. It is the most important hormone controlling hepatic glucose and VLDL-TG output.[34] Insulin is a 5.8 kDa hormone secreted by the β-cells of the islets of Langerhans in the pancreas. Insulin is secreted in a biphasic manner in response to an increase in blood glucose level. There is an initial burst of insulin secretion that lasts about 5-15 minutes, resulting from secretion by the preformed insulin secretory granules. This initial burst is followed by a more gradual and sustained insulin secretion resulting from biosynthesis of new insulin molecules.

The binding of insulin to the insulin receptor leads to a phosphorylation cascade eventually resulting in the induction of several target genes.[34] In addition to glucose, amino acids, long-chain FA and several hormones can induce insulin secretion. The main target organs for insulin are the liver, skeletal muscle and adipose tissue. The overall net result of insulin action is fuel storage of both glucose and lipids. Insulin resistance refers to the condition where a specific tissue is (or several tissues are) less sensitive to the effects of insulin.

Insulin exerts direct effects on VLDL-TG production, although the mechanism behind this phenomenon remains unclear.[35] Insulin is thought to accelerate the degradation of apoB[36] and VLDL-TG secretion is decreased when apoB availability is decreased. Insulin indirectly inhibits HVP by inhibiting HSL. The consequence of decreased TG-hydrolysis by HSL is a decreased flux of FA towards the liver resulting in decreased substrate availability for hepatic VLDL-TG output.

Hepatic glucose output (HGO) is determined by the rate of hepatic glycogen breakdown, which is regulated by G6P and by the rate of hepatic gluconeogenesis which is regulated by PEPCK. [37-39] In the fed state insulin inhibits HGO via inhibition of these two key regulatory enzymes. Insulin also stimulates glucose uptake by peripheral tissues (PGU) such as the muscle and adipose tissue. In these tissues insulin stimulates translocation of the glucose transporter-4 (Glut-4) mediating uptake of glucose.[40]

Interactions of glucose and lipid metabolism

In the liver, glucose and lipid metabolism are closely linked. In the presence of decreased glucose availability, glucose oxidation decreases and the need for the oxidation of FA increases.

Substrate availability

In 1963 the glucose/FA cycle was postulated by Randle.[41] Based on experimental evidence, this cycle states that the availability of FA determines the rate of FA oxidation and that FA oxidation directly inhibits glucose oxidation. The exact mechanism behind this interaction has not been elucidated. Several mechanisms have been proposed to explain this link between FA and glucose oxidation including the accumulation of intermediates in the FA and glucose metabolism.[42]

Some studies investigated the effects of increasing plasma FA by infusion but observed no effects on the intermediates such as citrate or G6P levels.[43-45] On the other hand the infusion of lipids or FA can induce insulin resistance leading to decreased uptake of glucose.[46] However, this does not automatically include decreased glucose oxidation. When plasma FA levels were increased during hyperinsulinemic hyperglycemic clamp conditions, no effects on glucose oxidation were observed.[47] Therefore, it was proposed that glucose availability may be the most important determinant for substrate utilisation.[48]

Transcription factors

FA derivatives can exert significant effects on transcription factors.[49] FA activate PPARα by direct binding, leading to the induction of hepatic FA oxidation.[50] FA can inhibit hepatic FA synthesis by indirectly suppressing sterol responsive element binding protein-1c (SREBP-1c), which can be induced by insulin. Fatty acid control of this transcription factor is not completely clear yet. On the other hand, glucose can activate carbohydrate responsive element binding protein (ChREBP).[51,52] Most lipogenic enzymes have response elements for binding ChREBP (ChRE) and SREBP (SRE). These two factors work synergistically to induce transcription of the lipogenic enzyme genes in the presence of glucose and insulin. Glucagon and FA can inhibit the activation of the ChRE and SRE. In this way the control of expression of lipogenic enzyme genes is regulated in an integrated manner by multiple nutrient and hormonal signals.

Taken together, FA and glucose control hepatic lipid composition and the type and quantity of lipids available for hepatic VLDL-TG production. Because the liver plays a central role in lipid metabolism these transcription factors can affect whole-body lipid composition. Ultimately, increased plasma levels of FA and glucose contribute to the onset and progression of chronic diseases such as atherosclerosis, diabetes and obesity.[53] It may well be that the interactions between glucose and FA metabolism may be dependent on the circumstances and on tissue specific mechanisms. Nevertheless, all evidence points towards important interactions between the glucose and FA metabolism.

Hepatic steatosis

TG from the diet are mainly stored in adipose tissue. These TG form the most important energy storage in mammals. In humans about 10-30% of the body weight is adipose tissue. It provides the body with a virtually limitless capacity to store TG, which is reflected by extreme forms of obesity. TG storage is evolutionary very important to allow survival during periods when food is scarce. In addition to adipocytes, the liver and skeletal muscle can accumulate TG, although to a much lesser extent. Hepatic steatosis, or fatty liver, is defined as a histopathological condition marked by increased accumulation of lipids within the hepatocytes. Several forms of hepatic steatosis can be distinguished, depending on the underlying condition and progression of the disease. Although it has been known for a long time that excessive alcohol use is associated with hepatic steatosis we focus on non-alcoholic steatosis in this thesis.

Facts and figures

In the body there is a continuous cycling and redistribution of non-oxidized FA between different organs especially in the post-absorptive state, with a central role for the interaction between the liver and the adipose tissue. When the input of FA into the liver exceeds the FA oxidation and output of VLDL-TG, hepatic steatosis occurs. During fasting or high fat feeding, hepatic steatosis can be readily induced in healthy subjects. The flexibility of the liver in the accommodation of TG had already been demonstrated in dogs in 1970.[54] Hepatic steatosis is observed frequently even in normal-weight and moderately overweight subjects.[55] The prevalence of fatty liver in the general population is estimated to be 3% to 24%, with most estimates in the 6%

to 14% range.[56] In obese and diabetic subjects the prevalence of hepatic steatosis is estimated to be much higher.[57] Patients eligible for bariatric surgery have a body mass index of ≥ 40 kg/m^2 or of ≥ 35 kg/m^2 with significant co-morbidities such as type 2 diabetes. In this population the prevalence of fatty liver is estimated to range even from 84% to 96%.[56] Nowadays, we know that in about half of the subjects hepatic steatosis can progress to fibrosis, 15% progress to cirrhosis and 3% eventually experience liver failure or need a liver transplant.[58] Apart from progression to these more severe stages of liver disease, hepatic steatosis is associated with a number of metabolic disturbances in glucose and lipid metabolism in the liver and even throughout the body. However, it remains unclear whether these disturbances are a cause and/or a consequence of the TG accumulation.

Causes of hepatic steatosis

The accumulation of TG can be caused by an increased mobilization and increased availability of FA in the circulation.[59] HSL activity in adipose tissue is regulated among others by insulin. In insulin resistant states insulin no longer (or to a lesser extent) inhibits HSL, causing too many FA to be released into the circulation.[34,60] The liver functions as a buffer and takes up the excess FA. Epinephrine and norepinephrine stimulate the mobilization of FA from adipose tissue by stimulating HSL.[61] A high fat diet or long term fasting can also cause an increased flux of FA to the liver. The CD36 knockout mouse is a mouse model that lacks the FA transporter CD36 in muscle and adipose tissue, causing an increased flux of FA to the liver. These mice show hepatic steatosis and have severely insulin resistant livers.[20]

Increased *de novo* lipogenesis in the liver can cause TG accumulation in hepatocytes.[19,59,62] In this process FA are produced from glucose. Rate-limiting enzymes in *de novo* lipogenesis are acetyl-coenzymeA carboxylase (ACC) and fatty acid synthase (FAS). These enzymes are stimulated under fed conditions by insulin and high carbohydrate diets. Glucagon inhibits endogenous FA synthesis. Cortisol inhibits FAS and endogenous FA synthesis in the liver while it stimulates TG lipolysis in the circulation by stimulating LPL activity.

Increased esterification of FA can also lead to TG accumulation.[59] TG in the hepatocytes are not an inert storage but are continuously recycled.[63] Intracellular TG are lipolyzed in larger quantities than necessary to form VLDL-TG. The FA that are

not build into VLDL are re-esterified into TG and are transported back into the cytoplasmatic pool. When this equilibrium is disturbed, TG accumulation can occur.
Decreased secretion of VLDL-TG by the liver can cause accumulation of TG. VLDL-TG production is regulated by several factors as has been discussed previously. An important factor regulating VLDL-TG production is the size of the intracellular TG pool, but limited synthesis or availability of any of the important components of VLDL-TG can inhibit the production of the particle. ApoE stimulates the secretion of VLDL-TG.[64]
Finally, decreased mitochondrial β-oxidation can be the cause of hepatic steatosis.[65] Studies in children with inborn deficiencies in one or more enzymes of the FA oxidation pathway have shown that, when there is a need for increased β-oxidation during short term fasting (for example during infection), this often ends fatal with an enormous accumulation of TG in the liver.[66] Insulin inhibits and glucagon stimulates mitochondrial β-oxidation in the liver.
Taken together, the accumulation of TG within hepatocytes is caused by a disturbed equilibrium between liver TG synthesis and secretion.[67] An increased flux of FA to the liver from adipose tissue, dietary intake or endogenous synthesis can lead to accumulation of TG in the hepatocytes when mitochondrial β-oxidation and VLDL-TG secretion and production are not capable of processing all incoming FA.

Metabolic consequences of hepatic steatosis

Hepatic steatosis is not only a consequence of metabolic disturbances, but steatosis *per se* can also have profound effects on lipid and glucose metabolism and cardiovascular risk factors. Accumulation of TG in the liver is strongly associated with hepatic insulin resistance.[68] This is associated with cardiovascular risk factors such as hyperglycemia, hypertriglyceridemia, and elevated levels of alanine transferase (ALT), fibrinogen, C-reactive protein (CRP), plasminogen activator inhibitor-1 (PAI-1) and factor VII (Figure 3).[68]

Effects on lipid metabolism

In insulin resistant states the liver becomes less sensitive to the inhibitory effect of insulin on hepatic VLDL-TG production. This contributes to dyslipidemia in insulin resistant states. The inability of insulin to accelerate the degradation of apoB results in the overproduction of VLDL-TG particles. This contributes to the frequently

observed diabetic hypertriglyceridemia. Insulin also has peripheral effects on the lipid metabolism, since it normally regulates the expression of LPL, resulting in a net storage of lipids into the adipose tissue. In insulin resistant states VLDL-TG particles remain longer in the circulation because insulin does not (or to a lesser extent) induce LPL-expression. This allows more transfer of TG to LDL and HDL particles by cholesteryl ester transfer protein (CETP).[69] When CE-depleted TG-rich LDL particles are hydrolyzed by LPL and HL, this leaves small dense LDL particles which are highly atherogenic. Eventually the frequently observed diabetic dyslipidemia is established that poses an increased risk for cardiovascular disease.

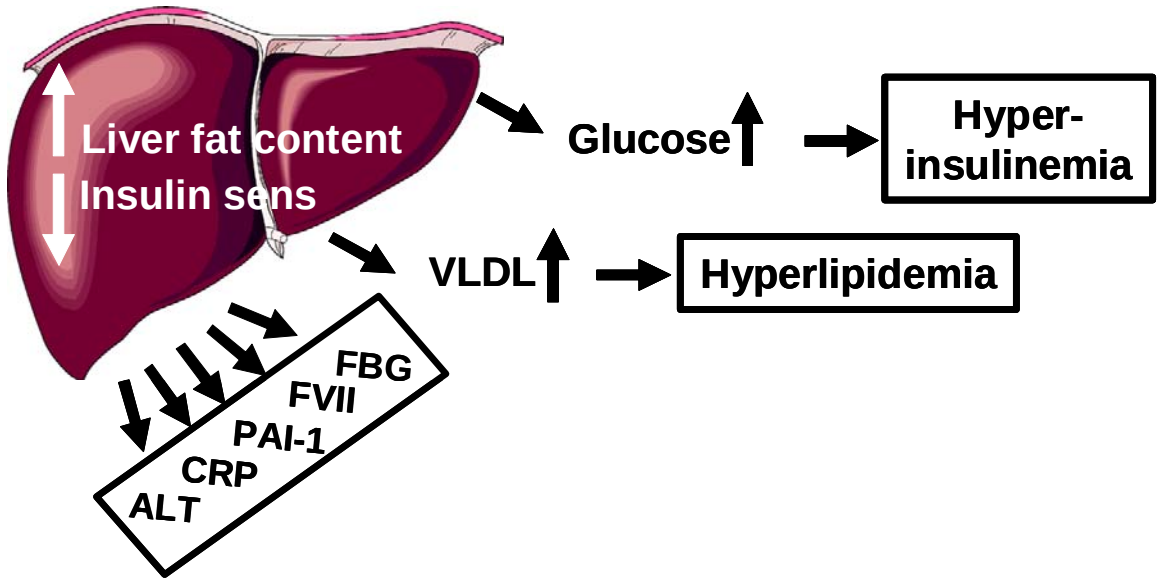

Figure 3. The fatty liver overproduces cardiovascular risk factors. Hepatic steatosis is strongly associated with insulin resistance. The insulin resistant liver overproduces glucose, VLDL-TG and other factors known to associate with enhanced cardiovascular risk such as alanine transferase (ALT), fibrinogen (FBG), C-reactive protein (CRP), plasminogen activator inhibitor-1 (PAI-1) and factor VII (FVII).

Effects on glucose metabolism

Decreased insulin sensitivity associated with increased hepatic lipid content has major effects on the glucose metabolism. The suppressive effect of insulin on G6Pase and PEPCK expression levels is decreased. This will lead to more glycogen breakdown and more gluconeogenesis and consequently increased hepatic glucose output. Postprandially insulin normally decreases the output of glucose and increases uptake of glucose by peripheral tissues. Consequently, plasma glucose levels are

well controlled. In subjects with fatty liver a higher output of glucose may exist.[68] Because insulin action is impaired postprandially (decreased suppression of liver output) the glucose output is less suppressed by insulin. Peripheral tissues still take up glucose, however the increased (relatively) steady glucose levels cause diabetic adverse effects. The pancreas compensates by increased insulin secretion (hyperinsulinemia). In time β-cell failure will occur and the body can no longer compensate for insulin resistance with extra insulin secretion. This results in hyperglycemia despite hyperinsulinemia and this state is referred to as type 2 diabetes mellitus.

Outline of this thesis

The studies described in this thesis focus on the metabolic consequences of hepatic steatosis on lipid and glucose metabolism. Many interactions between the glucose and lipid metabolism exist. Research usually tends to focus on glucose metabolism with regard to insulin resistance and type 2 diabetes mellitus. However, concomitant disturbances in lipid metabolism may be of great importance for the major clinical endpoints such as cardiovascular disease. In this thesis we investigated the integrated role of the glucose and lipid metabolism from the perspective of the liver.

Hepatic steatosis is frequently observed and is associated with a number of cardiovascular risk factors.[70-73] From observations in humans it remains unclear to what extent hepatic steatosis is a cause rather than a consequence of the metabolic syndrome. In this thesis several mouse models of steatosis with targeted disruptions of the fatty acid and TG metabolism are used to study these causes and consequences of fatty liver. We reviewed reported studies in rodent models of hepatic steatosis in Chapter 2.

Since in humans the liver is not readily accessible and study protocols can be limiting, mouse models are often used to investigate mechanisms of insulin resistance. The C57/Black 6 mouse is a wild type mouse model that is sensitive to diet-induced hyperlipidemia, obesity and insulin resistance.[74,75] In this model we compared the inhibitory effects of insulin on hepatic glucose and VLDL-TG production in Chapter 3.

In Chapter 4 the CD36-deficient mouse (*cd36* $^{-/-}$) is studied. This mouse completely lacks CD36 or Fatty Acid Transporter (FAT) in adipose tissue and muscle and cannot take up FA in these peripheral tissues.[83] Consequently, a large flux of plasma FA

towards the liver exists in these mice. Previously, our group has shown that this mouse model displays severe hepatic steatosis and has a very insulin resistant liver.[20] The peripheral tissues, however, are more sensitive to insulin-mediated stimulation of glucose uptake compared to wild type littermates. The CD36-deficient mice have increased plasma triglyceride levels. The mechanism behind the observed hypertriglyceridemia in *cd36* $^{-/-}$ mice was studied in Chapter 4.

Increased inflammatory cytokine expression levels such as IL-6 and TNFα are associated with insulin resistance.[76,77] Decreased plasma levels of IL-10 have been associated with insulin resistance in humans.[78,79] IL-10 is an immunoregulatory and anti-inflammatory cytokine that can reduce the IL-6 and TNFα production by macrophages.[80-82] To evaluate the possible effects of endogenous IL-10 secretion on insulin sensitivity we compared the effect of high fat feeding on hepatic steatosis and hepatic insulin sensitivity in wild type mouse *versus* the interleukin-10 knock-out mouse (IL-10$^{-/-}$), which completely lacks IL-10 production capacity. In this mouse model we studied the metabolic effects of the absence of IL-10 during high fat feeding (Chapter 5). Our hypothesis was that the IL-10$^{-/-}$ mice would be more insulin resistant.

In Chapters 6 and 7 we used the APOE*3-Leiden transgenic mouse to study the effects of the antiretroviral drug ritonavir (RTV) on the lipid metabolism and the development of atherosclerosis. RTV is a protease inhibitor, which is used in treatment of HIV-infection. The introduction of antiretroviral therapy has led to a significant reduction in the morbidity and mortality that was associated with HIV-infection. Unfortunately, these drugs are associated with severe adverse metabolic effects. Wasting of subcutaneous fat, with or without the accumulation of fat in the dorso-cervical and abdominal region, is frequently observed.[84] Interestingly, like in excess of adipose tissue (obesity), the wasting of subcutaneous fat (lipoatrophy) is also associated with hepatic steatosis.[68,85-87] The metabolic adverse effects also resemble the metabolic disturbances observed in obesity and include hyperlipidemia, hyperinsulinemia and hyperglycemia. RTV use specifically is renowned for the association with severe hypertriglyceridemia.[88] We used the APOE*3-Leiden transgenic mouse to study the underlying mechanisms of RTV-induced hypertriglyceridemia, because this transgenic mouse model is a very well characterized mouse model with a humanized lipoprotein profile.[89-91] Similar to humans, APOE*3-Leiden transgenic mice have a much lower clearance rate of

VLDL-TG than wild type mice. In contrast to wild type mice, these mice are susceptible to diet- and drug-induced hyperlipidemia and to obesity and atherosclerosis. Furthermore, these mice are sensitive to several lipid-lowering drugs such as statins, fibrates and PPARα- and PPARγ-agonists.[92] Consequently, the APOE*3-Leiden transgenic mouse represents a suitable model to investigate the mechanism underlying RTV-induced hypertriglyceridemia. In Chapter 6 we evaluated the cause of hypertriglyceridemia induced by RTV in APOE*3-Leiden transgenic mice. Finally, in Chapter 7, we evaluated the effect of RTV on atherosclerosis in these mice.

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Chapter 2

Hepatic Steatosis: a Mediator of the Metabolic Syndrome

Lessons from animal models

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Abstract

Epidemiological studies in humans, as well as experimental studies in animal models, have shown an association between visceral obesity and dyslipidemia, insulin resistance and type 2 diabetes mellitus. Recently, attention has been focused on the excessive accumulation of triglycerides (TG) in the liver as part of this syndrome. In this review important principles of the pathophysiological involvement of the liver in this metabolic syndrome obtained in rodent models are summarized. The current review focuses on non-alcoholic causes of steatosis, since the animal experiments we refer to, did not include alcohol as an experimental condition.

In general, there is continuous cycling and redistribution of non-oxidized fatty acids (FA) between different organs and the liver acts in concert with other organs, especially adipose tissue, in the orchestration of this inter-organ FA/TG partitioning. The amount of TG in an intrinsically normal liver is not fixed, but can readily be increased by nutritional, metabolic and endocrine interactions involving both TG/FA partitioning and TG/FA metabolism. Steatosis can also be induced by intrahepatic changes in glucose and FA/TG metabolism, independently of extrahepatic conditions. Steatosis is not merely a change in hepatic TG storage, but also reflects changes in the regulation of hepatic metabolic function. VLDL-TG production rates can be decreased, normal or increased in steatosis.

Several lines of evidence indicate that hepatic TG accumulation is also a causative factor involved in hepatic insulin resistance, defined by a decreased ability of insulin to suppress hepatic glucose production. Complex interactions between endocrine, metabolic and transcriptional pathways are involved in TG-induced hepatic insulin resistance. Therefore, the liver participates both passively and actively in the metabolic derangements of the metabolic syndrome. We speculate that similar mechanisms may also be involved in human pathophysiology.

Introduction

Epidemiological studies in humans have documented an association between visceral obesity and cardiovascular risk factors such as dyslipidemia, insulin resistance and type 2 diabetes mellitus.[1-4] Recently, attention has been focused on the excessive accumulation of triglycerides (TG) within the liver as part of this metabolic syndrome. It appears that fat accumulation in the liver is associated with several features of insulin resistance even in normal-weight and moderately overweight subjects.[5] Nonetheless, from these observations in humans it remains unclear to what extent hepatic steatosis is a cause rather than a consequence of the metabolic syndrome.

This issue is difficult to solve, since the liver is not readily accessible in humans. Therefore, we focus in the present review on mouse models with variations in liver TG content induced by targeted interventions, in order to elucidate the role of liver steatosis in metabolic diseases like dyslipidemia, insulin resistance and type 2 diabetes mellitus. Although alcohol-induced liver steatosis was already described by Thomas Addison in 1845, it is appreciated only since 1962 that steatosis can also occur without the use of alcohol, so-called non-alcoholic steatosis.[6] The current review focuses on non-alcoholic causes of steatosis, since the animal experiments we refer to, did not include alcohol as an experimental condition. We will briefly describe factors involved in body TG homeostasis, intra- and extrahepatic factors causing steatosis, the metabolic consequences of steatosis on VLDL-TG, and glucose production and potential molecular mechanisms mediating the effects of intrahepatic TG accumulation on hepatic metabolic function.

Whole-body TG homeostasis

The TG content of hepatocytes is regulated by the integrated activities of cellular molecules that facilitate hepatic TG uptake, FA synthesis, and esterification on the one hand ("input") and hepatic FA oxidation and TG export on the other ("output"). Steatosis occurs, when "input" exceeds the capacity for "output". The liver acts in concert with other organs in the orchestration of inter-organ FA/TG partitioning. Therefore, we will first describe whole body TG homeostasis.

In the absorptive state, dietary TG are transported by the blood to peripheral organs in the form of chylomicrons (Figure 1A). Lipoprotein lipase (LPL) is required for the intravascular hydrolysis of plasma chylomicron-, as well as VLDL-TG into FA.

Through the tissue-specific action of LPL the TG-derived FA are taken up mainly locally in peripheral tissues.[7] LPL is stimulated by insulin, especially in adipose tissue, and by exercise, especially in muscle. After the hydrolysis of a large part of the TGs in chylomicrons by LPL, remnant particles remain which are transported to and taken up by the liver.[8,9]

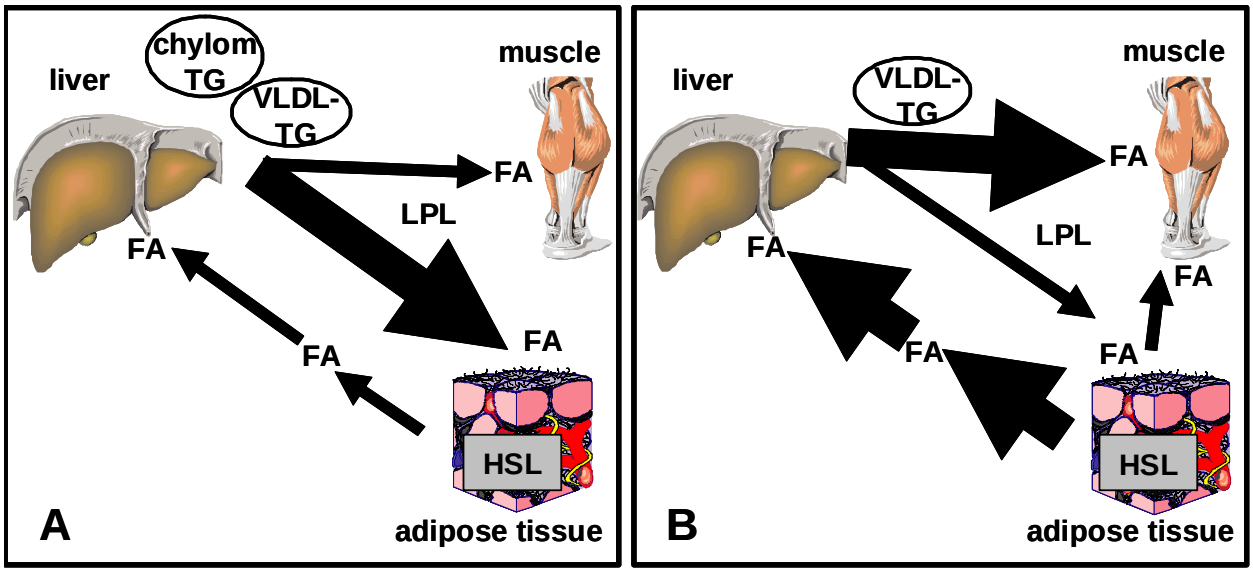

Figure 1. Diversion of fatty acids towards peripheral tissues. A. In the fed state chylomicron-triglycerides and VLDL-triglycerides are lipolyzed by lipoprotein lipase to generate fatty acids, that are mainly taken up by muscle and adipose tissue for oxidation and esterification into triglycerides, especially in the adipose tissue. **B.** In the fasting state triglycerides within the adipose tissue are lipolyzed by the enzyme hormone-sensitive lipase and fatty acids are released into the blood in excess of oxidative requirements. The excessive fatty acids can be taken up by the liver, for oxidation or for synthesis of VLDL-triglycerides. The arrows indicate the fluxes of fatty acids. FA = fatty acids, LPL = lipoprotein lipase, HSL = hormone-sensitive lipase, VLDL = very low density lipoprotein, chylom = chylomicrons derived from the intestine.

In the post-absorptive (fasting) state, whole-body TG metabolism differs from that of the absorptive state (Figure 1B). The TG contained within adipose tissue are continuously being hydrolyzed into FA and glycerol by the enzyme hormone-sensitive lipase (HSL).[10] Because HSL is inhibited by insulin, the activity of HSL increases in the low insulin state of fasting. Although some of the FA released by HSL are re-esterified within adipocytes, most FA are released into the blood and transported as free FA to other organs. In resting, i.e. non-exercise, conditions the amount of FA

released by adipose tissue is considerably larger than the amount required for oxidative purposes. In this respect the liver is of paramount importance, because the liver takes up a considerable part of these FA. Within the liver these FA are either oxidized or re-esterified into TG, which can be secreted into the blood in the form of VLDL-TG. The FA re-esterified by the liver into TG are derived almost exclusively from the FA initially released by adipose tissue.[11] In turn, VLDL-TG are directed towards different tissues, depending on the tissue-specific availability of LPL.

Thus, there is a continuous cycling and redistribution of non-oxidized FA between different organs especially in the post-absorptive state, with a central role for the liver and the adipose tissue (Figure 2).

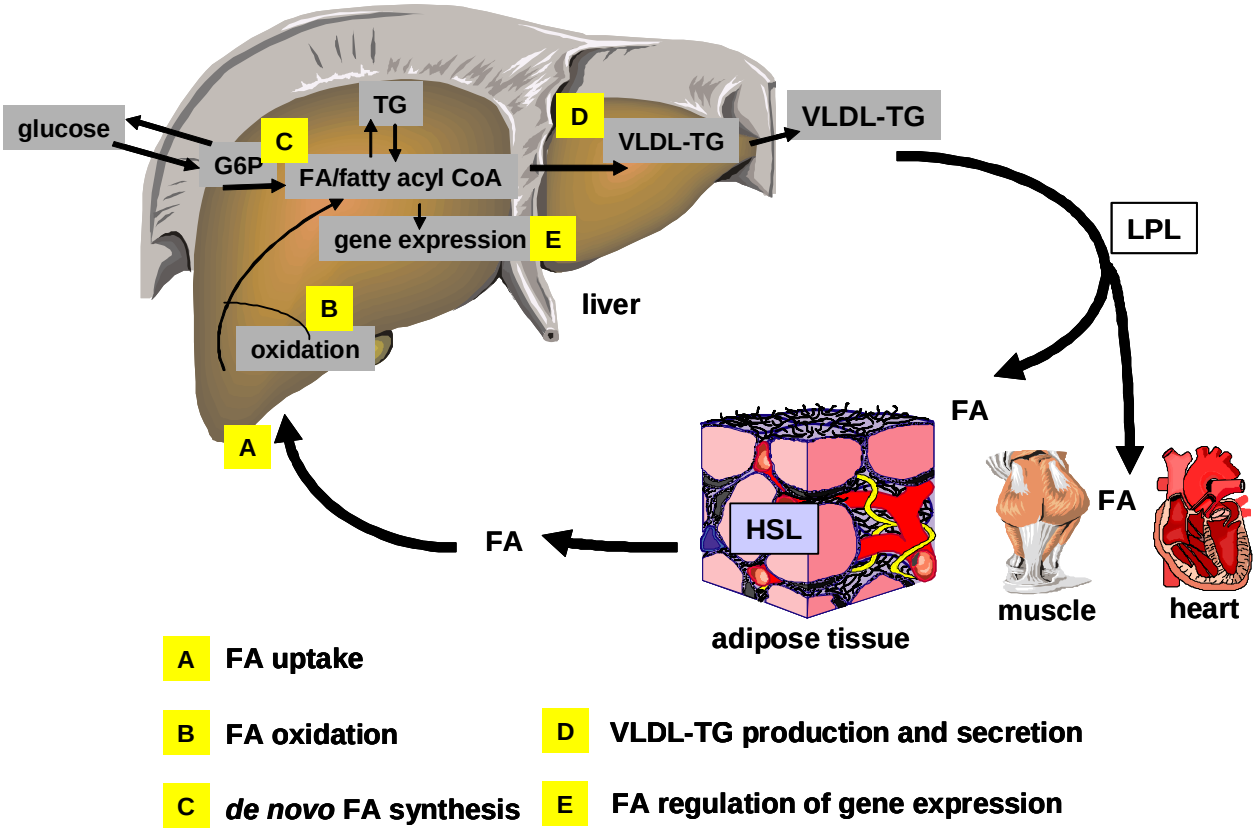

Figure 2. Major pathways of hepatic FA/TG metabolism in the liver. The liver plays a central role in lipid metabolism through **A** Uptake of fatty acids, **B** Fatty acid oxidation, **C** De *novo* fatty acid synthesis, **D** Assembly and secretion of VLDL-TG, **E** Effects of fatty acids on gene expression. FA = fatty acids, HSL = hormone sensitive lipase, LPL = lipoprotein lipase, G6P = glucose-6-phosphate.

Extrahepatic causes of steatosis

A major cause of steatosis is increased FA flux to the liver due to a high availability of plasma FA in relation to peripheral oxidative requirements. Several conditions increase the FA flux to the liver. An increase of exogenous fat, i.e. high-fat feeding, increases liver TG content.[12] This increase in hepatic TG content can occur within 10 days after starting the high fat diet in mice. Overnight fasting increases plasma FA to such an extent, that liver TG content increases in mice (unpublished observations). This flexibility of the liver to accommodate excessive plasma FA the form of hepatic TG after overnight fasting in was demonstrated already in 1970 in dogs.[13] These observations indicate that the amount of liver TG content is not fixed, but can readily be modulated by nutritional conditions in otherwise normal livers.

FA delivery to the liver can also be increased due to disturbances in FA/TG partitioning between different organs. This is illustrated by several observations. Mice lacking CD36, a FA transporter in muscle and adipose tissue, have increased plasma FA levels and show liver steatosis.[14,15] Conversely, mice lacking HSL have low plasma FA levels and low hepatic TG content.[16] Finally, muscle-specific modulation of lipoprotein lipase may result in altered distribution of tissue TG. In mice with muscle-specific LPL overexpression, muscle TG content is increased, whereas liver TG content is decreased compared to wild-type mice.[17] These observations in mouse models without excessive changes in adipose tissue mass prove that alterations in whole body FA/TG partitioning inversely modulate TG content in the liver.

The extrahepatic regulation of liver TG content is not merely a function of plasma FA delivery alone. Mouse models of lipodystrophy and models of its reverse condition, obesity, illustrate this. In both conditions, steatosis is present but can only partly be related to increased plasma FA and TG levels. However, lipodystrophy and obesity are complex conditions, with changes other than those reflected merely in the FA/TG metabolism. Adipose tissue is not only an organ designed for passive storage and release of TG. In addition, adipose tissue also actively participates in the integration of whole-body energy and fuel metabolism by the secretion of many hormones. Important hormones, which are derived from adipose tissue, and modulate hepatic TG content, are adiponectin, leptin and resistin.[18] Adiponectin decreases TG content in the muscle and liver of obese mice and decreased adiponectin levels have been implicated in the development of steatosis in mouse models of both obesity and lipodystrophy.[19] Leptin decreases the hepatic accumulation of TG in the A-ZIP/F-1

mouse, a model of severe lipodystrophy and low leptin levels.[20] Finally, tissue-specific overexpression of wild-type leptin receptors in the steatotic livers of obese (*fa/fa*) Zucker rats, which have an inactivating mutation in the leptin receptor, reduced TG accumulation in the liver but not in other non-adipose tissues. It has therefore been proposed that the physiologic role of leptinemia in conditions of caloric excess is to protect non-adipose tissue from steatosis by preventing the up-regulation of lipogenesis and increasing FA oxidation.[21] These examples indicate that an intrinsically normal liver may develop steatosis due to nutritional, metabolic and endocrine interactions involving both inter-organ TG/FA partitioning and TG/FA metabolism.

Intrahepatic causes of steatosis

Several intrahepatic mechanisms induce steatosis. These changes involve alterations in hepatic glucose and/or FA metabolism. Increased *de novo* hepatic synthesis of FA and subsequent esterification into TG is an important cause of steatosis. This is illustrated by several examples. Firstly, high sucrose feeding induces liver steatosis by increased *de novo* lipogenesis.[11,22] Secondly, inhibition of glucose-6-phosphatase by S4048 results in hepatic entrapment of glucose and *de novo* lipogenesis, leading to massive steatosis within several hours.[23] Thirdly, inhibition of FA oxidation in the liver is another intra-hepatic cause of the development of liver steatosis. For instance, etomoxir, a carnitine O-palmitoyltransferase-1 (CPT-1)-inhibitor, inhibits FA oxidation and induces steatosis.[24] These observations indicate that steatosis can be caused by intra-hepatic alterations in glucose and fat metabolism, independently of extrahepatic conditions. For a detailed summary of other rodent models with steatosis we refer to Koteish and Diehl.[24]

Steatosis and VLDL-TG secretion

A number of studies have addressed the relation between steatosis and basal VLDL-TG production in mice and rats, and *vice versa*. Inhibition of microsomal TG transfer protein (MTP) impairs the assembly and probably the secretion of VLDL-TG particles and results in intrahepatic accumulation of TG.[25] Although the inverse relation between steatosis and VLDL production is self-evident in the case of MTP blockers, in other conditions the relation between steatosis and VLDL-production is not

straightforward, which is illustrated by several examples. In obese *ob/ob* mice, which have steatosis, hepatic VLDL production is not increased, but rather even decreased.[26] This decrease in VLDL production despite the high FA flux to the liver contributes to the massive steatosis that is observed in these animals. In CD36-deficient mice the flux of FA towards the liver is increased, precipitating steatosis, but there is no evidence of an increase in hepatic VLDL production (unpublished observations). Thus, availability of FA is not the only determinant of the rate of hepatic VLDL-TG production.

In mice with increased *de novo* lipogenesis in the liver, VLDL-TG production can be either unaltered or increased probably depending on the cause of the increase in *de novo* lipogenesis and the capacity of the liver to increase FA ß-oxidation to get rid of the excess FA. The inhibition of glucose-6-phosphatase by S4048 results in an increase in *de novo* lipogenesis and hepatic TG content without any stimulation of hepatic VLDL-TG production.[23] In contrast, hamsters with increased *de novo* lipogenesis as a consequence of a diet high in fructose, have increased basal hepatic VLDL-TG production.[27] When lipogenesis is increased by pharmacological activation of the liver X receptor (LXR), hepatic VLDL-TG production is increased 2.5-fold and the liver produces large TG-rich VLDL particles.[28] Therefore, it is likely that different molecular mechanisms are involved to explain the relation between steatosis and the rate of basal VLDL production in different conditions.

Steatosis and hepatic insulin resistance

Steatosis is associated with hepatic insulin resistance, which means that the liver is less sensitive to the suppressive effects of insulin on hepatic glucose and VLDL-production.[29-32] If the ability of insulin to suppress the hepatic output of glucose and VLDL is decreased, this contributes to (postprandial) hyperglycemia and hyperlipidemia, intrinsic features of the metabolic syndrome. As such, steatosis is not only a consequence of, but also a major contributor to, the metabolic syndrome.

The inhibitory effects of insulin on VLDL production involve peripheral effects, because insulin inhibits FA release from adipose tissue, as well as the direct hepatic effects of insulin on hepatic VLDL-TG assembly/secretion.[33] Because the effects of steatosis on insulin sensitivity of hepatic VLDL-TG production are complex and have been less extensively studied than those of glucose metabolism, we focus on insulin resistance of the hepatic glucose metabolism.

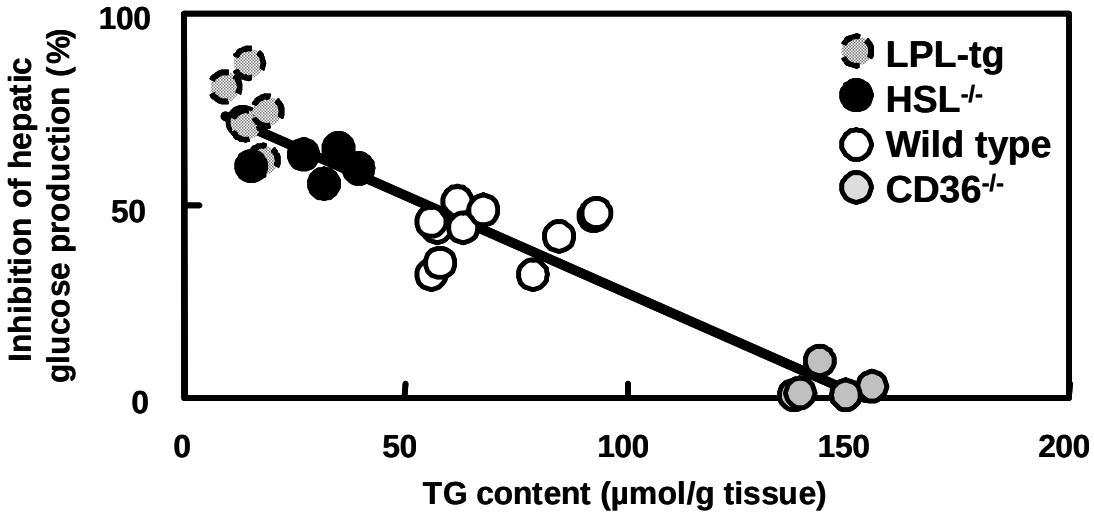

Figure 3. Insulin-mediated inhibition of hepatic glucose production is related to hepatic TG content. Muscle-specific LPL-overexpressing mice (LPL-tg) show increased TG content in the muscle, whereas liver TG content is decreased compared to wild-type mice. During a hyperinsulinemic euglycemic clamp the livers in these mice showed increased sensitivity to the suppressive effect of insulin on hepatic glucose production. Mice deficient in hormone-sensitive lipase ($HSL^{-/-}$) showed decreased hepatic TG content and increased inhibition of hepatic glucose production compared to wild-type mice. $CD36^{-/-}$ mice lacking the FA transporter that is normally present in muscle and adipose tissue, showed increased hepatic TG content and a decreased sensitivity of hepatic glucose production to insulin.[15-17]

There is an inverse relationship between hepatic TG content and hepatic insulin sensitivity (Figure 3). We observed this inverse relationship in transgenic mice with targeted disruptions in TG/FA partitioning. Interestingly, mice with decreased hepatic TG content compared to wild-type controls, such as mice with muscle-specific overexpression of LPL or $HSL^{-/-}$ mice, revealed increased insulin sensitivity.[16,17] Apparently, the relationship between hepatic TG content and insulin sensitivity holds true for both increased and decreased hepatic TG stores. The more complex mouse models of obesity, like the *ob/ob* mice, and its counterpart, the lipodystrophic mice, have steatosis with severe hepatic insulin resistance.[34-36] Adiponectin and leptin are not only capable of reversing steatosis, but also hepatic insulin resistance in these mice. These observations further strengthen the notion that hepatic TG accumulation is a causative factor involved in hepatic insulin resistance.

Paradoxically, this relationship between steatosis and insulin resistance is dissociated in some mouse models by treatment with thiazolidinediones. These PPARγ-activators improve hepatic insulin resistance despite the augmentation of steatosis in obese and diabetic mice, but not in lean controls.[37] The mechanisms that underlie this paradox have not yet been elucidated.

Molecular mechanisms involved in hepatic insulin sensitivity

Insulin acts by stimulating the insulin receptor, by sequential phosphorylation of proteins of the insulin-signaling pathway.[38] Through these proteins insulin exerts its metabolic effects, e.g. on glucose transport, glycogen synthesis and lipid synthesis. In addition, the insulin-signaling pathway interacts with transcription factors, resulting in altered transcription of a multitude of genes, involved in a variety of cellular functions.[39-41] Strong indications exist that alterations in hepatic FA/TG content modulate this insulin-signaling cascade. The expression of insulin receptors and phosphoinositol-3 kinase mediated protein kinase B (PKB) phosphorylation are considerably decreased in a mouse model with steatosis and hepatic insulin resistance, such as CD36 $^{-/-}$ mice.[15] Conversely, the expression of the insulin receptor and activation of phosphoinositol-3 kinase-mediated PKB-phosphorylation are increased in a mouse model of decreased hepatic TG content and increased hepatic insulin sensitivity, like in the HSL$^{-/-}$ mice.[16] Apparently, the inverse relationship between hepatic TG stores and insulin sensitivity is linked to the activity of the insulin-signaling cascade at a molecular level.

There are indications, that a direct interaction between FA derivatives and components of the insulin-signaling cascade are involved in the FA-induced insulin resistance.[42] FA intermediates like diacylglycerols are known to stimulate certain protein kinase Cs (PKC). PKCs promote threonine phosphorylation of the insulin receptor and its substrates, thereby blocking the insulin cascade. Furthermore, FA derivatives act as agonists and antagonists for nuclear transcription factors like PPARs, SREBPs and LXR. In addition to their regulation by different FA metabolites, these transcription factors are the targets for hormones, like insulin and leptin, growth factors, and inflammatory signals. Therefore, they appear to be a point of signaling convergence at a gene regulatory level.[43] These transcription factors profoundly alter the expression of enzymes and proteins that are involved in glucose and lipid metabolism. We postulate that these effects on gene expression include alterations in

the insulin-signaling cascade. Therefore, the understanding of the extremely complex interaction between FA derivatives and nuclear transcription factors is pivotal for understanding the relation between steatosis and the metabolic syndrome. This is illustrated by several observations in mice. PPARs are a family of nuclear receptors that have profound effects on gene expression and are involved in the modulation of glucose and lipid metabolism by complex mechanisms that are beyond the scope of this review. Nonetheless, several observations in mice point to a relationship between the activity of these receptors and hepatic insulin sensitivity. PPARα is mainly expressed in the liver. It is important in the regulation of several key enzymes in FA oxidation. PPARα$^{-/-}$ mice develop extensive hepatic steatosis after short-term fasting due to the considerably diminished hepatic oxidation capacity.[44] Drugs that activate PPARα, reduce liver TG content and improve hepatic insulin sensitivity in rodent models of liver steatosis.[45,46] Remarkably, PPARα$^{-/-}$ mice are protected against high fat induced insulin resistance.[47] This indicates that transcription factors like PPARα are involved in the interaction between hepatic FA metabolism and hepatic insulin resistance.

There are indications that inflammatory pathways are sub-clinically stimulated in insulin resistance. In tissues obtained from Zucker *fa/fa* rats, which have steatosis, basal IκB kinase β (IKKβ) activity was increased when compared to lean *fa/+* controls. IKKβ is a proximal activator of the transcription factor NF-κB. Inhibition of NF-κB by aspirin reverses hyperglycemia, hyperinsulinemia, and dyslipidemia in obese rodents by sensitizing insulin-signaling. The blunted insulin-stimulated phosphorylation of PKB in the livers of untreated Zucker rats was increased after salicylate treatment, providing a biochemical correlate for increased *in vivo* insulin sensitivity. Activation or overexpression of the IκB kinase β (IKKβ) attenuated insulin signaling in cultured cells, whereas IKKβ inhibition reversed insulin resistance.[48] These observations suggest that NF-κB may be another transcription factor, involved in steatosis-related hepatic insulin resistance.

To summarize, there are multiple endocrine, metabolic and transcriptionally active factors involved in the interaction between hepatic FA/TG metabolism and hepatic insulin sensitivity. The hierarchy between these different factors in modulating hepatic insulin sensitivity is at present unclear. Because the prevalence of the metabolic syndrome reaches endemic proportions, it is important to investigate the causes and consequences of this syndrome both in human and in animal studies. The

combination of these studies may lead to a better prevention and treatment of the metabolic syndrome.

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Chapter 3

Hepatic Glucose Production is More Sensitive to Insulin-mediated Inhibition than Hepatic VLDL-triglyceride Production

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Abstract

Insulin is an important inhibitor of both hepatic glucose output and hepatic VLDL-triglyceride (VLDL-TG) production. We investigated whether both processes are equally sensitive to insulin-mediated inhibition. To test this, we used euglycemic clamp studies with four increasing plasma concentrations of insulin in wild type C57Bl/6 mice. By extrapolation we estimated that half-maximal inhibition of hepatic glucose output and hepatic VLDL-TG production by insulin were obtained at plasma insulin levels of ~ 3.6 and ~ 6.8 ng/mL, respectively. In the same experiments, we measured that half-maximal decrease of plasma free fatty acid levels and half-maximal stimulation of peripheral glucose uptake were reached at plasma insulin levels of ~ 3.0 and ~ 6.0 ng/mL, respectively. We conclude that, in comparison to insulin sensitivity of hepatic glucose output, peripheral glucose uptake and hepatic VLDL-TG production are less sensitive to insulin.

Introduction

The liver is a very important regulator in the homeostasis of both glucose and lipid metabolism. Not only does the liver control the storage, production and secretion of glucose, it also produces and secretes very-low density lipoproteins (VLDL) and takes up VLDL-remnants, low density lipoproteins (LDL) and albumin-bound fatty acids (FA). Insulin inhibits both hepatic glucose and VLDL-TG production. It is not known, however, whether both processes are equally sensitive to insulin-mediated inhibition.

Hepatic glucose output (HGO) is determined by the rate of hepatic glycogen breakdown, which is regulated by glucose-6-phosphatase (G6Pase), and by the rate of hepatic gluconeogenesis, which is regulated by phosphoenolpyruvate carboxykinase (PEPCK). In the fed state insulin inhibits HGO via inhibition of these two key regulatory enzymes.[1-3] Insulin also stimulates glucose uptake by peripheral tissues, such as muscle and adipose tissue. In these tissues, insulin stimulates translocation of the glucose transporter-4 (Glut-4) mediating uptake of glucose.[4] Previous studies have documented different dose-response effects of insulin on the HGO and peripheral glucose uptake (PGU). Rizza *et al*.[5] showed that HGO is more sensitive to inhibition by insulin than peripheral glucose uptake is to stimulation by insulin.

Hepatic VLDL-TG production is commonly assumed to be primarily a substrate-driven process[6], but insulin also plays an important role in the regulation of this VLDL-TG production. Insulin can inhibit the hepatic VLDL-TG production via direct and indirect mechanisms. The exact mechanism remains unclear, but it is thought that insulin can directly accelerate the degradation of apoB which is necessary for VLDL-TG secretion.[7] An indirect effect of insulin is suggested to work via inhibition of hormone sensitive lipase (HSL) in adipose tissue, leading to decreased plasma levels of FA and thus, decreased flux of FA from the adipose tissue to the liver.[8] However, in a study in humans a metabolic relationship between insulin-mediated suppression of FA release from adipose tissue and FA flux to the liver on one hand, and the rate of hepatic VLDL-TG production on the other hand was not observed.[9] A study by Lewis *et al*.[10] showed that in normal individuals the acute inhibition of VLDL-TG production by insulin in vivo was only partly due to the suppression of plasma FA, and may also be due to an FA-independent process.

We investigated in wild type C57Bl/6 mice, whether HGO and hepatic VLDL-TG production are equally sensitive to insulin-mediated inhibition using the hyperinsulinemic euglycemic clamp technique[11] which was adapted to mice as described previously by our group.[12,13] We found that the HGO is much more sensitive to insulin-mediated inhibition than hepatic VLDL-TG production.

Materials and Methods

Animals

For our experiments we used 12-week old male C57Bl/6 mice that were housed under standard conditions. The mice were fed a standard mouse/rat chow diet (Hope Farms, Woerden, Netherlands) and water *ad libitum*. Mice were fasted for 2 h before the experiments and randomly assigned to respective groups which were infused with different amounts of insulin. Per group 5 to 6 animals were used. All animal experiments were approved by the Animal Ethics Committee from our institute.

Hyperinsulinemic euglycemic clamp

The clamp protocol was adapted from previously published studies performed by our group.[12,13] Food was withdrawn at 7 A.M. and at 9 A.M. the mice were anaesthetized with a combination of acetylpromazine (Vetranquil, Sanofi Santé Nutrition Animale, Libourne Cedex, France), midazolam (Dormicum, Roche, Woerden, Netherlands) and fentanyl (Fentanyl, Janssen-Cilag, Tilburg, Netherlands). An infusion needle was placed into the tail vein and basal glucose turnover rates were determined by infusion of D-[3-^{3}H]-glucose (0.6 µCi/kg.min, Amersham Biosciences, Little Chalfont, UK) alone during 45 minutes to achieve steady-state levels. After 45 and 60 minutes of infusion blood samples (60 µL) were drawn from the tip of the tail into chilled capillary tubes (Hawksley and Sons Limited, West Sussex, UK) coated with paraoxan (diethyl p-nitrophenyl phosphate, Sigma, St Louis, USA) to prevent *ex vivo* lipolysis.[14] These capillaries were kept on ice and spun for 5 min at 13.000 rpm to isolate the plasma which was snap-frozen in liquid nitrogen and stored at -20°C until analysis. After the basal period a hyperinsulinemic clamp was started with the continuous infusion of a combination of D-[3-^{3}H]glucose (0.6 µCi·kg^{-1}·min^{-1}) and insulin at the respective 4 concentrations (3.5, 7, 14 or 28 mU·h^{-1}). To maintain euglycemic blood glucose levels, exogenous glucose was infused via an adjustable infusion of a 20% D-glucose solution in phosphate-buffered saline (PBS). A blood sample (<5 µL) was taken every

10 min to monitor blood glucose (Freestyle, Disetronic Medical Systems BV, Vianen, Netherlands). When steady state blood glucose levels were reached, two blood samples (60 µL) were taken with 15 min intervals to measure hyperinsulinemic parameters of peripheral glucose uptake and HGO. After the last blood sample, Triton was injected which completely blocks lipolysis of plasma triglycerides (TG).[15] Plasma TG were measured before injection of Triton and at 30, 60 and 90 min after injection and related to the body mass of the mice. Hepatic TG production was calculated from the slope of the curve and expressed as $\mu mol \cdot h^{-1} \cdot kg\ bodyweight^{-1}$. The clamp experiments lasted approximately 4 h.

Plasma parameter analyses

Plasma glucose was measured using the glucose hexokinase method (Instruchemie, Delfzijl, Netherlands). FA and TG were determined using commercially available kits (#315 and #310-A Sigma GPO-Trinder kit, St. Louis, MA, USA) according to the manufacturer's instructions. Plasma insulin concentrations were measured by ELISA (ALPCO Diagnostics, Windham, NH, USA). To measure plasma [^{3}H]glucose, trichloroacetic acid (final concentration 10%) was added to 7.5 µL plasma to precipitate proteins using centrifugation. The supernatant was dried to remove water and resuspended in milliQ. The samples were counted by scintillation counting (Packard Instruments, Dowers Grove, IL, USA).

Calculations

Under steady-state conditions for plasma glucose concentrations, the rate of glucose disappearance equals the rate of glucose appearance. The latter ($\mu mol \cdot min^{-1} \cdot kg^{-1}$) was calculated during the basal period and under steady-state clamp conditions as the rate of tracer infusion (dpm/min) divided by the plasma specific activity of [^{3}H]glucose (dpm/µmol). The ratio was corrected for body weight. Hyperinsulinemic HGO was calculated as the difference between the tracer-derived rate of glucose appearance and the glucose infusion rate.

Statistical analysis

Results are presented as means ± SE for 5 animals per group. Differences between experimental groups were determined by the Mann-Whitney U test. The means per group were tested for linear trend (P_{trend}) with increasing insulin levels. The level of

statistical significance of the differences was set at $P < 0.05$. Analyses were performed using SPSS 12.0 for Windows software (SPSS, Chicago, USA) and Prism 4.0 (GraphPad).

Results

Plasma glucose and insulin levels and glucose infusion rates during the clamp analyses

Plasma glucose levels during the basal and hyperinsulinemic clamp period were not different between the groups (Table 1). At basal level plasma insulin levels were not different between the groups, averaging at ~1.4 ng/mL. At hyperinsulinemic conditions, steady state plasma insulin concentrations in the respective groups averaged at 2.4, 3.6, 9.3 and 22.4 ng/mL with increasing insulin infusion rates. In addition, to maintain euglycemia during the respective insulin infusion rates, glucose infusion rate (GIR) increased concomitantly, as expected (Table 1; $P_{trend} < 0.01$).

Dose-response effects of insulin on peripheral glucose uptake and hepatic glucose output

We observed no differences in basal peripheral glucose uptake between the groups (Table 2). During the hyperinsulinemic period insulin dose-dependently stimulated peripheral glucose uptake when compared to the respective basal levels, ($P_{trend} < 0.01$). Similarly, basal HGO did not differ between the groups, whereas HGO was dose-dependently inhibited by insulin during the hyperinsulinemic conditions ($P_{trend} < 0.01$).

Dose-response effects of insulin on plasma FA levels and hepatic VLDL-TG production

The decrease in plasma FA levels was determined as a measure of insulin sensitivity of adipose tissue lipolysis. Upon infusion of insulin plasma FA levels decreased dose-dependently ($P_{trend} < 0.01$; Table 3). To measure the effect of insulin infusion on hepatic VLDL-TG production at the end of the hyperinsulinemic period, all groups of mice were injected with Triton WR1339 to completely block plasma VLDL-TG lipolysis.[15] Table 3 presents that insulin infusion leads to a dose-dependent decrease in hepatic VLDL-TG production ($P_{trend} < 0.01$; Table 3).

Table 1. Plasma levels of glucose and insulin and glucose infusion rates.

Insulin Infusion ($mU \cdot h^{-1}$)	Bodyweight (g)	Plasma Glucose (mM)		Plasma Insulin (ng/mL)		GIR * ($\mu mol \cdot min^{-1} \cdot kg^{-1}$)
		Basal	*Hyper*	*Basal*	*Hyper **	
0	28.3 ± 0.9	7.5 ± 0.5	N.A.	1.1 ± 0.2	N.A.	0 ± 0
3.5	25.5 ± 0.5	7.9 ± 0.6	10.1 ±2.1	1.5 ± 0.4	2.4 ± 0.3#	13 ± 6
7	23.9 ± 0.7	8.9 ± 0.5	8.1 ± 1.3	1.1 ± 0.3	3.6 ± 0.5#	32 ± 11
14	26.0 ± 0.7	8.2 ± 0.3	8.2 ± 1.1	1.4 ± 0.3	9.3 ± 1.0#	104 ± 37#
28	27.2 ± 1.4	8.1 ± 0.5	7.3 ± 0.9	1.7 ± 0.4	22.4 ± 4.3#	152 ± 16#

Body weight was measured at the beginning of the experiment. Plasma glucose and insulin levels were measured during the basal and during the hyperinsulinemic (Hyper) period. GIR is the glucose infusion rate necessary to maintain euglycemia during hyperinsulinemia. Values represent means ± SE. (# $P < 0.05$ compared to basal group; * $P_{trend} < 0.01$; n=5-6 mice per group)

Table 2. Effects of insulin infusion on peripheral glucose uptake and hepatic glucose output.

Insulin Infusion ($mU \cdot h^{-1}$)	PGU ($\mu mol \cdot min^{-1} \cdot kg^{-1}$)		% of basal (%)*	HGO ($\mu mol \cdot min^{-1} \cdot kg^{-1}$)		% of basal (%)*
	Basal	*Hyper **		*Basal*	*Hyper **	
0	58.8±9.4	N.A.	N.A.	58.8 ±9.4	N.A.	N.A.
3.5	64.6 ±6.3	57.2 ±4.4	90 ±7.3	64.6 ±6.3	44.7 ±6.0	69 ±6.9
7	74.8 ±11.1	79.0 ±11.1	107 ±12.9	74.8 ±11.1	45.3 ±11.7	60 ±12.5
14	75.9 ±5.3	152.0 ±14.9#	202 ±19.1#	75.9 ±5.3	28.9 ±12.2#	39 ±17.3#
28	61.4 ±7.0	136.4 ±17.0#	221 ±8.1#	61.4 ±7.0	12.5 ±7.9#	21 ±13.8#

During the clamp experiment whole-body glucose uptake (PGU) and hepatic glucose output (HGO) were measured under basal and under hyperinsulinemic conditions. Values represent means ± SE. (# $P < 0.05$ compared to basal group; * $P_{trend} < 0.01$; n=5-6 mice per group)

Table 3. Effects of insulin infusion on plasma fatty acid levels and hepatic VLDL-TG production.

Insulin Infusion ($mU \cdot h^{-1}$)	FA * (mM)	% of basal (%)*	HVP * ($\mu mol \cdot h^{-1} \cdot kg^{-1}$)	% of basal (%)*
0	0.69 ± 0.06	100	159.6 ± 9.0	100
3.5	0.63 ± 0.05	91 ± 9	189.5 ± 19.5	119 ± 12
7	0.37 ± 0.05#	54 ± 9#	158.1 ± 17.6	99 ± 11
14	0.24 ± 0.03#	35 ± 4#	90.3 ± 6.0#	57 ± 4#
28	0.25 ± 0.02#	36 ± 3#	83.3 ± 7.7#	52 ± 5#

After the clamp experiment plasma fatty acid levels (FA) and hepatic VLDL-TG production-HVP) rate were measured under basal and under hyperinsulinemic conditions. Values represent means ± SE. (#$P < 0.05$ compared to basal group; * $P_{trend} < 0.01$; n=5-6 mice per group)

Table 4. Plasma insulin levels at half-maximal effect.

Parameter	Plasma insulin level (ng/mL)
FA	3.0
HGO	3.6
PGU	6.4
HVP	6.8

The half-maximal effect of insulin was determined for each parameter during hyperinsulinemic clamp studies. We estimated the half-maximal effect by extrapolation from the curves using the numbers presented in Table 2 and 3. FA = plasma FA, HGO = hepatic glucose output, PGU = peripheral glucose uptake, HVP = hepatic VLDL-TG production.

Comparison of peripheral glucose uptake, plasma FA decrease, HGO and hepatic VLDL-TG production regarding insulin sensitivity

Taken together, the data presented in Tables 2 and 3 clearly indicate that increasing plasma concentrations of insulin lead to a dose-dependent increase in peripheral glucose uptake (Figure 1A) and a dose-dependent decrease in adipose tissue FA

release (Figure 1B). Simultaneously, we measured in the same animals that in the liver the HGO (Figure 1C) and hepatic VLDL-TG production were inhibited dose-dependently (Figure 1D). For comparison of the dose-response characteristics of each of these effects of insulin, we estimated by extrapolation the insulin concentrations at which the half-maximal inhibitory or stimulatory effect was reached for these respective parameters (Table 4). It is obvious, that in the periphery FA release from adipose tissue is more sensitive to plasma insulin than peripheral glucose uptake. In the liver, HGO is more sensitive to plasma insulin levels than hepatic VLDL-TG production.

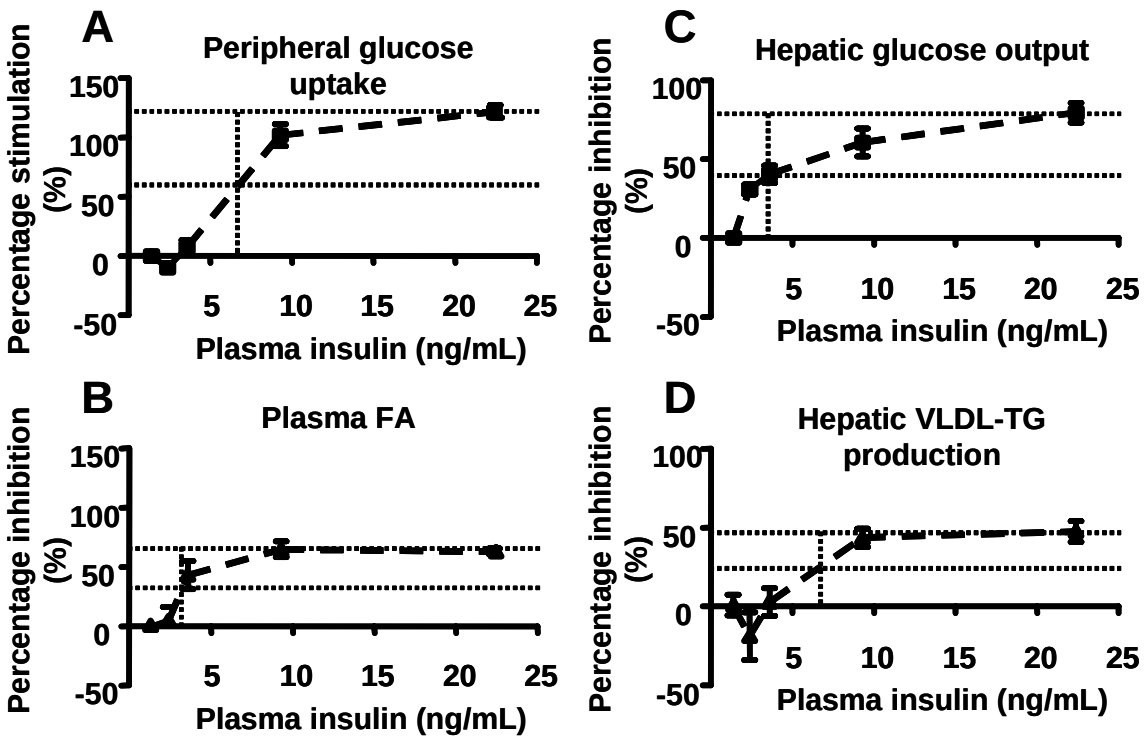

Figure 1. Hepatic glucose production is more sensitive to insulin-mediated inhibition than hepatic VLDL-TG production. During a hyperinsulinemic euglycemic clamp experiment with different plasma insulin concentrations per group we measured the stimulation of peripheral glucose uptake (**A**) and the decrease in plasma FA (**B**). Simultaneously we measured the insulin-mediated inhibition of hepatic glucose production (**C**) and of hepatic VLDL-TG production (**D**). The dotted lines indicate the maximal and half-maximal effect of insulin.

Discussion

Insulin inhibits both hepatic glucose output and VLDL-TG production. So far it was not known, whether both processes are equally sensitive to insulin-mediated inhibition. In the current study we addressed this question and found that the HGO is much more sensitive to insulin-mediated inhibition than hepatic VLDL-TG production.

Since in humans the liver is not readily accessible, mouse models are often used to investigate mechanisms of insulin resistance. The C57Black/6 mouse is a model that is sensitive to diet-induced obesity and insulin resistance.[16,17] Therefore we chose to use these mice for our studies of the glucose and lipid metabolism. In general there are three approaches to perform hyperinsulinemic clamp studies in mice *in vivo*. Some groups use free moving mice with preimplanted catheters [18], other groups use awake but restrained mice [19], and some groups use anesthetized mice.[12,13] Each approach has some limitations. In freely moving mice the effects of movement on the data of interest have to be taken into account. In restrained mice, the endocrine and neural effects of stress through restrainment will affect the data of interest. Finally, in anesthetized mice the effects of anesthetics on the parameters of interest have to be taken into account. Although formal studies comparing the three methods have not been published, it is clear from the publications that each approach is able to detect alterations in insulin effects induced by appropriate interventions. We performed the hyperinsulinemic euglycemic clamp experiments in anesthetized mice. Prior to the current study we compared different combinations of anesthetics. Using a combination of acetylpromazine, midazolam and fentanyl we observed no unwanted adverse effects of the anesthetics on glucose, lipid and insulin concentrations (den Boer *et al.* unpublished results). Nonetheless, we can not exclude the possibility that the exact dose-response relationships of insulin might be slightly different when one of the two other methods of hyperinsulinemic clamp experiments would have been used. However, our data on the relation between insulin concentrations and the parameters of glucose metabolism resemble those of previous studies.

By using this animal model we were able to measure the effect of insulin on HGO and hepatic VLDL-TG production, and in the same time also on peripheral glucose uptake and plasma FA levels, the latter as a measure for insulin sensitivity of adipose tissue lipolysis. Although an exact extrapolation for determination of half-maximal effect could not be made, Figure 1 shows that plasma FA levels, peripheral glucose uptake, HGO and hepatic VLDL-TG production differ in insulin sensitivity. By

comparison of the insulin levels at the half-maximal effect, we observed that HGO is more sensitive to insulin-mediated regulation than peripheral glucose uptake. This is in concordance with the study of Rizza *et al.*[5], who showed in humans that half-maximal suppression of HGO occurs at insulin levels of 29 µU/mL (~ 0.9 ng/mL), while half-maximal stimulation of peripheral glucose uptake occurs at 55 µU/mL (~ 1.8 ng/mL). Furthermore, the suppression of plasma FA appears to be much more sensitive to insulin than the stimulation of peripheral glucose uptake. In fact adipose tissue lipolysis and peripheral glucose uptake are two completely different processes. While lipolysis by HSL takes place in adipose tissue only, insulin-stimulated peripheral glucose uptake occurs both in adipose tissue and in muscle. Therefore, it is not possible to quantitatively compare these peripheral parameters regarding their regulation by insulin under these conditions.

The observation that hepatic VLDL-TG production is much less sensitive to the inhibitory effect of insulin than HGO suggests, that these two processes are regulated differentially. In the regulation of HGO insulin inhibits the forkhead box Other-1 (FoxO1) which binds to promoter regions of genes encoding the enzymes G6Pase and PEPCK[20], which are important regulators of glycolysis and gluconeogenesis respectively.[1-3] The molecular mechanism underlying the insulin-mediated suppression of hepatic VLDL-TG production is not completely clear. Studies have shown that insulin can inhibit the lipidation of pre-VLDL via inhibition of microsomal TG transfer protein (MTTP).[21,22] MTTP is the enzyme that catalyzes the fusion of the pre-VLDL with a lipid droplet, thereby rendering the pre-VLDL into a mature VLDL particle ready for secretion. In addition, *in vitro* studies have shown that insulin stimulates the degradation of apoB in hepatocytes.[23-25] Decreased intracellular apoB availability leads to a decreased hepatic VLDL-TG production. Furthermore, insulin is known to inhibit HSL in adipose tissue, leading to decreased plasma levels of FA and thus, to decreased flux of FA from adipose tissue to the liver, which will eventually decrease FA re-esterification into TG in hepatocytes.[8] It has indeed been shown, that in the presence of hyperinsulinemia the liver secretes less and smaller VLDL particles.[26] However, in a study in humans an association between insulin-mediated suppression of FA release from adipose tissue and FA flux to the liver on one hand, and the rate of hepatic VLDL-TG production (estimated from the mono-exponential slope of VLDL-TG [$^2H_5$]glycerol enrichment) on the other hand, was not observed.[9] Another semiquantitative study in humans also showed that in normal individuals the

acute inhibition of VLDL-TG production by insulin *in vivo* is only partly due to the suppression of plasma FA.[10] In accordance, in the current study, we could not find a significant correlation between decrease in plasma FA levels and decrease in hepatic VLDL-TG production during hyperinsulinemia. Apparently, plasma FA levels and FA availability to the liver *per se* do not determine hepatic VLDL-TG production. In accordance with this notion, we have previously shown that acute redirection of hepatic FA flux from β-oxidation to storage does not affect hepatic VLDL-TG production.[27] We suggest that under the conditions of our experiment insulin exerts direct effects on hepatic VLDL-TG production which are apparently of greater importance than the indirect effects via suppression of FA release from adipose tissue or FA availability in general, at least under the conditions of our experiments. We hypothesize that hepatic VLDL-TG production is inhibited by insulin via a combination of the three different mechanisms described above and may therefore be less sensitive to insulin.

Metabolic zonation may also be a factor involved in the difference in insulin sensitivity of HGO *versus* hepatic VLDL-TG production. Hepatic metabolic pathways are not uniformly distributed across the liver.[28] Within the liver acinus different zones exist. In the efferent perivenous zone more FA synthesis takes place and the activity of acetyl-CoA carboxylase is much higher than in the afferent periportal area. The perivenous zone also has a larger capacity to re-esterify exogenous FA into TG. Carbohydrate metabolism also differs between the two areas. Glucose uptake for glycogen synthesis mainly occurs in the perivenous zone, whereas the generation of glucose via glycogenolysis and gluconeogenesis occurs mainly periportally. Furthermore, although insulin receptor mRNA is homogenously distributed in the liver acinus, insulin receptor protein is mainly expressed in the perivenous area in rat liver.[29] How these differences in metabolic zonation may be reflected in differential regulation of HGO and hepatic VLDL-TG production by insulin is subject to speculation.

In summary, our study shows that HGO is much more sensitive to insulin-mediated inhibiton than hepatic VLDL-TG production. This is of major importance for the use of the golden standard of measuring insulin sensitivity: the hyperinsulinemic euglycemic clamp technique. A low insulin dose already suppresses HGO, while no effect on hepatic VLDL-TG production may be observed. Infusion of high insulin dosages may

lead to the overlooking of subtle differences in hepatic insulin sensitivity, especially with regard to the HGO.

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Chapter 4

CD36 Deficiency in Mice Impairs Lipoprotein Lipase-Mediated Triglyceride Clearance

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Abstract

CD36 is involved in high affinity peripheral fatty acid (FA) uptake. Mice lacking CD36 exhibit increased plasma FA and triglyceride (TG) levels. The aim of the present study was to elucidate the cause of the increased plasma TG levels in CD36-deficient (*cd36* $^{-/-}$) mice. *Cd36* $^{-/-}$ mice showed no differences in hepatic VLDL-TG production or intestinal [^{3}H]TG uptake as compared to wild type littermates. Importantly, the postprandial TG response upon an intragastric fat load was enhanced 2-fold in *cd36*$^{-/-}$ mice compared to wild type mice (13 ± 6 vs 7 ± 2 mM.h; $P < 0.05$), with a concomitant 2.5-fold increased FA response (20 ± 6 vs 8 ± 1 mM.h; $P < 0.05$), suggesting that the elevated FA in *cd36* $^{-/-}$ mice may impair LPL-mediated TG hydrolysis. Postheparin plasma lipoprotein lipase (LPL) levels were not different between *cd36* $^{-/-}$ and wild type mice. However, the *in vitro* LPL-mediated TG-hydrolysis rate as induced by postheparin plasma of *cd36* $^{-/-}$ mice in absence of excess FA-free BSA was reduced by 51% compared to wild type littermates (0.13 ± 0.06 vs 0.27 ± 0.07 nmol oleate/mL/min $P < 0.05$). This inhibition was relieved upon addition of excess FA-free BSA. To study whether LPL activity can be decreased *in vivo* via product inhibition by FA, we increased plasma FA in wild type mice by infusion and showed that the plasma half-life of glycerol tri[^{3}H]oleate-labeled VLDL-like emulsion particles was increased 2.5-fold (t½ = 17.5 ± 10.4 vs 7.0 ± 2.6 min, $P < 0.05$) as compared to vehicle-infused mice.

We conclude that the increased plasma TG levels observed in *cd36* $^{-/-}$ mice do not result from an increased hepatic VLDL-TG production or intestinal lipid absorption, but are caused by decreased LPL-mediated hydrolysis of TG-rich lipoproteins resulting from FA-induced product inhibition of LPL.

Introduction

CD36, also known as fatty acid translocase (FAT), is a receptor for several ligands, including oxidized LDL and long-chain FA.[1-5] Abumrad *et al*.[1] showed that CD36 is abundant in peripheral tissues active in FA metabolism, such as adipose tissue, skeletal muscle, and cardiac muscle, where it is involved in high-affinity uptake of FA.[1,6,7] To directly investigate a role for CD36 in lipid metabolism, mice lacking CD36 were generated by gene-targeting.[8] These CD36-deficient (*cd36* $^{-/-}$) mice exhibited increased plasma FA and triglyceride (TG) levels.[8] Coburn *et al*.[9] showed that FA uptake was considerably impaired in muscle and adipose tissue of CD36-deficient mice. Febbraio *et al*.[8] further showed that the increase in plasma TG levels in the absence of CD36 was primarily due to an increase in VLDL-sized particles. Although these data suggest a role for CD36 in TG metabolism in addition to FA metabolism, the exact mechanism(s) underlying the increased TG levels in *cd36* $^{-/-}$ mice is (are) unknown. It has been discussed by Hajri *et al.*[10] that the VLDL-TG production rate may be enhanced in CD36-deficient mice, but the increased plasma TG levels may also be due to increased intestinal lipid absorption or a decreased lipoprotein lipase (LPL)-mediated TG clearance from the circulation.

Therefore, the aim of the present study was to elucidate the cause of the hypertriglyceridemia in CD36-deficient mice *in vivo*. Our results show that the increased plasma TG levels in *cd36* $^{-/-}$ mice are caused by a decreased TG hydrolysis rate, rather than by differences in the production of hepatic VLDL-TG or intestinal lipid absorption. From the present study we conclude that the hypertriglyceridemia observed in *cd36* $^{-/-}$ mice is caused by decreased LPL-mediated hydrolysis of TG-rich lipoproteins resulting from FA-induced product inhibition.

Materials and Methods

Animals

CD36-deficient mice were generated by targeted homologous recombination and crossed back 6 times to C57Bl/6 background.[8] Male and female *cd36* $^{-/-}$ mice (4-6 months of age) were used with wild type littermates (*cd36* $^{+/+}$) as controls. They were housed under standard conditions with free access to water and food (standard rat-mouse chow diet, Standard Diet Services, Essex, UK). Principles of laboratory animal care were followed and the animal ethics committee of our institute approved all animal experiments.

Plasma TG and FA analysis

To determine plasma lipid levels, tail vein blood was collected from male *cd36* $^{-/-}$ and *cd36* $^{+/+}$ mice, after 4 h and 16 h fasting, into chilled paraoxon-coated capillary tubes to prevent ongoing lipolysis.[11] These tubes were placed on ice and immediately centrifuged at 4°C. Plasma levels of TG (without free glycerol) and FA were determined using the commercially available kits #337-B Sigma GPO-Trinder kit (Sigma, St. Louis, MA, USA) and Nefa-C kit (Wako Chemicals GmbH, Neuss, Germany), respectively.

Hepatic VLDL-TG production

After an overnight fast, *cd36* $^{-/-}$ and *cd36* $^{+/+}$ male mice were anesthetized (0.5 mL/kg hypnorm; Janssen Pharmaceutical, Beerse, Belgium and 12.5 mg/kg midazolam; Roche, Mijdrecht, The Netherlands), and injected intravenously into the tail vein with 500 mg Triton WR1339 per kg body weight as a 10% solution in 0.9% NaCl, which virtually completely inhibits serum lipoprotein clearance.[12] Blood samples were drawn at 0, 15, 30, 60, and 90 min after the Triton injection and TG concentrations were determined in plasma as described above and related to the body mass of the mice.

Intestinal lipid absorption

To study the intestinal lipid uptake, *cd36* $^{-/-}$ and *cd36* $^{+/+}$ female mice were injected intravenously with 500 mg Triton WR 1339 per kg body weight as a 10 % solution in 0.9% NaCl. Directly after the Triton injection, mice were given an intragastric 200 µL olive oil bolus with 7 µCi glycerol-tri[^{3}H]oleate ([^{3}H]triolein; Amersham, Little Chalfont, United Kingdom). Blood samples were drawn at 30, 60, 90, 120, 180, and 240 min after bolus administration, and the amount of ^{3}H-radioactivity was determined in plasma. TLC analysis revealed that > 90% of the label appeared in the TG fraction. Plasma volumes were calculated according to Rensen *et al.*[13]

Intragastric fat load

To investigate the handling of postprandial TG, male *cd36* $^{-/-}$ and *cd36* $^{+/+}$ mice, after 2 weeks on a high fat diet and an overnight fast, were given an intragastric 200 µL olive oil bolus. Blood samples were drawn at 0, 1, 2, 4, 6, and 8.5 h after bolus administration, and FA and TG concentrations were determined in plasma as described above and corrected for the plasma FA and TG levels at t=0.

Plasma LPL and hepatic lipase levels

Plasma was obtained from male *cd36* $^{-/-}$ and *cd36* $^{+/+}$ mice, after 2 weeks on a high fat diet (46.2% of the calories as fat, Hope Farms, Woerden, the Netherlands) and an overnight fast, at 10 min after a tail vein injection of heparin (0.1 U/g body weight, Leo Pharma BV, Weesp, The Netherlands). To prevent excessive plasma lipolysis the capillaries we used to sample the postheparin plasma were kept on ice, spun immediately at 4°C and snap-frozen in liquid nitrogen. Plasma LPL and hepatic lipase (HL) levels were determined in postheparin plasma as described.[14] In short, the lipolytic activity of plasma was assessed by determination of [^{3}H]oleate production upon incubation of plasma with a substrate mix containing an excess of both [^{3}H]triolein and FA-free BSA as FA-acceptor. HL and LPL activities were distinguished in the presence of 1 M NaCl, which specifically blocks LPL.

Modulated plasma LPL and HL activities

Plasma was obtained from male *cd36* $^{-/-}$ and *cd36* $^{+/+}$ mice, after 2 weeks on a high fat diet and an overnight fast, at 10 min after a tail vein injection of heparin (0.1 U/g). The effect of the FA content of plasma on the activity of LPL and HL in postheparin plasma was determined by [^{3}H]oleate production during incubation of plasma with [^{3}H]triolein-labeled 75 nm-sized VLDL mimicking protein-free emulsion particles essentially as described previously.[15] Hereto, mouse plasma (final concentration 2.5%, v/v) was incubated with emulsion particles (final concentration 0.5 mg TG/mL) in the absence and presence of excess FA-free BSA (final concentration 60 mg/mL) in a total volume of 200 µL of 0.1 M Tris pH 8.5. Generated [^{3}H]oleate was quantified after extraction.[15] Under these assay conditions, TG derived from mouse plasma contributed only marginally to the total TG present in the incubations (approx. 1%).

Clearance of TG-rich VLDL-like emulsion particles

[^{3}H]Triolein-labeled VLDL-like emulsion particles were prepared as described previously.[15] Fed wild type male mice were anaesthetized and an infusion needle was placed into the tail vein. The infusion of FA (0.75 µmol [^{3}H]oleate/min/mouse) or vehicle was started and after 30 min and 1 h blood samples were drawn to determine plasma FA and TG. One hour after the start of infusion of FA or vehicle a bolus of [^{3}H]triolein-labeled VLDL-like emulsion particles was injected. At 2, 5, 10 and 15 min after the bolus injection blood samples were drawn and the clearance of ^{3}H-activity

from the plasma was determined by scintillation counting and corrected for plasma volumes.[13]

Statistical analysis

The Mann-Whitney nonparametric test for 2 independent samples was used to define differences between *cd36* $^{-/-}$ and *cd36* $^{+/+}$ mice. The criterion for significance was set at $P<0.05$. All data are presented as means ± SD.

Results

Increased plasma TG levels in cd36 $^{-/-}$ *mice*

In accordance with previously published data[8,9], *cd36* $^{-/-}$ mice bred at our local facility exhibited significantly increased fasting plasma FA levels compared to wild type littermates (0.89 ± 0.07 and 0.52 ± 0.09 mM, respectively; $P<0.05$). Table 1 summarizes the plasma TG levels in male *cd36* $^{-/-}$ and wild type mice as observed by us and others.[8,10] On average, *cd36* $^{-/-}$ mice exhibited significantly 1.3-1.4-fold increased plasma TG levels compared to wild type mice after various fasting periods and dietary treatments (Table 1).

Table 1. Effect of CD36-deficiency on plasma TG levels (mM)

Diet	**chow**	**chow**	**chow** [8]	**chow** [10]	**fructose** [10]	**high fat** [10]
Fasting period	**4h**	**16h**	**8-12h**	**16h**	**16h**	**16h**
cd36 $^{+/+}$	0.32 ± 0.08	0.16 ± 0.04	1.12 ± 0.21	0.56 ± 0.18	0.61 ± 0.13	0.40 ± 0.03
cd36 $^{-/-}$	0.41 ± 0.03*	0.39 ± 0.10*	1.58 ± 0.38*	0.76 ± 0.16*	0.88 ± 0.17*	0.50 ± 0.03*

Triglyceride (TG) levels were measured in plasma of *cd36* $^{+/+}$ and *cd36* $^{-/-}$ male mice after various fasting periods, and compared with data obtained by Febbraio *et al.*[8] and Hajri *et al.*[10] after correction for molecular weight (*MW* 870) and conversion of SE into SD values. The fructose diet consisted of 60% fructose, 20% protein, and 7% fat as soybean oil.[10] The high fat diet contained 18.2% sucrose, 33% casein, and 32% safflower oil.[10] Mice were fed fructose and high fat diets for 12 and 16 weeks, respectively.[10] Values represent the mean ± SD per group, *$P < 0.05$

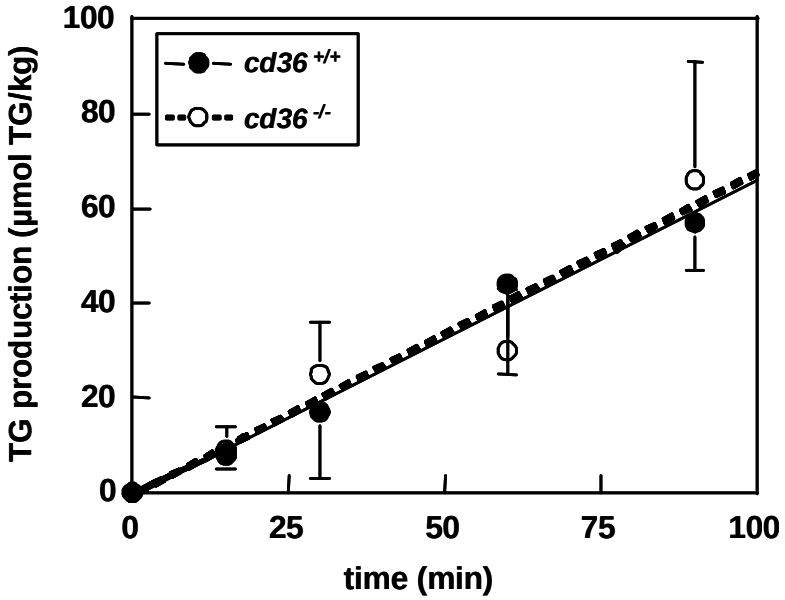

Figure 1. Effect of CD36 deficiency on VLDL-TG production rate. Triton WR 1339 (500 mg/kg body weight) was injected iv into mice which had fasted overnight. Plasma triglyceride (TG) levels were determined at 15, 30, 60, and 90 minutes and related to the body mass of the mice. Values represent means ± SD of 3 *cd36* $^{+/+}$ and 4 *cd36* $^{-/-}$ mice.

Hepatic VLDL-TG production is not affected in CD36 deficiency

The increased plasma TG levels in *cd36* $^{-/-}$ mice can be due either to i) increased hepatic VLDL-TG production, ii) increased intestinal lipid absorption, or iii) decreased lipolysis and/or clearance of TG from the circulation. To evaluate the effect of CD36-deficiency on hepatic VLDL-TG production, *cd36* $^{-/-}$ mice and wild type mice were injected with Triton WR1339 to block LPL-mediated TG hydrolysis, and the accumulation of endogenous VLDL-TG in plasma was monitored over time. Figure 1 shows that CD36 deficiency did not affect the VLDL-TG production rate (40.9 ± 12.9 versus 40.2 ± 1.9 µmol TG·kg^{-1}·h^{-1}). Consistently, we did not observe any difference in the composition of nascent VLDL-TG that was isolated at 90 min after Triton WR1339 treatment (not shown).

Intestinal lipid absorption is not affected in CD36 deficiency

We next investigated whether the increased plasma TG levels in CD36 deficiency could be due to increased intestinal lipid absorption. Hereto, *cd36* $^{-/-}$ and wild type mice were administered an intragastric load of [^{3}H]triolein-containing olive oil after injection of Triton WR1339, and the appearance of ^{3}H-label in plasma was monitored

over time (Figure 2). It appeared that, after a lag-phase of approximately 30 min, ^{3}H-label gradually appeared in plasma of *cd36* $^{-/-}$ and wild type mice at a similar rate of 4.1 ± 1.4 and 3.5 ± 1.3% of bolus·h^{-1}, respectively.

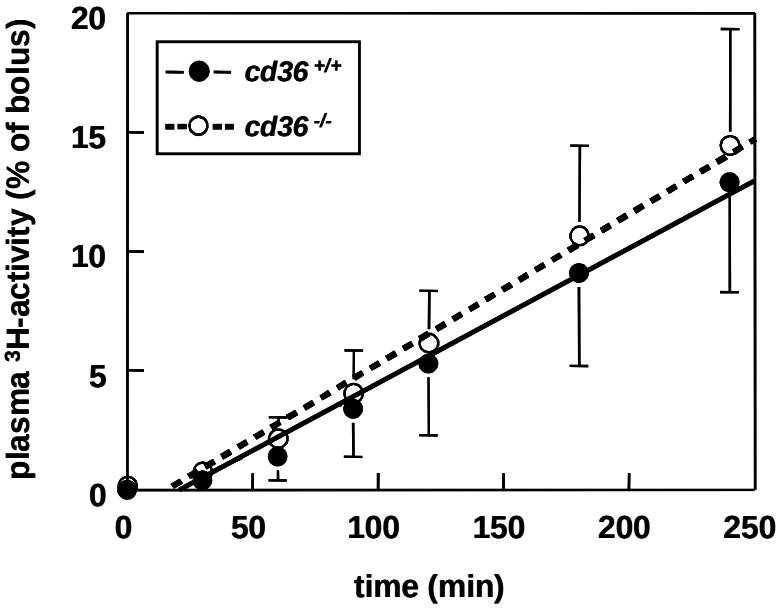

Figure 2. Effect of CD36 deficiency on intestinal lipid absorption. Triton WR 1339 (500 mg/kg body weight) was injected i.v. into mice which were fasted overnight. Directly after the Triton injection, mice were given an olive oil bolus including [^{3}H]triolein by intragastric gavage. The amount of plasma ^{3}H-radioactivity was determined, and depicted as a percentage of the given bolus. Values represent means ± SD of 5 *cd36* $^{+/+}$ and 4 *cd36* $^{-/-}$ mice.

Increased postprandial TG response in cd36 $^{-/-}$ *mice*

Apparently, the elevated TG levels in *cd36* $^{-/-}$ mice cannot be explained by an increased VLDL-TG production or intestinal TG absorption. Therefore, to get more insight into the underlying mechanism, we severely stressed TG metabolism by giving mice an intragastric fat load, and monitored the appearance of TG and FA in plasma (Figure 3). Remarkably, the postprandial TG response was 2-fold enhanced in *cd36* $^{-/-}$ mice as compared to wild type littermates ($AUC_{0\text{-}8.5\ h}$: 13 ± 6 and 7 ± 2 mM·h, respectively; $P < 0.05$), which suggests that CD36 deficiency results in impaired lipolytic conversion of postprandial TG in plasma (Figure 3A). Interestingly, FA levels were also 2.5-fold elevated as compared to wild type littermates ($AUC_{0\text{-}8.5\ h}$: 20 ± 6 and 8 ± 1 mM·h, respectively; $P < 0.05$; Figure 3B).

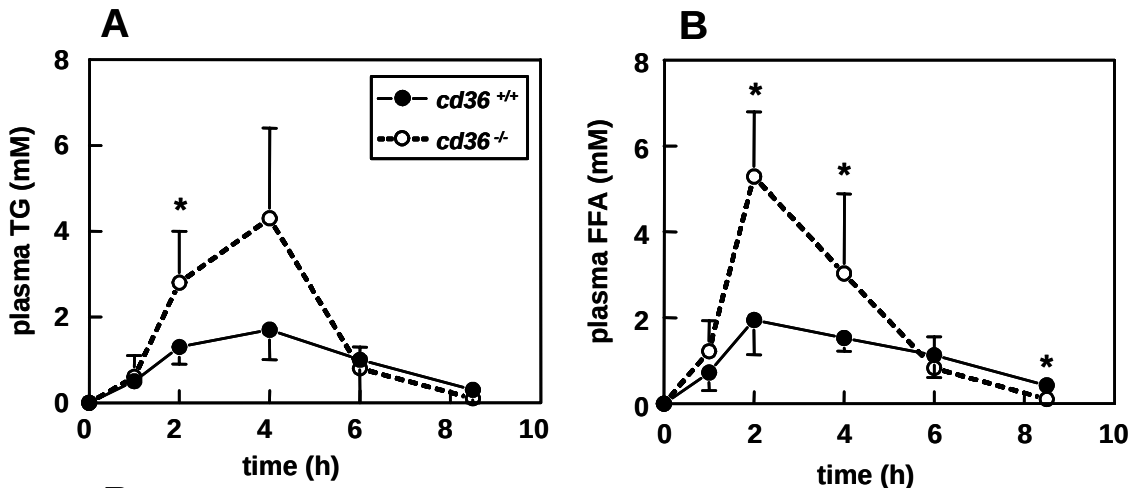

Figure 3. Effect of CD36 deficiency on postprandial response. After 2 weeks on a high fat diet and an overnight fast, *cd36* $^{+/+}$ and *cd36* $^{-/-}$ mice were given an intragastric olive oil bolus. Blood samples were drawn at 0, 1, 2, 4, 6 and 8.5 h after the bolus and plasma triglyceride (A) and FA (B) concentrations were determined in plasma and corrected for plasma TG or FA concentrations at t=0. Values represent means ± SD of 6 mice per group, **P* < 0.05.

CD36 deficiency does not modulate plasma LPL levels

Since the elevated plasma TG levels in *cd36* $^{-/-}$ mice may thus be explained by a decreased LPL-mediated TG hydrolysis, the levels of LPL and HL were measured in postheparin plasma of *cd36* $^{-/-}$ and wild type mice (Figure 4). However, CD36 deficiency did not affect the total plasma LPL or HL levels as determined by their TG hydrolase activity.

Increased plasma FA levels in CD36 deficiency decreases LPL activity

Since CD36-deficient mice have elevated FA levels, which are severely increased to approximately 5 mM after an intragastric fat load (Figure 3), we speculated that these elevated FA might interfere with the activity of LPL in plasma. Therefore, we determined the FA-modulated LPL and HL activities of plasma from *cd36* $^{-/-}$ and wild type mice in the absence of excess albumin (Figure 5A). In this setting, although the total lipolysis of [^{3}H]triolein-labeled emulsion particles as induced by plasma of *cd36* $^{-/-}$ mice was not significantly decreased, the LPL activity was indeed decreased by 51% (0.13 ± 0.06 vs 0.27 ± 0.07 nmol oleate/mL/min; $P < 0.05$). However, as shown in Figure 5B, the addition of excess FA-free albumin relieved this inhibition of LPL activity in *cd36* $^{-/-}$ mice (1.31 ± 0.32 vs 0.80 ± 0.40 nmol oleate/mL/min; $P = 0.055$).

Cd36 $^{-/-}$ mice even show an increased total TG hydrolase activity probably due to the increased plasma TG levels (1.54 ± 0.25 vs 1.10 ± 0.32 nmol oleate/mL/min; *P*<0.05). Collectively, these data suggest that the increased (postprandial) TG levels are caused by a decreased TG hydrolysis rate *in vivo* caused by product-inhibition of LPL resulting from increased plasma FA levels, rather than by an altered production of hepatic VLDL-TG or intestinal lipid absorption.

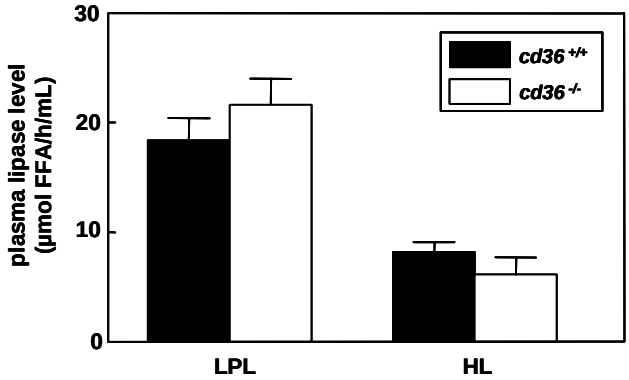

Figure 4. Effect of CD36 deficiency on plasma LPL and hepatic lipase (HL) levels. After 2 weeks on a high fat diet, postheparin plasma was obtained after an overnight fast from *cd36* $^{+/+}$ and *cd36* $^{-/-}$ mice. Total triglyceride hydrolase activity was measured in the absence (i.e. LPL and HL) or presence (i.e. HL) of 1 M NaCl. Values represent means ± SD of 5 mice per group, **P* < 0.05.

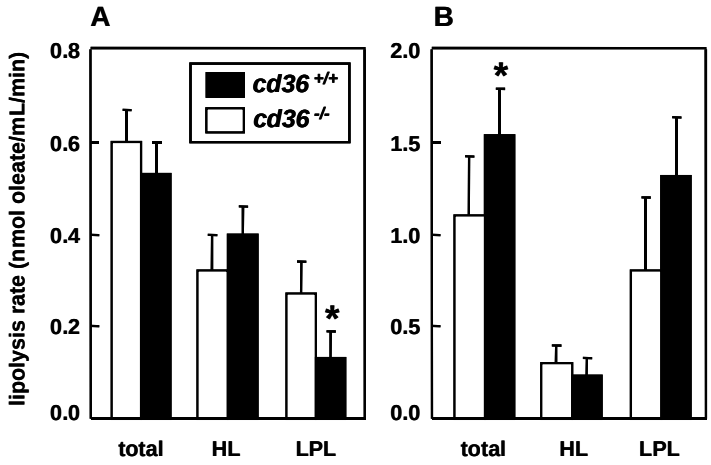

Figure 5. Effect of CD36 deficiency on the TG hydrolase activity of plasma. After 2 weeks on a high fat diet, postheparin plasma was obtained after an overnight fast from *cd36* $^{+/+}$ and *cd36* $^{-/-}$ mice. LPL and HL activities were determined by [^{3}H]oleate production during incubation of plasma with [^{3}H]triolein-labeled 75 nm-sized VLDL mimicking protein-free emulsion particles in the absence (A) and presence (B) of excess FA-free BSA. Values represent means ± SD of 5 mice per group, **P* < 0.05.

Increased plasma FA levels in wild type mice decreases LPL-mediated TG clearance

To provide direct *in vivo* evidence showing that increased FA levels indeed cause a decrease in LPL-dependent plasma TG clearance independent of *cd36*$^{-/-}$ background, we increased plasma FA levels by FA infusion in fed wild type male mice and determined the clearance of TG-rich VLDL-like emulsion particles. After 1 h of infusion, plasma FA were steadily increased approximately 1.4-fold compared to vehicle infused animals (1.93 ± 0.41 vs 1.38 ± 0.16 mM) while plasma TG levels were not increased yet. In mice with increased plasma FA levels the plasma half-life of [^{3}H]triolein-labeled TG-rich VLDL-like particles was 2.5-fold increased ($t_{½}$ = 17.5 ± 10.4 vs 7.0 ± 2.6 min, $P < 0.05$) compared to mice infused with vehicle (Figure 6) indicating a profound *in vivo* effect of plasma FA levels on LPL-dependent clearance of TG-rich lipoprotein particles.

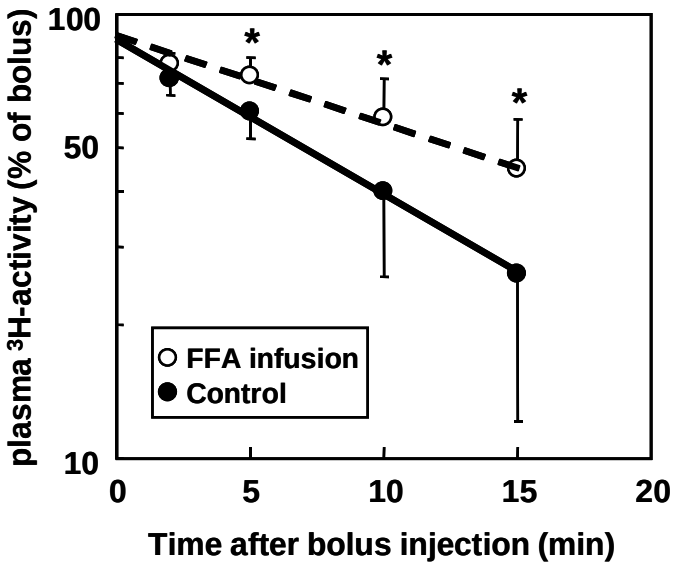

Figure 6. Effect of increased plasma FA on the clearance of [3H]TG-labeled VLDL-like emulsion particles. Fed male wild type mice were infused with FA or vehicle to increase plasma FA. During steady state plasma FA levels [^{3}H]triolein-labeled VLDL-like emulsion particles were injected and the clearance of ^{3}H-activity from the plasma was followed in time. Values represent means ± SD of 5 mice in the FA-infused group and 4 mice in the vehicle-infused group, *$P < 0.05$.

Discussion

In agreement with observations by others[8-10], we have shown that absence of the fatty acid translocase CD36 in mice leads to increased plasma FA levels concomitant with 30-40% increased TG levels. The effect of CD36 deficiency on increased plasma FA levels can easily be explained by an impaired peripheral uptake.[9] Although it has been postulated that the VLDL-TG production rate may be enhanced in CD36-deficient mice[10], the mechanism underlying the effect of CD36 on TG metabolism had not been addressed yet. The results of the present study clearly show that the hypertriglyceridemia observed in *cd36* $^{-/-}$ mice is caused by a decreased LPL-mediated TG hydrolysis rate induced by increased plasma FA levels, rather than by an increased production of hepatic VLDL-TG or increased intestinal lipid absorption.

Recently, we have shown that the increased plasma FA levels in CD36-deficient mice lead to an enhanced FA flux towards the liver, resulting in increased TG storage (hepatic steatosis).[16] Hepatic VLDL-TG production is thought to be primarily a substrate-driven process, regulated by the availability of FA (reviewed by Lewis *et al.*[17]). Furthermore, acute elevation of plasma FA levels stimulates VLDL-TG production in humans.[18] Therefore, the increased FA flux to the liver in CD36 deficiency[16] may result in an enhanced hepatic VLDL-TG production. Hajri *et al*.[10] hypothesized that such a mechanism may account for the hypertriglyceridemic effect of CD36 deficiency, but no experimental proof has been provided. Although we have observed the occurrence of elevated plasma FA levels and hepatic steatosis in *cd36* $^{-/-}$ mice, we did not detect any effect of CD36 deficiency on expression of genes involved in transcriptional regulation (*pparα*, *pparγ*, *srebp1c*) or VLDL-TG synthesis (*apob*, *apobec*, *apoe*, *mttp*) (not shown). Importantly, CD36 deficiency did not affect the actual VLDL-TG production rate or composition of nascent VLDL-TG. Similar to CD36-deficient mice, genetically obese *ob/ob* mice[19] and human apoCI-overexpressing mice[20] also have increased plasma FA levels and hepatic steatosis, but display normal hepatic VLDL-TG production. Apparently, increased plasma FA levels and hepatic steatosis *per se* do not necessarily lead to increased VLDL-TG production.

CD36 is highly expressed in the apical membrane of enterocytes in the intestinal jejunal villi.[1,21] Since this location is the main site of FA (lipid) absorption and CD36 does act as a FA transporter, CD36 is thought to play a role in the intestinal uptake of FA.[21,22] Therefore, increased intestinal lipid absorption as a result of CD36 deficiency

seemed highly unlikely. Indeed, the present study showed that in the absence of CD36, lipid absorption is not affected *in vivo* in mice, confirming observations from our earlier study.[23]

To get more insight into the mechanism underlying the observed hypertriglyceridemia in CD36-deficient mice, we severely stressed TG metabolism by giving mice an intragastric fat load, resulting in a rapid and extensive generation of chylomicrons. Remarkably, the postprandial TG response was 2-fold enhanced in *cd36*$^{-/-}$ mice as compared to wild type littermates. Concomitantly, the plasma FA concentrations also increased to approximately 5 mM in *cd36*$^{-/-}$ mice, as compared to only 2 mM in control littermates. Mouse plasma contains approximately 0.5 mM albumin, which under normal circumstances carries the major part of plasma FA. Since albumin has 4 high-affinity binding sites for FA[24], albumin is capable of binding about 2 mM FA in plasma. Apparently, the dramatically increased FA levels upon the intragastric fat load in *cd36*$^{-/-}$ mice to a maximum of 5 mM exceed the maximum albumin-binding capacity. Since the amphiphilic nature of FA precludes its presence in plasma in an unbound state, it is likely that the FA generated by TG hydrolysis will accumulate in the lipoprotein shell and interfere with LPL-mediated lipolysis. Indeed, it appeared that, although the total levels of LPL (and HL) were not affected by CD36 deficiency, LPL in postheparin plasma obtained from *cd36*$^{-/-}$ mice was less able to lipolyse VLDL-like emulsion particles in the absence of excess BSA as FA acceptor. Upon addition of an excess of FA-free BSA the inhibition of LPL-mediated lipolysis was relieved. These *in vitro* data thus confirm that the increased plasma TG levels in the absence of CD36 are caused by inhibition of lipases (mainly LPL) due to elevated plasma FA levels. We have indeed observed that a reduction of LPL activity in heterozygous LPL-deficient (*lpl* $^{+/-}$) mice (i.e. 40%) markedly elevated the postprandial TG response after an intragastric olive oil load as compared to wild type littermates ($AUC_{0\text{-}6}$: 43 ± 27 vs 3.5 ± 0.6, *lpl* $^{+/-}$, respectively; $P < 0.05$).

In our study we also show *in vivo* that in wild type mice 1.4-fold increased plasma FA levels lead to a decreased capacity of LPL to lipolyze VLDL-TG. In the short time frame in which the experiment was performed it is very unlikely that other LPL modulators such as apoCII or apoCIII have changed between groups and impair the LPL-mediated TG clearance. Slight changes in plasma concentrations of these modulators cannot be excluded in the case of the *cd36*$^{-/-}$ mice. However, our collective findings that 1) the inhibition of LPL activity by plasma from CD36-deficient

mice is relieved by addition of the FA-sequestrant BSA, and 2) elevation of plasma FA levels by infusion impairs TG clearance, strongly suggest that the hypertriglyceridemic phenotype of CD36-deficient mice is indeed mainly explained by increased FA levels.

These effects of increased plasma FA on tissue LPL activity may be explained by several mechanisms. Binding of FA to the active site of LPL might cause classical product inhibition of LPL activity. We and others[25] showed *in vitro* that the rate at which LPL hydrolyzes TG in lipoproteins or emulsions particles decreases sharply with the amount of FA formed unless albumin is present. An alternative mechanism has been proposed by Saxena and Goldberg[26] who showed *in vitro* that plasma FA levels may be important modulators of LPL interaction with the endothelial cell surface and apoCII. *In vivo* evidence for a role of plasma FA in the control of LPL was proposed in humans. Peterson ***et al.***[27] suggested that LPL is subject to feedback control by FA, involving an unusual mechanism that FA may regulate not only the catalytic activity of the enzyme but also its distribution between endothelial sites.[27]

In summary, in the present study we show that the increased plasma TG levels in CD36 deficiency are not due to a previously hypothesized enhancing effect on VLDL-TG production or to an effect on intestinal lipid absorption. Instead, CD36 deficiency resulted in hypertriglyceridemia caused by decreased LPL-mediated hydrolysis of TG-rich lipoproteins resulting from FA-induced product inhibition.

Acknowledgements

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Chapter 5

Endogenous IL-10 Protects Against Hepatic Steatosis, but Does Not Improve Insulin Sensitivity During High Fat Feeding in Mice

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Abstract

Several studies have demonstrated an association in humans between plasma levels or production capacity of the anti-inflammatory cytokine IL-10, and insulin sensitivity. The aim of our study was to investigate the protective role of endogenous IL-10 availability in the development of diet-induced insulin resistance. We compared parameters of glucose and lipid metabolism between IL-$10^{-/-}$ mice and wild type (wt) mice fed a high fat diet for 6 weeks. This diet has previously been shown to induce steatosis and insulin resistance. After 6 weeks on the high fat diet no differences in bodyweight, basal metabolism (measured by indirect calorimetry) and plasma levels of glucose, triglycerides (TG) or cholesterol were observed between IL-$10^{-/-}$ and wt mice. Nonetheless, in IL-$10^{-/-}$ mice plasma fatty acid levels were 75% increased compared to wt mice after overnight fasting ($P < 0.05$). In addition, hepatic TG content was 54% increased in IL-$10^{-/-}$ mice ($P < 0.05$). During a hyperinsulinemic euglycemic clamp no differences were observed in whole-body or hepatic insulin sensitivity between both groups.

We conclude that basal IL-10 production protects against hepatic steatosis, but does not improve hepatic or whole-body insulin sensitivity, during high fat feeding.

Introduction

In epidemiological studies insulin resistance is associated with chronic low-grade inflammation.[1] This is reflected in associations between the degree of insulin sensitivity and plasma levels of several cytokines, such as tumor necrosis factor (TNF)α and interleukin-(IL)6.[2,3] In addition, administration of exogenous TNFα and IL-6 induces insulin resistance *in vivo*.[4,5] Conversely, IL-6 depletion improves hepatic insulin action in an animal model of obesity.[6]

IL-10 is a potent anti-inflammatory cytokine, which is produced by T-cells, B-cells, monocytes and macrophages and plays a crucial role in the innate immune system.[7,8] IL-10 potently inhibits the production of pro-inflammatory cytokines, including TNFα and IL-6.[9] Several lines of evidence point to a beneficial effect of IL-10 on insulin sensitivity. A recent epidemiological study showed a positive correlation between IL-10 levels and insulin sensitivity in healthy subjects.[10] In the Leiden 85-plus study the IL-10 production capacity of whole blood was investigated using lipopolysaccharide as a stimulus. The IL-10 production capacity was found to be inversely associated with blood glucose and HBA1c levels.[11] Finally, administration of IL-10 in mice prevented IL-6–induced defects in hepatic insulin action and signalling activity.[12] Although these studies suggest a potentially beneficial role of IL-10 in insulin resistant conditions, the beneficial role of endogenous IL-10 secretion in insulin resistant states has not been proven.

To determine whether endogenous IL-10 production can protect against diet-induced insulin resistance, we compared metabolic characteristics of IL-10$^{-/-}$ mice and wild type (wt) control mice. We fed the mice a high fat diet for 6 weeks and subsequently analyzed parameters of lipid and glucose metabolism. Previous studies have documented, that high fat feeding induces accumulation of TG in the liver and hepatic insulin resistance.[13] We phenotyped the interaction between genotypes and diet by using the metabolic cages and by assessing insulin sensitivity with the hyperinsulinemic euglycemic clamp method. Our data indicate that, in contrast to our expectations, basal IL-10 production protects against hepatic steatosis during high fat feeding, but does not improve hepatic or whole-body insulin sensitivity.

Materials and Methods

Animals

Ten weeks old male C57Bl6/J mice (wt) and IL-10$^{-/-}$ mice on the same background were purchased from Charles River (Maastricht, Netherlands). Mice had free access to water and a normal chow diet (Technilab BMI, Someren, Netherlands) until 12 weeks of age. Subsequently, mice were fed a high fat diet for 6 weeks (40% of calories from bovine lard; Hope Farms, Woerden, Netherlands). A previous study showed a 2.5-fold increased liver lipid content on this high fat diet with a concurrent decrease in hepatic insulin sensitivity.[13] Mice were weighed every week and at t=0 and after 6 weeks on the high fat diet a blood sample was taken to determine plasma triglyceride (TG), cholesterol and glucose levels. Principles of laboratory animal care were followed and the animal ethics committee of our institute approved all animal experiments.

Plasma lipid and glucose analysis

In all experiments, tail vein blood was collected into chilled paraoxon-coated capillary tubes to prevent *in vitro* lipolysis.[14] These tubes were placed on ice and immediately centrifuged at 4°C. Plasma was isolated, snap-frozen in liquid nitrogen and stored at –20°C until analysis. The levels of plasma TG, total cholesterol, free fatty acids (FA) and glucose were determined enzymatically using commercially available kits and standards (#310-A Sigma GPO-Trinder kit, St. Louis, MA, USA; CHOL MPR3, Boehringer, Mannheim, Germany; #315 Sigma NEFA-C kit, St. Louis, MA, USA; Hexokinase method, Instruchemie, Netherlands).

Metabolic cages

After 6 weeks on the high fat diet basal metabolism in the IL-10$^{-/-}$ and wt mice was studied using the Comprehensive Laboratory Animal Monitoring System (CLAMS; Columbus Instruments, Columbus, USA). Metabolic rates were measured using an eight-chamber open-circuit system. Animals were maintained at approximately 24°C under a 12 h light/dark cycle. Food and water were freely available. The mice were housed individually in plexiglass cages through which 0.6 L of air was passed per min. Each chamber was sampled for 45 seconds at 7 min intervals for a 24 h period. The O_2 and CO_2 content of the exhaust air was compared to the O_2 and CO_2 content

of the standardized sample air. Before the start of the actual 24 h measurements mice were weighed and acclimatized to the cages for 24 h.

Hyperinsulinemic euglycemic clamp experiments

After 6 weeks on the high fat diet, clamp experiments were performed as described previously[15,16] after an overnight fast. Animals were anaesthetized by intraperitoneal injection with a combination of 6.25 mg/kg acetylpromazine (Sanofi Santé Nutrition Animale, Libourne Cedex, France) 6.25 mg/kg midazolam (Roche, Mijdrecht, Netherlands) and 0.3125 mg/kg fentanyl (Janssen-Cilag, Tilburg, Netherlands). An infusion needle was placed into the tail vein. After 45 min infusion of D-[3-^{3}H]glucose at a rate of 0.8 µCi/h (specific activity: 620 GBq/mmol, Amersham, Little Chalfont, UK) to achieve steady state levels, basal parameters were determined with 15 min intervals. Thereafter a bolus of insulin (4.5 mU, Actrapid, Novo Nordisk, Chartres, France) was administered and the hyperinsulinemic clamp was started. Insulin was infused at a constant rate of 6.8 mU/h and D-[3-^{3}H]glucose was infused at a rate of 0.8 µCi/h. A variable infusion of 12.5% D-glucose (in PBS) was also started to maintain blood glucose at approximately 7 mM. Blood glucose was measured with the FreeStyle hand glucose measurer (Therasense, Disetronic Medical Systems, Vianen, Netherlands) every 10 min to monitor glucose levels and adjust the glucose pump. After reaching steady state, blood samples were taken at 10 min time intervals during 30 min to determine steady state levels of [^{3}H]glucose. After the last blood sample mice were sacrificed by cervical dislocation and the organs were dissected. An average clamp experiment took approximately 3 h and anaesthesia was maintained throughout the procedure.

Analysis of clamp samples

Plasma insulin concentrations were measured by ELISA (ALPCO Diagnostics, Windham, NH, USA). To measure plasma [^{3}H]glucose trichloroacetic acid (final concentration 2%) was added to 7.5 µL plasma to precipitate proteins using centrifugation. The supernatant was dried to remove water and resuspended in milliQ. The samples were counted using scintillation counting (Packard Instruments, Dowers Grove, IL, USA).

Calculations

The glucose turnover rate (μmol·min^{-1}·kg^{-1}) was calculated during the basal period and under steady-state clamp conditions as the rate of tracer infusion (dpm/min) divided by the plasma specific activity of [^{3}H]glucose (dpm/μmol). The ratio was corrected for body weight. The hyperinsulinemic hepatic glucose production (HGP) was calculated as the difference between the tracer-derived rate of glucose appearance and the glucose infusion rate.

Determination of Akt phosphorylation in liver samples

To investigate hepatic insulin signalling, liver samples (100 mg) from clamped mice (n=4-5 mice/group) were homogenized in a buffer containing: 30 mM Tris, 2.5 mM EDTA, 150 mM NaCl, 0.5 mM Na_3VO_4, 5 mM NaF, 5 mM $MgCl_2$, glycerol, NP40, and protease inhibitors. The samples were homogenized using Ultra-Turrax for 20 s. After centrifugation (14 000 rpm, 15 min, 4°C) the supernatant was clarified from the pellet and its protein content was determined (Pierce, Rockford, IL, USA). For detecting protein levels of phosphorylated protein kinase B (pAkt), Akt and insulin receptor (IR) equal amounts of protein (25 μg) were solubilized in 5 x Laemmli sample buffer. Proteins were separated by SDS-PAGE, transferred to Immobilon-P membranes, blocked, incubated with polyclonal anti-IR (Santa Cruz, CA), anti-pAkt, -Akt and -IR (Cell Signalling, Beverley, MA) primary antibodies (1:1000) and detected by enhanced chemiluminescence after the incubation with HRP-linked secondary antibodies (1:5000). The protein bands were quantified using ImageGauge software (version 3.12, Fuji Photo Film, Tokyo, Japan).

Liver lipid analysis using high performance thin layer chromatography

For analysis of lipid content, livers were homogenized in PBS. Lipids were extracted with Bligh and Dyer's method as described.[17] Lipids were separated by high performance thin layer chromatography (HPTLC) on silica-gel-60 pre-coated plates (Alltech) as described.[18] The amount of lipid (free cholesterol, TG and cholesterylesters) was determined with TINA software (Raytest Isotopen meßgeräte GmbH, Straubenhardt, Germany).

Determination of fibrinogen and serum amyloid-A

Plasma fibrinogen and serum amyloid-A (SAA) levels were determined after 6 weeks on the high fat diet by ELISA as previously described.[19]

Statistical analysis

Results are presented as means ± SD for the number of animals indicated. Differences between experimental groups were determined by the Mann-Whitney U test. The level of statistical significance of the differences was set at $P < 0.05$. Analyses were performed using SPSS 12.0.1 for Windows software (SPSS, Chicago).

Results

Plasma lipid parameters and basal energy metabolism

We observed no differences in body weight between the IL-$10^{-/-}$ and wt mice before and after 6 weeks on a high fat diet. Blood samples taken after 4 h fasting showed no differences between the two groups in plasma TG and cholesterol levels before and after 6 weeks on a high fat diet (Figure 1). To study basal energy metabolism, IL-$10^{-/-}$ mice and wt controls were studied in the metabolic cages after 6 weeks on the high fat diet. Figure 2 shows metabolic characteristics during both the active (night) and inactive (day) periods. We observed no differences in O_2 consumption (3394 ± 636 vs 3201 ± 635 mL/kg/h at night), heat production (0.45 ± 0.09 vs 0.44 ± 0.09 kcal/h at night) or respiratory exchange ratio (RER; 0.83 ± 0.05 vs 0.82 ± 0.05 at night) after 6 weeks on the high fat diet.

Hyperinsulinemic euglycemic clamp studies

We performed hyperinsulinemic euglycemic clamp studies in IL-$10^{-/-}$ mice and wt controls after an overnight fast after 6 weeks on a high fat diet. After overnight fasting no difference in body weight was observed (Table 1). The plasma values of glucose, insulin and FA before and during hyperinsulinemia are shown in Table 1. During hyperinsulinemia glucose levels were maintained at approximately 7 mM and plasma insulin levels were ~ 5 to 10-fold higher when compared to basal conditions. Strikingly, during the hyperinsulinemic period plasma insulin concentrations were ~ 55% lower in IL-$10^{-/-}$ mice compared to wt control mice (1.8 ± 0.8 vs 4.1 ± 1.5 ng/mL;

P < 0.05) despite the infusion of identical amounts of insulin. Basal hepatic glucose production, which equals whole body glucose uptake in the basal state, was not different between IL-10$^{-/-}$ mice and wt controls (47.3 ± 7.0 vs 50.1 ± 4.0 µmol·min^{-1}·kg^{-1}; Figure 3A and B). During hyperinsulinemia whole-body glucose disposal (WGD) increased to a similar level in the two groups (74.6 ± 17.1 *vs* 83.8 ± 25.9 µmol·min^{-1}·kg^{-1}; Figure 3A). No differences in hepatic glucose production (HGP) were observed during hyperinsulinemia (26.5 ± 9.6 vs 26.5 ± 12.0 µmol·min^{-1}·kg^{-1}; Figure 3B). However, after correction for the 55% lower hyperinsulinemic plasma insulin levels, IL-10$^{-/-}$ mice showed a larger increase in insulin-stimulated whole-body glucose uptake. The corrected insulin-mediated decrease (CID) in hepatic glucose production was significantly larger in IL-10$^{-/-}$ mice compared to wt control mice (14.5 ± 6.5 vs 5.0 ± 2.6 µmol·min^{-1}·kg^{-1}/ng plasma insulin; *P* < 0.05; Figure 3C).

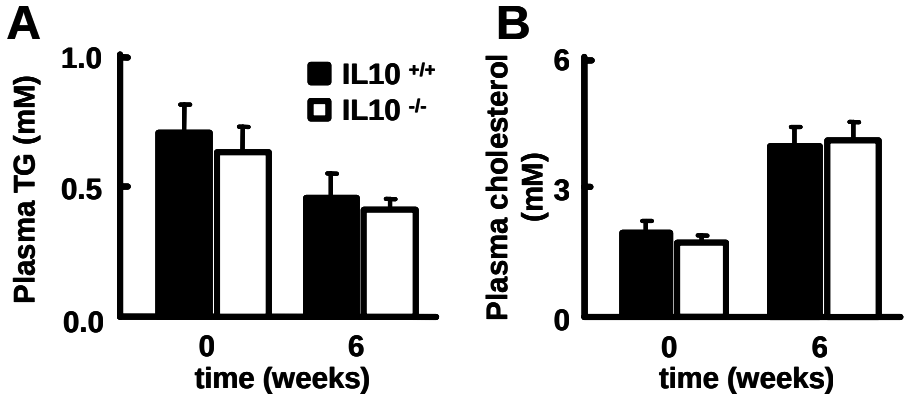

Figure 1. IL-10 deficiency does not affect plasma lipid levels. Plasma lipid levels were measured after 4 h fasting. **A**. Plasma triglycerides (TG). **B**. Plasma cholesterol. (n=12 mice/group)

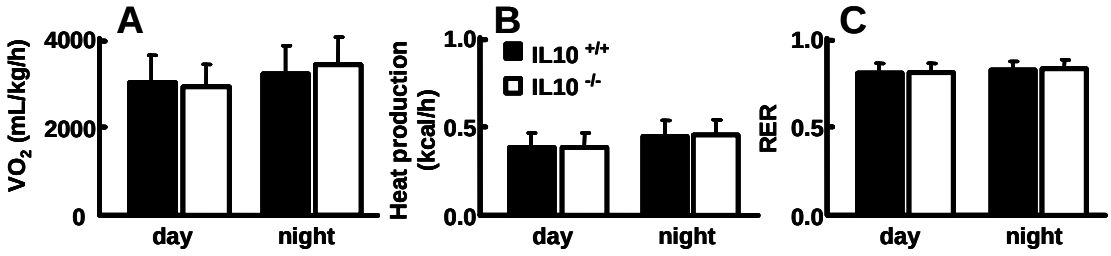

Figure 2. IL-10 deficiency does not affect basal energy metabolism. A. VO2 of IL-10$^{-/-}$ mice and wt controls after 6 weeks on the high fat diet. (n=4) **B**. Heat production of IL-10$^{-/-}$ mice and wt controls after 6 weeks on the high fat diet. (n=4) **C**. RER of the IL-10$^{-/-}$ mice and wt controls after 6 weeks on the high fat diet as measured by indirect calorimetry. (n=4 mice/group)

Table 1. Plasma parameters and glucose infusion rate during the clamp.

	BW	Glucose (mM)		Insulin (ng/mL)		FA (mM)		GIR
		basal	hyper	basal	hyper	basal	hyper	(µmol·min^{-1}·kg^{-1})
IL-10$^{+/+}$	**26.0**	**6.1**	**7.7**	**0.36**	**4.05**	**0.65**	**0.29**	**57.3**
	1.5	*0.5*	*0.8*	*0.13*	*1.50*	*0.09*	*0.05*	*17.9*
IL-10$^{-/-}$	**27.2**	**5.8**	**7.0**	**0.36**	**1.83***	**1.14***	**0.52***	**45.7**
	1.8	*0.5*	*0.9*	*0.04*	*0.63*	*0.14*	*0.31*	*10.8*

The clamp procedure was performed on IL-10$^{-/-}$ mice and wt controls after overnight fasting. Hyperinsulinemia and euglycemia were indeed established during the hyperinsulinemic period (hyper). Plasma insulin levels were ~ 55% lower in the IL-10$^{-/-}$ mice while infusing an identical amount of insulin. Plasma FA were significantly increased in the IL-10$^{-/-}$ mice during the clamp. Under hyperinsulinemia the plasma FA were decreased ~ 40% in both groups, but remained elevated in the IL-10$^{-/-}$ mice. The glucose infusion rate was not different between the groups. (*P < 0.05; n=6-7 mice/group) BW = body weight, GIR = Glucose infusion rate.

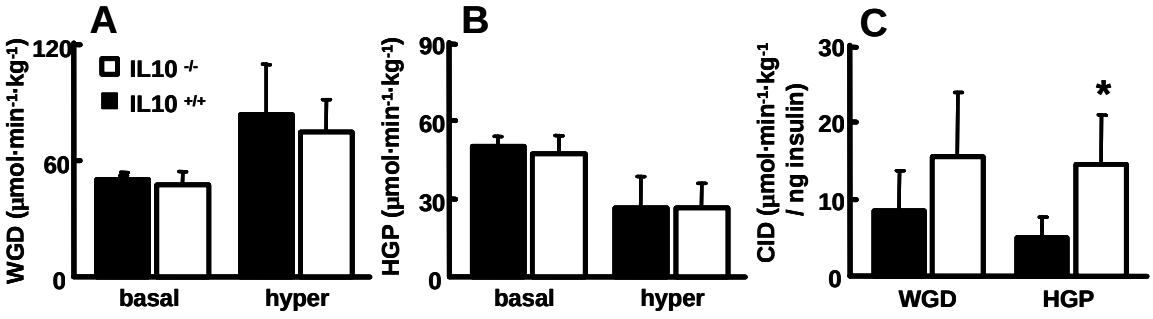

Figure 3. IL-10 deficiency does not affect peripheral or hepatic insulin sensitivity as measured during a hyperinsulinemic euglycemic clamp. Whole-body glucose disposal (WGD; panel **A**.) and hepatic glucose production (HGP; panel **B**.) were measured during the basal period and under hyperinsulinemic conditions using the hyperinsulinemic euglycemic clamp method in both groups. **C**. The insulin-mediated stimulation of whole-body glucose disposal and the inhibition of hepatic glucose production were corrected for the plasma insulin levels (CID) because in the IL-10$^{-/-}$ mice the plasma insulin levels were ~ 55% lower compared to wt mice. (* P < 0.05; n= 6-7 mice/group)

Basal plasma FA levels were significantly increased in the IL-10$^{-/-}$ mice compared to the wt mice after overnight fasting (1.14 ± 0.14 vs 0.65 ± 0.09 mM; $P < 0.05$; Table 1). During hyperinsulinemia plasma FA levels decreased in both groups by about 40% as compared to the respective levels under basal conditions. Nonetheless, plasma FA levels remained significantly higher in IL-10$^{-/-}$ mice (0.52 ± 0.31 *vs* 0.29 ± 0.05 mM; $P < 0.05$).

Hepatic pAkt protein expression levels

To investigate the effect of the hyperinsulinemic euglycemic clamp conditions on insulin signalling in the liver, we measured phosphorylation and protein expression of Akt and IR protein expression. We performed immunoblotting on liver samples from mice, which had undergone the euglycemic hyperinsulinemic clamp. Despite decreased plasma insulin levels, we found increased phosphorylation of Akt in IL-10$^{-/-}$ mice upon insulin stimulation during the clamp compared to wt mice (Figure 4, 12.5 ± 1.4 vs 9.3 ± 2.0 arbitrary units (AU); $P < 0.05$), while Akt and IR protein levels were not changed (2.7 ± 0.3 vs 2.2 ± 0.1 and 8.4 ± 0.5 vs 7.8 ± 1.4 AU, respectively).

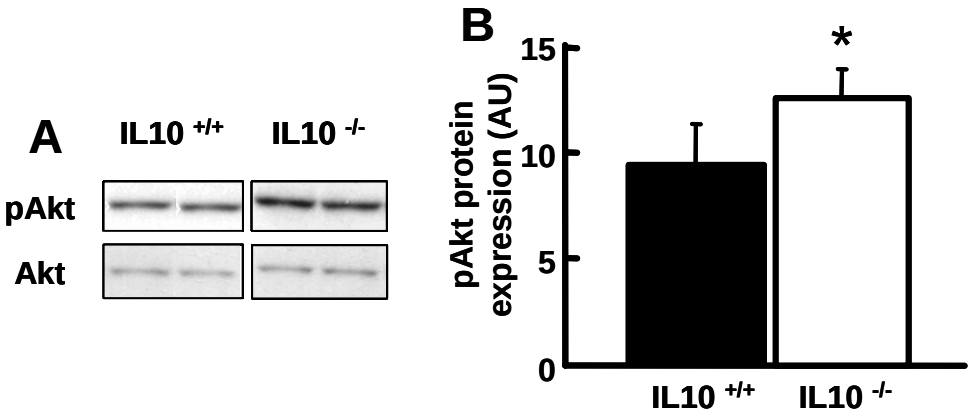

Figure 4. Increased hepatic Akt phosphorylation in IL-10$^{-/-}$ mice during euglycemic hyperinsulinemic clamp conditions. pAkt protein levels were determined using western blotting. Equal amounts of protein (25 µg) for pAkt and Akt expression were loaded, quantified and corrected for loading differences. **A.** Western blot. **B.** Quantification of pAkt protein levels corrected for loading differences (* $P < 0.05$; n=4).

Liver lipid content

Hepatic TG content is inversely related to hepatic insulin sensitivity in some mouse models.[20] In IL-10$^{-/-}$ mice we observed a ~ 54% increase in hepatic TG content

compared to wt mice (168.7 ± 42.3 vs 109.4 ± 42.3 µg TG/mg protein; $P < 0.05$; Figure 5), even though IL-10-deficiency does not alter plasma TG levels (see Figure 1). Liver free cholesterol (FC) content was also increased in IL-10$^{-/-}$ mice (17.7 ± 5.2 vs 11.9 ± 2.7 µg FC/mg protein; $P < 0.05$), whereas the amount of cholesterylesters (CE) was decreased (3.8 ± 1.8 vs 6.3 ± 2.8 µg CE/mg protein; $P = 0.063$). Consequently, the FC/CE ratio was significantly larger in the IL-10 $^{-/-}$ mice (5.9 ± 3.3 vs 2.3 ± 1.4; $P < 0.05$).

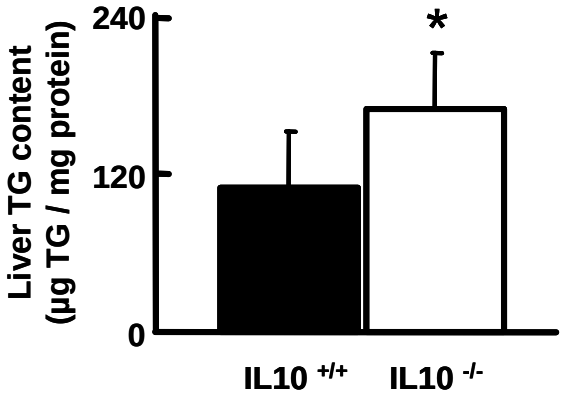

Figure 5. IL-10 protects against hepatic steatosis. Hepatic triglyceride content was determined using high performance thin layer chromatography. (* $P < 0.05$; n= 6-7 mice/group)

Adipose tissue mass

Fatty liver and increased plasma FA are associated with increased visceral adipose tissue mass.[21] Therefore we measured subcutaneous and visceral adipose tissue mass in the IL-10$^{-/-}$ and wt mice and related it to the body weight of the mice. We found that visceral adipose tissue mass was significantly increased in IL-10$^{-/-}$ compared to wt mice (2.0 ± 0.5 vs 1.5 ± 0.7 % of total body weight; $P < 0.05$; Figure 6). The subcutaneous adipose tissue mass was not changed between IL-10$^{-/-}$ and wt mice (0.8 ± 0.2 vs 0.7 ± 0.2 % of total body weight).

Plasma fibrinogen and SAA

To exclude differences in systemic or hepatic inflammation we measured plasma fibrinogen and SAA levels after 6 weeks on the high fat diet in both mouse groups. No differences in fibrinogen (2.9 ± 1.1 vs 2.6 ± 0.8 mg/mL) or SAA levels (182 ± 170 vs 217 ± 291 µg/mL) were observed.

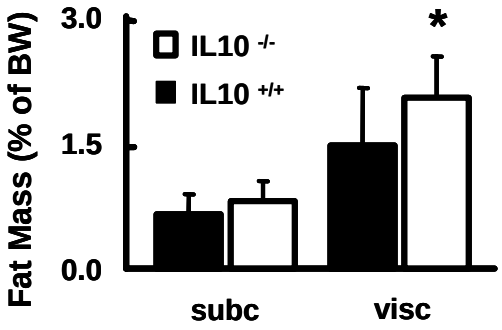

Figure 6. Increased visceral adipose tissue mass in IL-10$^{-/-}$ mice. Visceral and subcutaneous adipose tissue was quantified as a percentage of total body weight. (* P < 0.05; n= 6-7 mice/group)

Discussion

Our study is the first to establish the direct consequences of IL-10 deficiency on hepatic and peripheral insulin sensitivity. Our data show, that basal IL-10 production protects against hepatic steatosis during high fat feeding. However, endogenous IL-10 secretion does not improve hepatic or whole-body insulin sensitivity during high fat feeding as assessed by the hyperinsulinemic euglycemic clamp technique. These observations argue against a simple protective role of endogenous IL-10 secretion in insulin resistant states, at least within our mouse model. Nonetheless, our data also indicate that endogenous IL-10 secretion is not metabolically inert, since we documented clear effects of IL-10 deficiency on hepatic and peripheral lipid metabolism.

We observed no differences in the plasma levels of TG and total cholesterol between high fat-fed IL-10$^{-/-}$ and wt mice. This is in concordance with a previous study [22] in IL-10$^{-/-}$ mice on an apolipoprotein E-deficient background. In those mice a shift of cholesterol from VLDL to LDL was observed, although total cholesterol levels remained unchanged. Conversely, over-expression of IL-10 in mice on a LDLr$^{-/-}$ background led to a significant decrease in total cholesterol.[23] In that study a high correlation between plasma total cholesterol levels and plasma IL-10 concentration was found. In accordance, several studies in humans documented an inverse association between plasma IL-10 and lipid levels.[11,24] In contrast, this association does not hold in the complete absence of IL-10, as we show in our study in IL-10$^{-/-}$ mice on a Black6 background and is shown by others in apolipoprotein E knockout

mice.[11,22] We can not exclude the possibility that, in the absence of any IL-10 production capacity, compensatory mechanisms prevent dysregulation of the lipid metabolism.

We expected mice lacking IL-10 to be more catabolic in comparison to wild type mice, since they lack this anti-inflammatory cytokine. Interestingly, when we compared basal metabolic characteristics we found absolutely no differences in heat production, food intake, VO_2, VCO_2 and respiratory exchange ratio. Apparently, under basal conditions IL-10 is not a crucial cytokine in energy metabolism. LPS-mediated activation of the immune system may elucidate a more important role for IL-10. However, that would be a model of infection rather than a model of metabolic regulation *per se*.

Strikingly, we found decreased hyperinsulinemic plasma insulin concentrations in the IL-$10^{-/-}$ mice compared to the wild type controls although we infused identical amounts of exogenous insulin. The amount of insulin infused in our study protocols normally results in plasma insulin levels of ~ 4-6 ng/mL as were observed our wild type control mice.[25,26] Thus, the absence of any difference in hepatic glucose production and peripheral glucose uptake between IL-$10^{-/-}$ mice and wt controls during the clamp experiment occurred despite lower plasma insulin levels in the IL-$10^{-/-}$ mice. This combination of data suggests improved insulin sensitivity in IL-$10^{-/-}$ mice, rather than the initially hypothesized decreased insulin sensitivity. In addition, the data indicate that IL-10 deficiency is associated with a higher rate of plasma clearance of insulin, for reasons presently unknown.

We subsequently evaluated the activity of important markers of the hepatic insulin signalling cascade in livers obtained from hyperinsulinemic IL-$10^{-/-}$ mice and wt controls. We found, that phosphorylation of Akt was significantly increased in IL-$10^{-/-}$ mice despite lower plasma insulin concentrations under hyperinsulinemia, although Akt and insulin receptor expression were not changed. Therefore, both the *in vivo* glucose kinetic data obtained during hyperinsulinemia, as well as these markers of the insulin signalling cascade point to increased hepatic insulin sensitivity, rather than the expected hepatic insulin resistance in IL-$10^{-/-}$ mice.

IL-10 deficiency is associated with major changes in hepatic lipid content, reflected in increased TG content upon high fat feeding. In many mouse models and in humans, positive correlations exist between hepatic steatosis and hepatic insulin

resistance.[20,27-30] However, there are also many examples of steatosis, that are not associated with hepatic insulin resistance, including the treatment of mice with thiazolidinediones or LXR agonists or the inhibition of fatty acid oxidation.[31-33] Obviously, the relation between steatosis and hepatic insulin resistance is not straightforward, because other factors with complex interactions may be involved. The increased liver TG content may be due to increased plasma FA flux into the liver after overnight fast. Plasma FA levels were significantly increased in the IL-10$^{-/-}$ mice both in the basal state and under hyperinsulinemia (Table 1). This may result from increased lipolysis and release of FA from the increased visceral adipose tissue store in the IL-10$^{-/-}$ mice compared to control mice. In both groups of mice plasma FA as a measure of adipose tissue lipolysis is deceased by ~ 40% under hyperinsulinemia, suggesting no change in adipose tissue insulin sensitivity. However, in the IL-10$^{-/-}$ mice the plasma FA level remains significantly increased compared to controls. Increased visceral adipose tissue mass is associated with increased plasma FA and fatty liver in humans.[21] A potential explanation for this association may be the portal delivery of FA to the liver.[34] Subsequently, upon uptake by the liver these FA may be esterified into TG that may accumulate within the liver, since hepatic VLDL-TG production is not increased in IL-10$^{-/-}$ mice. [23] Alternatively, we cannot exclude the involvement of other changes in intra-hepatic fatty acid metabolism like an increase in the expression of lipogenic enzymes, or a decrease in fatty acid oxidation. Although the increase in hepatic cholesterol content could be due to increased cholesterol synthesis in the liver, the increased FC/CE ratio indicates an impairment of the esterification of cholesterol into cholesteryl esters. The mechanism behind this observation is beyond the scope of this paper.

We measured plasma fibrinogen and SAA in the IL-10$^{-/-}$ and the wt control mice. Although fibrinogen and SAA levels increased in both groups in time on the high fat diet, no difference in the plasma levels of these markers of systemic and hepatic inflammation were observed between the two genotypes. Therefore, we conclude that the effects in IL-10 deficient mice do not simply reflect a higher state of chronic (hepatic) inflammation.

In summary, IL-10 deficiency alters peripheral and hepatic lipid metabolism. However, this study does not support a causal role of IL-10 in the protection against diet-induced hepatic insulin resistance and other metabolic disturbances.

Ackowledgements

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Chapter 6

Ritonavir Impairs LPL-mediated Lipolysis And Decreases Uptake of Fatty Acids in Adipose Tissue

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Abstract

The use of the HIV protease inhibitor ritonavir (RTV) is frequently associated with hypertriglyceridemia and lipodystrophy. The aim of our study was to determine the mechanism underlying the observed hypertriglyceridemia.

Feeding female APOE*3-Leiden transgenic mice a western-type diet supplemented with RTV (35 mg/kg/day) for 2 weeks resulted in a 2-fold increase in fasting plasma triglyceride (TG) levels, which was specific for VLDL. RTV did not change the hepatic VLDL-TG production. Instead, RTV did increase the postprandial TG response to an oral fat load (AUC 25.5 ± 12.1 vs 13.8 ± 6.8 mM.h in controls; $P < 0.05$). Likewise, RTV hampered the plasma clearance of intravenously injected glycerol tri[^{3}H]oleate-labeled VLDL-like emulsion particles ($t_{½}$ 19.3±10.5 vs 5.0±1.3 min in controls; $P < 0.05$), associated with a decrease of 44% in plasma LPL activity. Accordingly, RTV decreased the uptake of TG-derived fatty acids (FA) into adipose tissue, as well as the uptake of albumin-bound FA.

We conclude that RTV causes hypertriglyceridemia via decreased LPL-mediated clearance of VLDL-TG. In addition, RTV specifically impairs the uptake of FA in adipose tissue which may contribute to the lipodystrophy that is frequently observed in HIV-infected subjects on antiretroviral therapy.

Introduction

The introduction of highly active antiretroviral therapy (HAART) has considerably decreased morbidity and mortality associated with HIV-infection. This therapy, however, is associated with a lipodystrophy syndrome, which is characterized by changes in body fat distribution and metabolic abnormalities, such as hyperlipidemia and insulin resistance.[1,2] Studies in humans investigating the mechanism of HAART-induced hypertriglyceridemia reveal inconclusive results.[3-11] Some of these studies suggested that HAART increased VLDL-triglyceride (TG) production rates, whereas others suggested that antiretroviral treatment results in defective removal of VLDL-TG from plasma, either exclusively or in combination with increased VLDL-TG production rates. This discrepancy is difficult to resolve in humans, because the combination of drugs used in HAART does not permit a distinction between the effects of individual antiretroviral drugs. Since the HIV protease inhibitor ritonavir (RTV) is the antiretroviral drug that is associated with the most severe hypertriglyceridemic effects when used at therapeutic doses,[2,12] we aimed at conclusively elucidating the mechanism underlying hypertriglyceridemia induced by RTV. We used the APOE*3-Leiden transgenic mouse as an experimental model, because these mice have a humanized lipoprotein profile and are susceptible to diet- and drug-induced hyperlipidemia, obesity and atherosclerosis.[13-15] In contrast to wild-type mice, APOE*3-Leiden transgenic mice are highly sensitive to treatment with hypolipidemic drugs, such as statins, fibrates, and PPARα and PPARγ-agonists.[16] Similar to humans, APOE*3-Leiden transgenic mice have a much lower clearance rate of VLDL-TG than wild type mice. As a consequence, APOE*3-Leiden mice represent a suitable animal model for RTV-associated hyperlipidemia.

The first aim of the present study was to assess the effects of RTV on both VLDL-TG production and clearance rates. We used a low dosage of RTV that induced hypertriglyceridemia without causing toxicity, as measured by plasma alanine amino transferase (ALAT) levels. The second aim was to evaluate the effects of RTV on tissue-specific uptake of fatty acids (FA) derived from VLDL-TG and from the plasma free FA pool, by applying our recently described method using differentially labeled FA to quantify tissue-specific uptake of FA derived from VLDL-TG and from plasma free FA.[17] We found that RTV 1) decreased the clearance of VLDL-TG from plasma by decreasing lipoprotein lipase (LPL) activity, and 2) decreased the uptake of FA

derived from VLDL-TG and of albumin-bound FA in adipose tissue, but not in other organs.

Materials and methods

Animals

Female APOE*3-Leiden transgenic mice, housed under standard conditions with free access to water and food, were used for the experiments. Mice were fed a standard mouse chow diet (Hope Farms, Woerden, Netherlands) until 2 months of age. After this period they were fed a semi-synthetic western type diet (Hope Farms, Woerden, Netherlands) containing 15% saturated fat, 0.2% cholesterol and 40% sucrose for a 5 weeks run-in period. Mice were randomized and divided into 2 groups. One group was fed the western type diet with RTV (Norvir, Abbott, Kent, United Kingdom) added at a concentration of 35 mg/kg body weight/day for 2 weeks. The other group of APOE*3-Leiden transgenic mice was fed the western type diet without addition of RTV to serve as appropriate controls. On the basis of two papers investigating pharmacokinetic properties of HIV-protease inhibitors in mice[18,19] we designed a dose-finding study in which we showed that 35 mg/kg body weight/day did induce hypertriglyceridemia without causing liver damage as measured by plasma ALAT levels. Principles of laboratory animal care were followed and the animal ethics committee of our institute approved all animal experiments.

Plasma lipid analysis

In all experiments, tail vein blood was collected into chilled paraoxon-coated capillary tubes to prevent in vitro lipolysis.[20] These tubes were placed on ice and immediately centrifuged at 4°C. Plasma levels of TG, total cholesterol and free FA were determined enzymatically using commercially available kits and standards (#310-A Sigma GPO-Trinder kit, St. Louis, MA, USA; CHOL MPR3, Boehringer, Mannheim, Germany; #315 Sigma NEFA-C kit, St. Louis, MA, USA). FPLC analysis was performed on pooled plasma to determine the distribution of TG and cholesterol over the lipoprotein fractions using the AKTA purifier supplied with a Superose-6 column (Amersham Pharmacia Biotech).

Hepatic VLDL-TG production by Triton WR1339 injection

After the diet period mice were fasted overnight, anaesthetized (0.5 mL/kg Hypnorm; Janssen Pharmaceutica, Beerse, Belgium and 12.5 mg/kg midazolam; Roche, Mijdrecht, The Netherlands) and subsequently injected with Triton WR1339 (500 mg/kg body weight, 15% solution in 0.9% NaCl). Plasma VLDL clearance is completely inhibited under these circumstances.[21] Plasma TG were measured before injection of Triton and at 30, 60 and 90 min after injection and related to the body mass of the mice. Production of hepatic TG was calculated from the slope of the curve and expressed as µmol/h/kg body weight.

Postprandial TG response

After an overnight fast, mice were administered a 200 µL olive oil bolus through intra-gastric gavage. Blood samples were drawn just before and 1, 2, 4 and 8 h after olive oil bolus administration. TG concentrations were determined in plasma as described above and corrected for the plasma TG levels at t = 0.

In vivo clearance of VLDL-like TG-rich emulsion particles

The preparation and characterization of glycerol tri[^{3}H]oleate-labeled 80-nm-sized protein-free VLDL-like emulsion particles have previously been described.[22] This emulsion was stored at 4°C under argon and was used within 3 days. To study the in vivo serum clearance of the glycerol tri[^{3}H]oleate-labeled emulsions, fed mice were anaesthetized, the abdomen was opened and the emulsion (1 mg of TG) was injected intravenously via the vena cava inferior. Blood samples were taken via the vena cava inferior at 2, 5 and 10 min after bolus administration and the radioactivity in serum was determined by scintillation counting (Packard Instruments, Dowers Grove, IL). From these data the serum half-life of the glycerol tri[^{3}H]oleate was determined. The total plasma volumes of the mice were calculated from the equation: V (mL) = 0.04706 x body weight (g) as determined from ^{125}I-BSA clearance studies as previously described.[23]

Total plasma LPL activity

To determine the total LPL activity present in plasma, 4 h fasted RTV-treated mice and their controls were injected intravenously with heparin (0.1 U/g BW; Leo Pharmaceutical Products B.V., Weesp, Netherlands) and blood was collected after

10 min. The capillaries were kept on ice and were spun immediately at 4°C. The plasma was snap-frozen in liquid nitrogen and stored at -80°C until analysis of the LPL activity, as modified from Zechner.[24] A TG substrate mixture containing triolein (TO; 4.6 mg/mL), [^{3}H]TO (2.5 µCi/mL) essentially FA-free BSA (20 mg/mL; Sigma), Triton X-100 (0.1%; Sigma) and heat-inactivated (30 min at 56 °C) human serum (20%) in 0.1 M Tris-HCl, pH 8.6, was generated by 6 sonication periods of 1 min using a Soniprep 150 at 7 µm output, with 1 min intervals on ice. Ten µL of post-heparin plasma was added to 0.2 mL of substrate mixture and incubated for 30 min at 37 °C in the presence or absence of 1 M NaCl which completely inhibits LPL activity, to estimate both the LPL and HL levels. The reaction was stopped by the addition of 3.25 mL of heptane-methanol-chloroform (1:1.28:1.37, v/v/v), and 1 mL of 0.1 M K_2CO_3 in saturated H_3BO_3 (pH 10.5) was added. To quantify the [^{3}H]oleate generated, 0.5 mL of the aqueous phase obtained after vigorous mixing (20 s) and centrifugation (15 min at 3,600 rpm) was counted in 4.5 mL of Ultima Gold (Packard Bioscience, Meriden, CT). The LPL activity was calculated as the fraction of total lipolytic activity inhibited by 1 M NaCl and expressed as the amount of FA released per h per mL of plasma.

Modulated lipolytic activity in plasma

To study the effect of RTV on LPL activity in plasma in situ, post-heparin mouse plasma (2.5% of the incubation volume) was incubated with a mix of [^{3}H]triolein-labeled 80 nm-sized VLDL-mimicking protein-free emulsion particles (0.25 µg TG/mL, prepared as described previously[22]) and excess FFA-free BSA (60 mg/mL) in 0.1 M Tris, pH 8.5. After 1 h of incubation 50 µL samples from the total 200 µL incubation volume were taken and added to 1.5 mL of extraction liquid (methanol-chloroform-heptane-oleic acid; 1404:1245:1001:1; v/v/v/v) and 0.5 mL of 0.2 N NaOH was added to terminate lipolysis. Generated [^{3}H]oleate was counted as described above and expressed as the amount of FA released per h per mL. In this assay, the lipolytic activity of plasma is determined towards a relatively low amount of emulsion particles instead of an excess of solubilized TG. Hereby, the modulated lipolytic activity of plasma is assessed, by allowing interference of the endogenous activators (e.g. apoCII) and inhibitors (e.g. apoCI and apoCIII) with the activity of LPL.

Tissue-specific FA uptake

To determine the effect of RTV on the uptake of FA from VLDL-TG by peripheral tissues in the fed state we used a steady-state approach, as described previously by Teusink et al.[17] In short, glycerol tri[^{3}H]oleate-labeled 80-nm-sized protein-free VLDL-like emulsion particles which are known to mimic endogenous VLDL-TG particles[22] and [^{14}C]oleate bound to albumin were continuously infused for 2 h. Blood samples were drawn at 1.5 h and at 2 h to determine steady-state specific activity in plasma. After 2 h infusion the mice were sacrificed and the liver, muscle, heart, and subcutaneous adipose tissue were taken out to determine the retention of [^{3}H]oleate and [^{14}C]oleate in these tissues as a measure for the uptake of FA from VLDL-TG and from albumin-bound FA, respectively. Values were corrected for specific activity of FA in the plasma and are expressed as retention of total plasma FA in nmol/mg tissue protein.

Statistical analysis

Results are presented as means ± SD for the number of animals indicated. Differences between experimental groups were determined by the Mann-Whitney U test. The level of statistical significance of the differences was set at $P < 0.05$. Analyses were performed using SPSS 12.0 for Windows software (SPSS, Chicago).

Results

*Ritonavir increases plasma TG specifically in the VLDL fraction in APOE*3-Leiden transgenic mice*

Plasma TG, cholesterol and free FA were measured in APOE*3-Leiden transgenic mice after a five-week run-in period on the western type diet (t=0) and, subsequently, again after 2 weeks of feeding the same diet with or without the addition of RTV (t=2 weeks). In RTV-treated mice plasma TG increased from 2.7 to 5.4 mM (Fig. 1A, $P < 0.05$) and plasma cholesterol from 12.7 to 15.3 mM (Fig. 1B, $P < 0.05$), whereas plasma lipid levels remained unchanged in the control group. The increase in plasma TG was mainly due to an increase in VLDL-TG (Fig. 1C), while cholesterol was mainly increased in the VLDL and IDL/LDL lipoprotein fractions (Fig. 1D). Plasma free FA increased significantly from 0.70 to 0.93 mM ($P < 0.05$) after 2 weeks on the western type diet with RTV added as is shown in Figure 2.

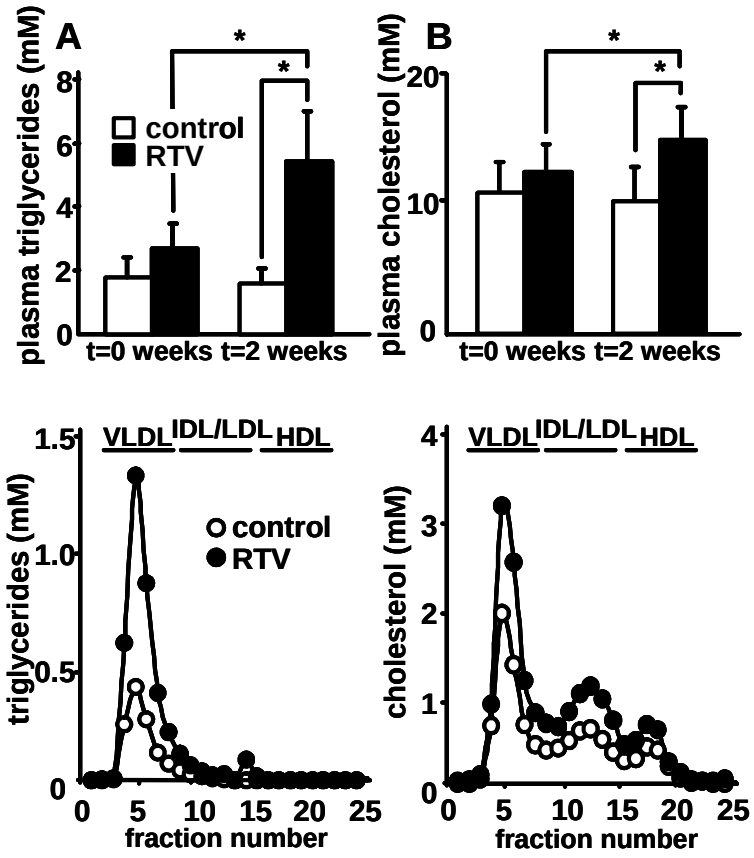

Figure 1. Ritonavir increases plasma TG and cholesterol. Plasma levels of TG (**A**) and cholesterol (**B**) were measured after a five-week run-in period and after 2 weeks of subsequent feeding with or without RTV administration through the diet. Values represent means ± SD of 8 mice per group. Lipoproteins in pooled plasma were fractionated by FPLC and eluted fractions were analyzed for TG (**C**) and cholesterol (**D**) distribution over the lipoproteins. * $P < 0.05$

Ritonavir does not change in vivo VLDL-TG production

To investigate whether the increase in plasma TG levels was due to increased hepatic VLDL-TG production, we injected fasted mice with Triton WR 1339, which completely inhibits lipolysis of VLDL-TG. However, as is shown in Figure 3A, after 2 weeks of dietary RTV administration no significant difference was observed in the rate of VLDL-TG production, when the RTV-treated mice were compared to the controls (139 ± 41 vs 177 ± 60 µmol TG/kg/h).

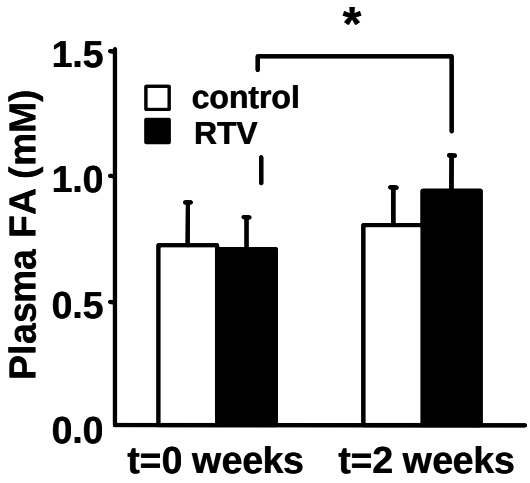

Figure 2. Ritonavir increases plasma free FA. Plasma levels of free FA were measured after a five-week run-in period and after 2 weeks of subsequent feeding with or without RTV administration through the diet. Values represent means ± SD of 8 mice per group. $P < 0.05$

Ritonavir increases postprandial TG response

Subsequently, we investigated whether the increase in postprandial plasma TG levels was caused by impaired postprandial clearance of TG. For this purpose, an intra-gastric bolus of olive oil was administered and subsequently plasma TG levels were determined. Figure 3B shows that RTV treatment caused a 2-fold increment in the postprandial TG response upon an intragastric olive oil administration (area under the curve 25.5 ± 12.1 vs 13.8 ± 6.8 mM.h; $P < 0.05$), which indeed suggests impaired TG clearance.

Ritonavir increases plasma half-life of TG-rich VLDL-like emulsion particles

To investigate whether the decreased clearance of TG indeed contributes to the hypertriglyceridemia observed in RTV-treated mice, mice were i.v. injected with glycerol tri[^{3}H]oleate-labeled protein-free VLDL-like emulsion particles. These particles mimic the metabolic behavior of TG-rich lipoproteins.[22,25] Because LPL is more abundantly expressed on the adipose tissue in the postprandial state compared to the fasted state[26], we used fed mice for this study. As is shown in Figure 4, the clearance of glycerol tri[^{3}H]oleate was markedly decreased in RTV treated mice when compared to the control group, which is evident from an approximately 4-fold

increase in serum half-life of glycerol tri[^{3}H]oleate ($t_{½}$ 19.3 ± 10.5 vs 5.0 ± 1.3 min; P < 0.05).

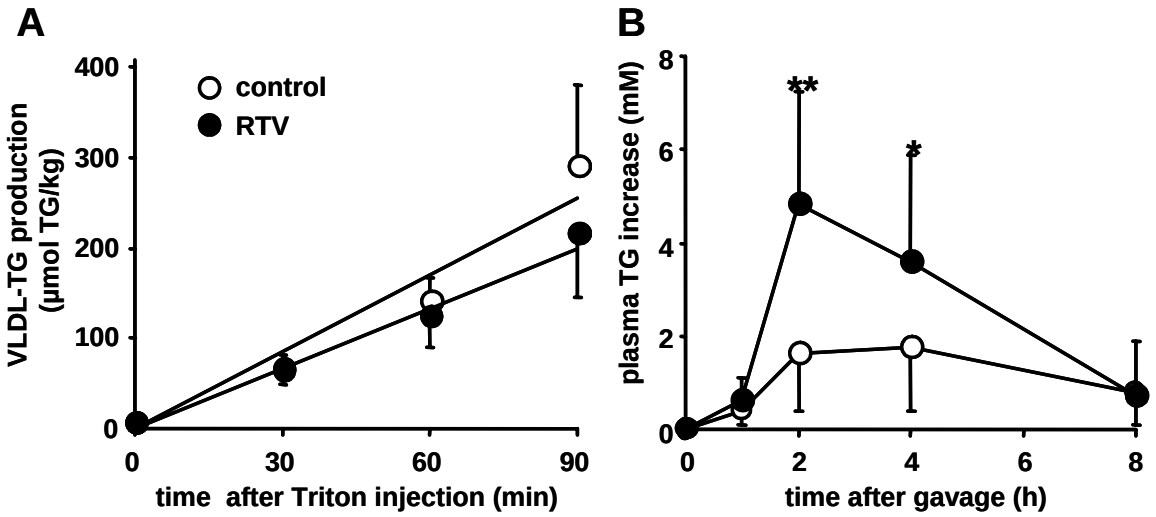

Figure 3. Ritonavir does not affect hepatic VLDL-TG production but increases the postprandial plasma TG response. **A**. After overnight fast, mice were anaesthetized and injected i.v. with Triton WR1339 (500 mg/kg BW) to completely block the peripheral lipolysis of VLDL-TG. Before and 30, 60 and 90 min after Triton injection blood samples were drawn. Plasma TG were determined and corrected for body weight and the values at T = 0. The slopes of the curves were calculated by linear regression to determine the rate of hepatic VLDL-TG production. Values represent means ± SD of 7 mice per group**.** **B**. After an overnight fast, mice were administered a 200 µL olive oil bolus through intra-gastric gavage. Blood samples were drawn before and at 1, 2, 4 and 8 h after the olive oil bolus and the levels of plasma TG were determined and corrected for the values at T=0. Values represent means ± SD of 8 mice per group. * P < 0.05, ** P < 0.01

Ritonavir decreases total LPL activity in post-heparin plasma

Impaired LPL-mediated TG hydrolysis can be due to decreased expression of LPL and/or by a direct effect of RTV on LPL activity. Therefore, we determined the effect of RTV on the total lipolytic activity in post-heparin plasma by incubation with a glycerol tri[^{3}H]oleate-containing substrate mixture. As shown in Figure 5A, the post-heparin HL activity in RTV-treated mice did not differ significantly from that of control mice (15.1 ± 3.7 vs 12.5 ± 3.7 µmol FA/h/mL). The post-heparin LPL activity, however, was significantly decreased by 44% in RTV-treated mice versus control mice (11.2 ± 3.3 vs 19.9 ± 11.1 µmol FA/h/mL; P < 0.05). This observation shows that

RTV impairs LPL-mediated TG lipolysis by lowering the total LPL activity present in plasma.

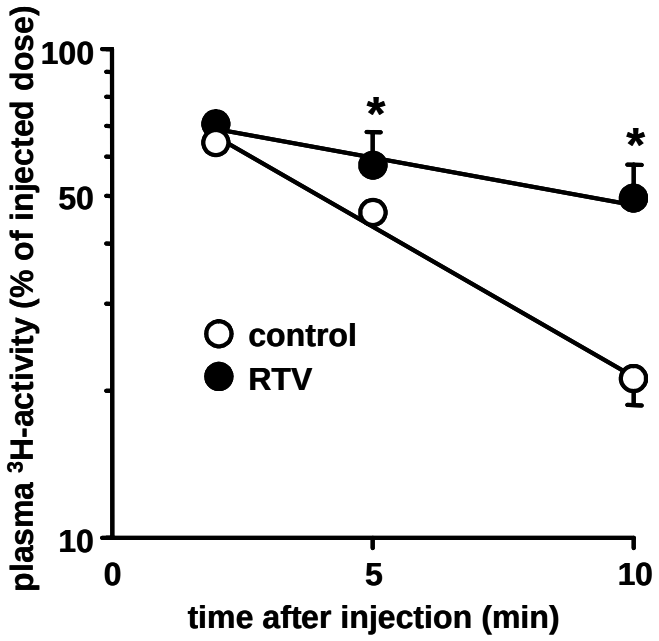

Figure 4. Ritonavir increases the plasma half-life of [3H]TG-labeled VLDL-like emulsion particles. Fed mice were injected via the vena cava inferior with glycerol tri[^{3}H]oleate-labeled VLDL-like emulsion particles to investigate the plasma clearance. Blood samples were drawn at 2, 5 and 10 min after bolus administration and the amount of ^{3}H-activity in plasma was detemined. Values represent means ± SD of 3 mice per group. * P < 0.05

Ritonavir decreases the modulated lipolytic activity in post-heparin plasma

To study the modulated lipolytic activity in plasma, by allowing interference of the endogenous activators (e.g. apoCII) and inhibitors (e.g. apoCI and apoCIII) with the activity of LPL, we performed an additional assay in which the lipolytic activity of plasma is determined towards a relatively low amount of well-defined emulsion particles instead of an excess of solubilized TG. As is shown in Figure 5B the post-heparin modulated lipolytic activity is decreased significantly by 55% in plasma of RTV-treated mice as compared to control mice (19.0 ± 3.7 vs 42.8 ± 12.7 nmol FFA/h/mL; P < 0.05).

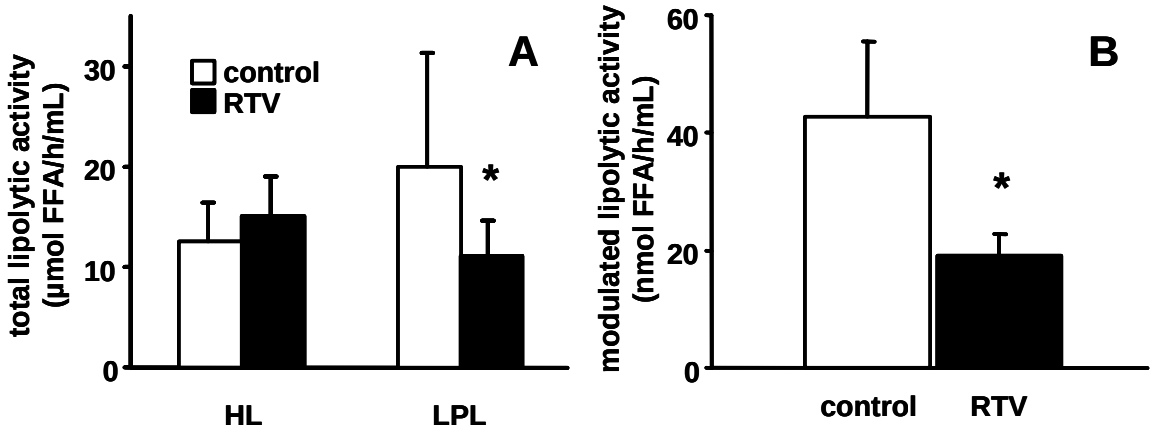

Figure 5. Ritonavir decreases total and modulated lipolytic activity in post-heparin plasma. Mice were fasted for 4 h and injected i.v. with heparin. After 10 min blood samples were drawn. A. The total lipolytic activity of post-heparin plasma was assessed by determination of [^{3}H]oleate production upon incubation of plasma with a substrate mix containing an excess of both [^{3}H]triolein and FA-free BSA as FA-acceptor. HL and LPL activities were distinguished in the presence of 1 M NaCl, which specifically blocks LPL. Values represent means ± SD of 9 mice in the RTV group and 10 mice in the control group. B. The modulated lipolytic activity of post-heparin plasma was assessed by incubation of plasma (2.5%) with [^{3}H]triolein-labeled VLDL-mimicking protein-free emulsion particles and excess FA-free BSA. After 1 h of incubation samples were taken and the modulated lipolytic activity was calculated as the amount of generated [^{3}H]oleate released per h per mL. Values represent means ± SD of 7 mice in the RTV group and 6 mice in the control group. * $P < 0.05$

Ritonavir decreases FA uptake in adipose tissue

The effect of RTV on the uptake of FA from VLDL-TG and albumin-bound FA by various tissues was studied during steady state infusion of glycerol tri[^{3}H]oleate TG-rich VLDL-like emulsion particles. RTV-treatment did not affect VLDL-TG derived FA uptake by the liver, skeletal muscle and the heart (Figure 6A). In adipose tissue, however, the uptake of VLDL-TG derived FA was significantly decreased (639 ± 220 vs 986 ± 80 nmol FA/mg tissue protein; $P < 0.05$). The uptake of FA bound to albumin was also decreased in adipose tissue of RTV-treated mice (514 ± 176 vs 1078 ± 194 nmol FA/mg tissue protein; $P < 0.05$), and not in the liver, skeletal muscle and the heart when compared to control mice (Figure 6B).

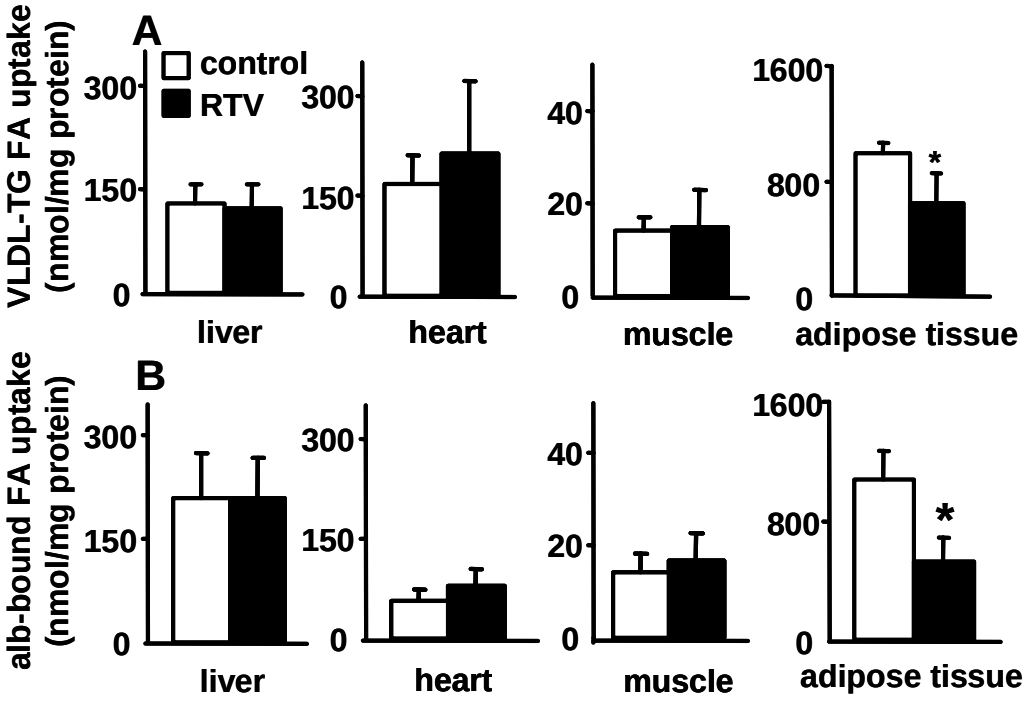

Figure 6. Ritonavir specifically decreases the uptake of FA by adipose tissue. Fed mice were anaesthetized and infused with a mixture of glycerol tri[^{3}H]oleate-labeled VLDL-like emulsion particles and [^{14}C]oleate bound to albumin for 2 h to reach steady state specific activity in the plasma. After 2 h of infusion mice were bled and the organs were dissected to determine the uptake of VLDL-TG derived and albumin-bound FA. Values represent means ± SD of 7 mice per group. * $P < 0.05$

Discussion

In this study, we investigated the mechanism underlying the hypertriglyceridemia caused by RTV administration in APOE*3-Leiden transgenic mice with a human-like lipoprotein profile. Our data demonstrate that RTV clearly inhibits LPL-mediated TG clearance, which is supported by multiple lines of evidence. First, RTV increased postprandial hypertriglyceridemia indicating defective clearance of TG-rich lipoproteins. Second, RTV decreased the plasma clearance of i.v. injected TG-rich VLDL-like emulsion particles. Third, RTV decreased post-heparin plasma total LPL activity. In addition, the uptake of FA derived from VLDL-TG, as well as albumin-bound FA, was decreased selectively in adipose tissue where LPL is highly expressed in the postprandial state.

Human studies remain inconclusive with respect to the underlying mechanism of RTV-induced hypertriglyceridemia.[3-11] Purnell *et al.* showed that RTV decreased hepatic lipase activity, although there was no difference in post-heparin LPL levels

between RTV- and placebo-treated healthy subjects.[27] In contrast, a study by Baril *et al*.[3] showed that RTV caused decreased LPL activity while no differences in the amount of apolipoprotein CII (cofactor for LPL) or apolipoprotein CIII (inhibitor of LPL) were found, indicating a direct effect of RTV on the LPL enzyme as we now conclusively show in our study. Shahmanesh *et al*.[10] showed a significant decrease in the fractional catabolic rate of VLDL-TG in individuals treated with RTV either alone or in combination with other antiretroviral drugs, due to a decreased activity of LPL even in the postabsorptive state. Another study in HIV-negative subjects treated with RTV showed a trend towards decreased fat clearance as measured by an intravenous fat tolerance test after a 10 h fast.[5] A recent study by Sekhar *et al*.[9] revealed marked abnormalities in the ability of HIV lipodystrophy patients to metabolize dietary TG suggesting an impairment of the function of LPL. In humans it is impossible to conclusively show the direct effects of the individual drugs on the lipid metabolism, because HAART-treated patients are usually on a therapy regimen of at least three drugs. Moreover, in humans there is considerable heterogeneity in both environmental and genetic background.

To conclusively determine the mechanism underlying RTV-induced hypertriglyceridemia we used the APOE*3-Leiden transgenic mouse as our model. Studies in AKR/J mice[28] and in C57BL/6 wild type[29] mice showed an effect of RTV only on hepatic VLDL-TG production rate. In contrast to AKR/J and wild type mice, the APOE*3-Leiden transgenic mouse has a lipoprotein profile with close resemblance to the human profile.[13-15] In these mice plasma cholesterol levels can be titrated to any desired level by varying the amount of cholesterol in the diet. In contrast to wild-type mice, APOE*3-Leiden transgenic mice are highly sensitive to treatment with hypolipidemic drugs, such as statins, fibrates, and PPAR-α and γ-agonists.[16] These observations imply that the APOE*3-Leiden transgenic mice on a western type diet represent a suitable animal model for hyperlipidemia.

An *in vitro* study in human and rat hepatoma cells and primary hepatocytes from mice showed that protease inhibitor treatment inhibits proteasomal degradation of nascent apoB.[30] However, protease inhibitors also inhibited secretion of apoB. The concentrations of drugs used in these *in vitro* studies are much higher than the maximal plasma concentrations in subjects taking these drugs.[31] RTV may affect different components of the lipid metabolism depending on the dosage used. The dosage we used in our mice was 2 times higher than what an average adult would

receive per kg/day. Taking into account the much faster metabolic rate in mice it is clear that we used a low physiological dosage in our mice. Unfortunately, we did not have the opportunity to assess plasma RTV concentrations. It may be that at superphysiological concentrations RTV affects VLDL-TG production rate as well.

In the present study, RTV impaired FA uptake in adipose tissue under steady state conditions while infusing glycerol tri[^{3}H]oleate-labeled VLDL-like particles together with albumin-bound ^{14}C-labeled FA. Before tissues can take up FA derived from VLDL-TG, these TG have to be lipolyzed by LPL. In the current study we show that RTV decreased plasma LPL activity by 44%. As expected, due to decreased LPL activity the adipose tissue of RTV-treated mice took up significantly less FA derived from VLDL-TG compared to control mice under fed conditions. In the fed state LPL is more abundant in adipose tissue than in muscle[17,26] explaining why no change is seen in the uptake of VLDL-TG derived FA in muscle. In addition to decreased uptake of FA derived from VLDL-TG, the adipose tissue of RTV-treated mice also took up less albumin-bound FA, a process independent of LPL. The active transport of FA into tissues occurs mainly via CD36. CD36 functions as a high affinity transporter of long-chain FA in adipose tissue and the muscle.[32,33] Serghides *et al*.[34] have shown that CD36 deficiency was induced by antiretroviral therapy both in healthy humans and in HIV-infected subjects. They also showed that RTV significantly decreased CD36 levels in THP1 and C32 cells. The observed decrease in the uptake of albumin-bound FA in adipose tissue as we observed is in accordance with a decrease in CD36 levels. Another study showed that in murine peritoneal macrophages CD36 can be upregulated by protease inhibitor therapy leading to increased uptake of cholesterol and cholesteryl esters.[35] The difference in outcome of these studies may be a matter of different concentrations that are used in the *in vitro* studies. Many protease inhibitors, especially RTV, are very poorly soluble and difficult to handle in an *in vitro* assay.[36] Alternatively, it may be that the same drug exerts different effects in different types of cells.

In accordance with decreased FA uptake by peripheral tissues we found an increase of ~16% in plasma FA levels in RTV-treated mice. As we have shown recently[37], increased plasma FA levels can directly impair LPL activity most probably via product inhibition, because free FA can bind to the active site of LPL. In the present study plasma free FA levels are slightly but significantly increased, therefore, in addition to

direct impairment of LPL activity RTV may also be contributing indirectly to decreased LPL-mediated lipolysis via increased plasma FA.

Lipodystrophic HAART-treated HIV-infected patients showed an increased postprandial TG and FA response compared to non-lipodystrophic HIV-infected patients and healthy controls most likely caused by inadequate trapping of FA into adipose tissue.[38] Decreased postprandial adipose tissue FA uptake was already observed in our study after 2 weeks of drug administration, even though no obvious lipodystrophy as measured by weighing fat pads was observed yet. The flux of FA to adipose tissue mediated by LPL is an important determinant of adipogenesis. Deletion of LPL in adipose tissue in leptin-deficient *ob/ob* mice has been shown to prevent excessive storage of TG in the adipose tissue.[39] In contrast, the absence of apoCIII, the natural LPL inhibitor, enhances fatty acid uptake from plasma triglycerides in adipose tissue, which leads to higher susceptibility to diet-induced obesity.[40] In mice that were administered RTV for a much longer period generalized lipoatrophy was shown in male mice, while this lipodystrophy was restricted to the gonadal depot in female mice.[41] The investigators proposed that the lipodystrophy in these mice is caused, at least in part, by reduced PPARγ function. PPARγ transcriptionally activates a number of genes that are essential for adipogenesis, lipid storage and metabolism, including CD36.

The cause of the HAART-associated hypertriglyceridemia as observed in humans may be multifactorial in nature due to the use of different protease inhibitors simultaneously in combination with antiretroviral drugs of other classes. We propose that the main mechanism by which RTV increases plasma TG is by decreasing the LPL-mediated clearance of TG-rich lipoproteins. In the present study we directly show that RTV decreases the uptake of VLDL-TG derived FA and albumin-bound FA specifically in adipose tissue, an effect that may well contribute to HAART-associated lipodystrophy.

Ackowledgements

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Chapter 7

Ritonavir Protects Against the Development of Atherosclerosis Despite an Atherogenic Lipoprotein Profile in APOE*3-Leiden Transgenic Mice

In preparation

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Abstract

The use of the HIV protease inhibitor ritonavir (RTV) is associated with the induction of cardiovascular risk factors such as dyslipidemia and insulin resistance. It is not clear whether this increase in cardiovascular risk factors may lead to an epidemic of premature cardiovascular disease in HIV-infected patients treated with antiretroviral drugs.

To investigate the potential effects of RTV administration on atherosclerosis development, we fed APOE*3-Leiden mice, which have a human-like lipoprotein profile, a Western-type diet with or without the addition of RTV (35 mg/kg/day). Every 4 weeks, plasma triglyceride (TG) and total cholesterol levels were measured. RTV administration increased plasma TG levels when compared to control mice ($P < 0.05$), but did not alter total cholesterol levels. Unexpectedly, after 19 weeks on the diet, the mean atherosclerotic lesion area in the aortic root was decreased by ~52 % in RTV-treated mice compared to control mice ($P < 0.05$), which was reflected by decreased lesion severity. In contrast, *in vitro* studies with peritoneal macrophages showed that RTV dose-dependently increased oxLDL and lipid association.

In conclusion, RTV decreased atherosclerotic lesion area and severity, even though RTV induced hypertriglyceridemia. We speculate that RTV may decrease atherosclerotic lesion formation via an alternative (*e.g.* cholesterol efflux-enhancing or anti-inflammatory) pathway on the cellular or molecular level.

Introduction

The introduction of highly active antiretroviral therapy (HAART) has considerably decreased morbidity and mortality associated with HIV-infection. This therapy, however, is associated with a lipodystrophy syndrome, which is characterized by changes in body fat distribution and increased cardiovascular risk factors, such as hyperlipidemia and insulin resistance.[1,2] At present, the relationship between HAART and the development of premature atherosclerosis in HIV-infected patients is unclear. Studies measuring intima-media thickness (IMT) as a surrogate marker for the development of atherosclerosis do not conclusively show a correlation between HAART and IMT.[3-6] Some recent studies observed a slightly increased risk for HIV-infected individuals treated with HAART for the development of atherosclerosis.[5,7,8] It should be noted that the characteristics of study cohorts bias results, since HIV-infected subjects have in general more cardiovascular risk factors such as opportunistic infections and smoking compared to the general population.[9] However, a large prospective observational study showed that HAART was independently associated with a 26% relative increase in the rate of myocardial infarction per year of exposure during the first 4-6 years of use.[10]

Since it is difficult to study the effect of specific antiretroviral drugs on the development of atherosclerosis in HIV-infected subjects, several mouse models have been used. A study in male apoE knockout (apoE$^{-/-}$) and low density lipoprotein receptor knockout (LDLr$^{-/-}$) mice showed promotion of atherosclerotic lesion formation by the HIV protease inhibitor ritonavir (RTV) accompanied by CD36-dependent cholesterylester (CE) accumulation in macrophages.[11] In female LDLr$^{-/-}$ mice this effect was significantly less pronounced[12] even though in general female LDLr$^{-/-}$ mice are more susceptible to development of atherosclerosis.[13] This observation in transgenic mice partly supports the hypothesis that the metabolic effects of RTV may ultimately translate into an increased incidence of cardiovascular disease in HAART-treated subjects.

In accordance with the studies in humans and mice, we observed in a previous study that RTV causes hypertriglyceridemia in APOE*3-Leiden mice.[14] This atherogenic lipoprotein profile was caused via inhibition of LPL-mediated lipolysis. APOE*3-Leiden transgenic mice have an attenuated clearance rate of VLDL-TG, which resembles the VLDL-TG metabolism of humans, rather than wild type mice. As a consequence, the APOE*3-Leiden mouse represents a suitable animal model to

study the effects of dyslipidemia on atherosclerosis development.[15] Therefore, the aim of the present study was to determine the effects of RTV on the development of atherosclerosis in this APOE*3-Leiden transgenic mouse model. In contrast to our expectations, we observed that RTV significantly decreased atherosclerotic lesion area and severity in APOE*3-Leiden mice, compared to control mice, independent of plasma cholesterol levels.

Materials and Methods

Animals

Female APOE*3-Leiden transgenic mice, housed under standard conditions with free access to water and food, were used for the experiment. Mice were fed a standard mouse chow diet (Hope Farms, Woerden, Netherlands) until 2 months of age. After this period they were fed a semi-synthetic Western type diet (Hope Farms, Woerden, Netherlands) containing 15% saturated fat, 0.2% cholesterol and 40% sucrose for a 5 weeks run-in period. Mice were randomized and divided into 2 groups (n=14). One group of APOE*3-Leiden mice was fed the Western type diet with RTV (Norvir, Abbott, Kent, United Kingdom) added at a concentration of 35 mg/kg body weight/day for 19 weeks. The other group was fed the Western type diet without addition of RTV to serve as appropriate controls. On the basis of two papers investigating pharmacokinetic properties of HIV-protease inhibitors in mice[16,17], we previously designed a dose-finding study in which we observed that RTV at a dose of 35 mg/kg body weight/day induced hypertriglyceridemia without causing liver damage as reflected by increased plasma levels of ALAT.[14] Principles of laboratory animal care were followed and the animal ethics committee of our institute approved all animal experiments.

Plasma lipid analysis

Every 4 weeks tail vein blood was collected into chilled paraoxon-coated capillary tubes to prevent *in vitro* lipolysis.[18] These tubes were placed on ice and immediately centrifuged at 4°C. Plasma levels of TG and total cholesterol were determined enzymatically using commercially available kits and standards (#310-A Sigma GPO-Trinder kit, St. Louis, MA, USA; CHOL MPR3, Boehringer, Mannheim, Germany).

Atherosclerosis analysis

After 19 weeks on the Western type diet, with or without the addition of RTV, mice were sacrificed. The hearts were perfused with ice-cold PBS, isolated, fixed in phosphate-buffered 4% formaldehyde, dehydrated and embedded in paraffin. The embedded hearts were cross-sectioned (5 µm) throughout the entire aortic root area. Sections were stained with hematoxylin-phloxine-saffron (HPS). Per mouse, 4 sections at 40 µm intervals within the valve area were used for quantification of atherosclerotic lesion area and characterization of lesion severity. Lesion area was determined using Image-Pro Plus version 3.0 analysis software (Media Cybernetics, U.S.). The atherosclerotic lesions were categorized for severity according to the American Heart System for humans[19], which has been adapted to categorize lesions in mice.[20] Three categories were discerned: no or very early lesions (type 0-1 lesions), moderate lesions that are fatty streaks containing only foam cells (type 2-3 lesions) or advanced lesions showing foam cells in the media and presence of fibrosis, cholesterol clefts, mineralization and/or necrosis (type 4-5 lesions). The number observed in each lesion category is expressed as a percentage of the total number of lesions present within one group of mice.[15]

In vitro lipid association studies with peritoneal macrophages

Four days after i.p. injection of Brewer's thioglycollate, peritoneal cells from APOE*3-Leiden transgenic mice were harvested into PBS. The cells were recovered after centrifugation, and resuspended in DMEM (Invitrogen) containing 10% fetal calf serum (Cambrex) and 1% penicillin and streptomycin. The cells were plated onto 24-wells plates (Costar, Corning Inc., Corning, NY, USA) at a density of $6.0*10^5$ cells/well. After incubation at 37°C under 5% CO_2 humidified air for 2 h, cells were washed to remove non-adhering cells. After o/n culturing, the macrophages were pre-incubated with RTV (0.1 or 1 µg/mL) or vehicle (0.5% ethanol) for 24 h. To determine the effect of RTV on the association of oxLDL with the macrophages, after 24 h of pre-incubation, cells were subsequently incubated with 50 µg/mL oxLDL[21] and 2 µCi/mL [1α,2α(n)-^{3}H]cholesterol (Amersham Biosciences, UK) as a tracer in presence of RTV or vehicle for another 24 h. Alternatively, to determine the effect of RTV on the association of 80 nm-sized TG-rich emulsion particles[22], after 24 h of pre-incubation with RTV or vehicle, cells were incubated with 380 µg TG/mL [^{3}H]TG and

[^{14}C]cholesteryl oleate (CO) labeled VLDL-like emulsion particles (380 µg TG/mL) for 3 h. After incubation with either oxLDL or TG-rich particles, cells were washed three times with ice-cold PBS, lyzed with 0.1 M NaOH and subsequently the amount of cell-associated radioactivity was determined.

Statistical analysis

Differences between experimental groups were determined by the Mann-Whitney U test for two independent samples. The differences in lesion severity were statistically tested using the Chi-Square test. The level of statistical significance of the differences was set at $P < 0.05$. Analyses were performed using SPSS 12.0 for Windows software (SPSS, Chicago).

Results

Ritonavir increases plasma TG

At the start of the experiment, and every 4 weeks thereafter, blood samples were taken to determine plasma levels of TG and total cholesterol. Throughout the whole study period, RTV administration significantly increased plasma TG levels approximately 2-fold compared to control mice and this effect was sustained until the end of the experiment ($P < 0.05$ for all time points; Figure 1A). In contrast, RTV did not affect plasma total cholesterol levels throughout the study period (Figure 1B).

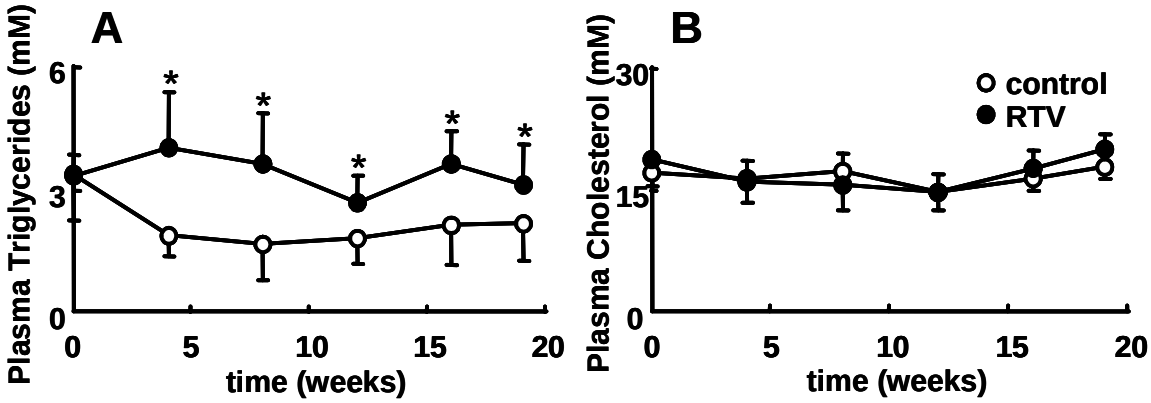

Figure 1. RTV increases plasma TG. Mice were fed a Western type diet without or with RTV added (35 mg/kg bodyweight/day). At baseline and every 4 weeks thereafter, plasma levels of TG (1A) and total cholesterol (1B) were measured after 4 h fasting. (n=14; * $P < 0.05$)

Ritonavir decreases the development of atherosclerotic lesions

After 19 weeks on the Western type diet, we sacrificed the mice to quantify the atherosclerotic lesion area and to determine atherosclerotic lesion severity in the aortic root (Figure 2A). RTV attenuated the development of atherosclerosis as indicated by a ~52% decrease in atherosclerotic lesion area compared to control mice (39.3 ± 41.4 x 10^3 μm^2 vs 82.6 ± 46.3 x 10^3 μm^2; $P < 0.05$; Figure 2B). This was reflected by a reduction in moderate (type 2-3) and advanced (type 4-5) lesions, concomitant with an increase in the percentage of segments with early (type 1) lesions, or no lesions at all (type 0) ($P < 0.05$; Figure 2C).

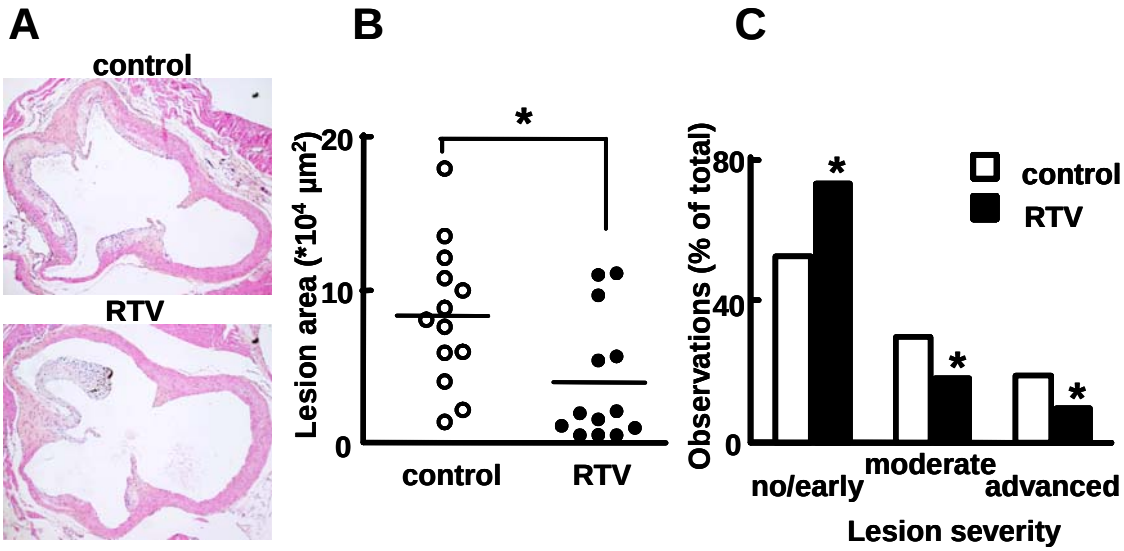

Figure 2. RTV decreases atherosclerotic lesion area and severity. After 19 weeks on the Western type diet with or without the addition of RTV (35 mg/kg body weight/day) mice were sacrificed and lesion area as well as lesion severity was determined. **A.** Representative overviews of the aortic root area of a control and a RTV-treated mouse. **B.** The lesion area was quantified in the aortic root area. **C.** The severity of lesions was determined in the aortic root area. (n=14; * $P < 0.05$)

Ritonavir increases the association of oxLDL with macrophages

To determine whether RTV induces decreased CD36 expression leading to decreased oxLDL uptake, and consequently, decreased atherosclerosis, we investigated oxLDL uptake by peritoneal macrophages. RTV dose-dependently enhanced the cell-association of oxLDL up to 28% at 1 µg/mL RTV ($P < 0.05$; Figure 3).

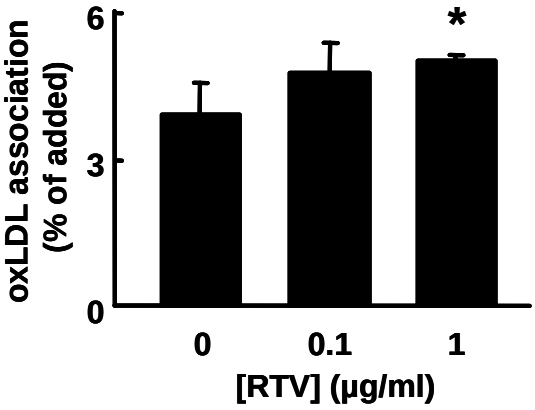

Figure 3. Ritonavir increases oxLDL association with peritoneal macrophages. After 24 h of pre-incubation with RTV or vehicle, cells were incubated with 50 μg/ml oxLDL and 2 μCi/ml [1α,2α(n)-^{3}H]cholesterol as a tracer in presence of RTV or vehicle for another 24 h. The amount of cell-associated [^{3}H]cholesterol was determined. (n=3; * *P* < 0.05)

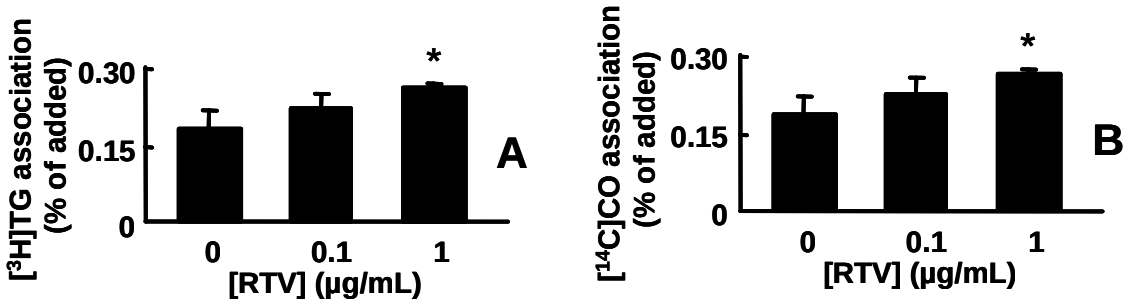

Figure 4. Ritonavir increases the association of TG-rich VLDL-like particles with peritoneal macrophages. After 24 h of pre-incubation with RTV or vehicle, cells were incubated with 380 μg TG/ml [^{3}H]TG/[^{14}C]cholesteryl oleate (CO) labeled VLDL-like particles for 3 h. The amount of cell-associated [^{3}H]TG and [^{14}C]CO was determined. (n=4; * *P* < 0.05)

Ritonavir increases the association of TG-rich VLDL-like particles with macrophages

We have previously observed that RTV-treatment of APOE*3-Leiden mice reduced the systemic expression of LPL, as reflected by reduced postheparin LPL levels.[14] To evaluate whether RTV would also reduce LPL expression specifically in macrophages, thereby reducing lipid uptake, we incubated peritoneal macrophages with TG-rich VLDL-like emulsion particles. However, RTV dose-dependently increased the association of both [^{3}H]TG and [^{14}C]CO (*P* < 0.05 ; Figure 4). Since the ratio between the uptake of [^{3}H]TG and [^{14}C]CO was similar for all conditions, and

was equal to their ratio in the emulsion itself, we conclude that RTV increases whole-particle association rather than selectively inducing the uptake of TG-derived fatty acids.

Discussion

The introduction of antiretroviral drug therapy has considerably increased the life span of HIV infected subjects. Consequently, long-term adverse drug effects become more clinically relevant in the considerations for the most optimal drug regimens. In this study we have conclusively shown that RTV decreases atherosclerosis in the aortic root in APOE*3-Leiden mice, despite the induction of dyslipidemia. Because there were no differences in plasma cholesterol levels between RTV-treated and control APOE*3-Leiden transgenic mice, this paradoxical effect of RTV was independent of plasma cholesterol levels.

We have previously shown that RTV induced hypertriglyceridemia, which was mainly confined to the VLDL fraction.[14] In that study we also found a small increase in plasma cholesterol after 2 weeks of RTV administration, also confined to VLDL. This initial increase in plasma cholesterol was probably secondary to the decreased clearance of VLDL, to which adaptation occurred during long-term administration of RTV (at the dose of 35 mg/kg body weight/day).

Previous studies in male apoE$^{-/-}$ and LDLr$^{-/-}$ mice showed, that HIV protease inhibitors such as RTV, promoted atherosclerotic lesion formation independent of dyslipidemia, which was explained by an increased CD36 expression in macrophages, thereby enhancing CD36-dependent cholesterylester accumulation.[11] On the other hand, a study in healthy volunteers, treatment-naive HIV-infected subjects and in human cell lines showed that antiretroviral therapy induced CD36 deficiency in monocytes.[23] Because we found that RTV decreased the formation of atherosclerotic lesions in our mouse model, we speculated that decreased CD36 expression on the macrophages could be the cause of decreased oxLDL uptake, and consequently of decreased development of atherosclerosis. Unexpectedly, we found that RTV dose-dependently increased oxLDL association with peritoneal macrophages, which is in accordance with the macrophage studies of Dressman *et al.*[11] In female LDLr$^{-/-}$ mice a much less pronounced effect of RTV administration on atherosclerosis development was observed.[12] In most animal models female mice are more susceptible to atherosclerosis than male mice.[13] Allred *et al*. suggested that the dissociation from

the usual gender difference in their RTV study is due to the pharmacological initiation of atherosclerosis.[12]

Another possible mechanism underlying the observed decrease in atherosclerosis could be decreased LPL expression. In our previous study, we observed that in postheparin plasma total LPL activity was considerably decreased by RTV administration (*i.e.* 50%).[14] Therefore, we speculated that decreased LPL activity on the macrophages in the vascular wall could lead to decreased lipid uptake and accumulation by macrophages. Interestingly, when we investigated this hypothesis *in vitro*, we found that RTV dose-dependently increased the association of both TG and cholesterylesters with macrophages. These findings indicate that RTV increases the whole-particle uptake of VLDL-like emulsion particles by macrophages. RTV apparently does not decrease LPL activity in all tissues, at least not in macrophages.

It is tempting to speculate about the mechanism(s) through which RTV decreases atherosclerotic lesion formation in the APOE*3-Leiden transgenic mouse model. It is possible, that RTV has anti-atherosclerotic effects at the cellular level in the arterial wall that overshadow the increased atherosclerotic risk induced by hypertriglyceridemia. For instance, an *in vitro* study with vascular smooth muscle cells showed that RTV inhibits platelet derived growth factor (PDGF)-induced DNA synthesis and chemotaxis.[24] PDGF is a major contributor to atherogenesis.[25] Furthermore, RTV inhibited PDGF-dependent downstream signaling such as Erk activation and these effects were not due to cytotoxicity of apoptosis.[24]

The upregulation of CD36 which was observed during RTV-treatment was shown to be accompanied by an increase in peroxisome proliferator-activated receptor-γ (PPARγ).[11] PPARγ is a ligand-activated nuclear transcription factor with pleiotropic effects on lipid metabolism and inflammation.[26] A study in apoE$^{-/-}$ mice showed that although the PPARγ agonist troglitazone upregulated the expression of CD36 in macrophage foam cells, this PPARγ agonist inhibited fatty streak lesion formation.[27] PPARγ has anti-atherogenic effects because it promotes cholesterol efflux via upregulation of ATP-binding cassette A1 (ABCA1) and ABCG1 and indirectly via upregulation of liver X receptor-α (LXRα) leading to decreased foam cell formation.[28-31] Furthermore, PPARγ agonists have anti-inflammatory effects on the macrophage[32-35], protecting against atherosclerosis. Taken together, the PPARγ-increasing activity of RTV could be involved in the paradoxical decrease in atherosclerotic lesion formation despite the presence of hypertriglyceridemia and upregulation of CD36 in

the APOE*3-Leiden mice.[27,36-38] We speculate that this anti-atherogenic effect of RTV is not observed in the apoE$^{-/-}$ mice because part of the anti-inflammatory and efflux-enhancing effects of PPARγ are caused by increased apoE expression via LXR activation.[39]

The Data Collection on Adverse Events of Anti-HIV Drugs (DAD) study showed that HIV-infected HAART-treated subjects are at a significantly greater risk of myocardial infarction.[10] In HAART-treated patients, however, the high prevalence of cardiovascular risk factors might overshadow the beneficial inhibitory effects of RTV on atherosclerosis.[8] The prevalence of the most significant risk factor, i.e. cigarette smoking, is high among HIV-infected patients with CHD (69 %).[9] Patients may already have some atherosclerotic lesion formation due to ageing.[9] In contrast, treatment of our mice with RTV started at young adulthood. More importantly, prior to initiation of HAART treatment, HIV-infected subjects may have been exposed to chronic systemic inflammation due to HIV-infection for many years. Several opportunistic infections may play a role in the pathophysiology of CHD. It has been suggested that Cytomegalovirus and *Chlamydiae pneumoniae* may promote atherosclerosis.[40] The APOE*3-Leiden mouse provides a good model to study the molecular effects of specific drugs such as RTV in a human-like lipoprotein metabolism setting, independent of the many complicating genetic and environmental factors that can influence the results in human studies.

In conclusion, RTV decreases the development of atherosclerosis in the aortic root of APOE*3-Leiden transgenic mice, despite the induction of hypertriglyceridemia. Because there were no differences in plasma cholesterol levels between RTV treated and control APOE*3-Leiden transgenic mice, this paradoxical effect of RTV was independent of plasma cholesterol levels. This observation indicates that plasma cardiovascular risk factors may not translate into the development of atherosclerosis under all conditions.

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Chapter 8

General Discussion and Future Perspectives

In this thesis the metabolic causes and consequences of hepatic steatosis are described. Hepatic steatosis is characterized by excessive accumulation of triglycerides (TG). The prevalence of hepatic steatosis will certainly increase in the near future, associated with the expected exponential increase in the prevalence of obesity and type 2 diabetes mellitus.[1-3] At present, hepatic steatosis is observed already in 3-24 % of healthy subjects and even in 84-96 % of morbidly obese subjects.[4] Hepatic steatosis was considered a benign, histological condition, until it was discovered that a fatty liver is associated with cardiovascular risk factors such as increased levels of VLDL-TG, glucose, PAI and fibrinogen.[5] Because the liver is the central organ in the disturbances in glucose and lipid metabolism, the main questions are: Are these associations links in a chain or spokes on a wheel and what could then be the common feature or cause connecting the spokes? Although many studies have shown strong associations between hepatic TG content and hepatic insulin resistance[6,7], only few studies have investigated the mechanisms underlying this association. We consider fatty liver as a mediator in the perturbations of glucose and lipid metabolism. Hepatic steatosis can be both actively and passively involved in these metabolic disturbances.

Comments on the measurements of hepatic insulin sensitivity

A glucose tolerance test can not discriminate between whole-body and liver-specific insulin sensitivity. Therefore, in our studies we used the golden standard for measuring whole body and liver-specific insulin sensitivity: the hyperinsulinemic euglycemic clamp technique. By primed continuous infusion of D-[3-^{3}H]glucose and the measurement of the specific activity of this tracer, we can discriminate between the amount of glucose produced by the liver and the amount of glucose taken up by peripheral tissues. In Chapter 3 we have compared the dose-dependent effects of insulin on glucose production and VLDL-TG production by the liver under hyperinsulinemic euglycemic conditions with different insulin concentrations. Interestingly, although the liver plays a central role in both glucose and lipid metabolism, these two processes are differentially regulated by insulin. We found that hepatic glucose output (HGO) is much more sensitive to insulin-mediated inhibition than hepatic VLDL-TG production. The mechanism behind this difference in insulin sensitivity remains unclear.

The mammalian body, especially the brain, largely depends on glucose as an energy substrate. From a teleological perspective it is tempting to speculate that maybe therefore, plasma glucose levels are tightly regulated, even after a carbohydrate containing meal. In contrast, after a fat containing meal, a large increase in plasma fatty acids (FA) and TG can be observed. This may be due to the fact that the hepatic VLDL-TG production is less sensitive to insulin-mediated inhibition than HGO. Normally insulin-mediated suppression of HGO is used as a measure of hepatic insulin sensitivity, but it is also relevant to consider insulin sensitivity of hepatic VLDL-TG production. It appears that these two processes do not change in parallel. For instance, we found in our CD36-deficient mice that although HGO is severely insulin resistant, the hepatic VLDL-TG production was not different under hyperinsulinemic conditions between *cd36*$^{-/-}$ mice and control littermates (83 ± 2 vs 94 ± 3 µmol TG/kg bodyweight/h; unpublished observations). It would be interesting to determine whether this dissociation between insulin sensitivity of HGO and of hepatic VLDL-TG production also occurs in other conditions.

The amount of insulin that is infused and the resulting plasma insulin levels are of major importance for the implementation and interpretation of the hyperinsulinemic euglycemic clamp analysis. A low insulin dose already suppresses HGO, whereas no effect on hepatic VLDL-TG production may be observed. Infusion of high insulin dosages may lead to the overlooking of subtle differences in hepatic insulin sensitivity, especially with regard to HGO. In the ideal situation plasma insulin levels are always similar in experimental groups to allow comparison of the clamp results. For different reasons, however, the resulting plasma insulin levels sometimes differ between groups, despite the infusion of identical amounts of insulin. Some studies correct for plasma insulin levels in their results, but should this be allowed? In Chapter 5 we also found a difference in plasma insulin levels between groups, despite the infusion of identical amounts of insulin. We decided not to correct for this observation, since we do not know the underlying cause of this difference in plasma insulin levels. Insulin can be cleared faster, with or without having an impact on insulin signaling. Therefore, we suggest that when the cause and/or consequence of different plasma insulin levels is not clear, corrections should not be used.

Another important aspect, that needs to be considered in the design of hyperinsulinemic euglycemic clamp experiments, is the use of anesthetics. In this thesis all clamp studies are performed in mice anaesthetized with acetylpromazine,

midazolam and fentanyl (VDF). Early on in our studies we were forced to switch from one combination of anesthetics to another combination for practical considerations, i.e. the availability of the anesthetics. To validate the new anesthetics we compared the old regimen (fluanisone, midazolam and fentanyl; HM) with two new combinations: VDF versus medetomodine, midazolam and fentanyl (MMF) on parameters obtained during clamp experiments. We found that MMF caused severe insulin resistance, whereas HM and VDF did not affect insulin sensitivity. Therefore, it is of great importance to validate anesthetics in all physiological experiments, to exclude possible interference of these drugs with normal metabolism.

Hepatic steatosis with hepatic insulin resistance

In this thesis we have used several murine models with targeted disruptions of the FA metabolism. The *cd36*$^{-/-}$ mice and the ritonavir- (RTV-)treated mice confirm the inverse association between increased liver lipid content and decreased hepatic insulin sensitivity. In these two models we investigated the mechanisms behind the disturbances in the lipid metabolism, leading to increased plasma FA and TG levels.

CD36-deficient mice

CD36, or fatty acid translocase (FAT), is involved in the high affinity uptake of FA in the periphery. Mice lacking CD36 have considerably impaired FA uptake in muscle and in adipose tissue.[8] These mice exhibit increased plasma FA and TG levels and show decreased plasma glucose levels.[9] In the liver plasma membrane FA-binding protein (FABPpm), but not CD36, is the main FA transporter.[10] Consequently, in *cd36*$^{-/-}$ mice the increased plasma FA level leads to increased uptake of FA by the liver. This increased flux of FA leads to an increase in β-oxidation, reflected in increased plasma levels of ketone bodies. The increased FA flux, however, largely exceeds β-oxidation capacity. These excess FA, that cannot be oxidized, are stored as TG and steatosis develops. Previously, Goudriaan *et al.* showed that *cd36*$^{-/-}$ mice exhibit hepatic steatosis and severely decreased hepatic insulin sensitivity.[11] If the liver would have been able to increase the production of VLDL-TG, this increase of hepatic TG content could have been prevented. We showed in Chapter 4 that the increased plasma TG levels in CD36 deficiency were not due to a previously hypothesized enhancing effect on hepatic VLDL-TG production or an effect on intestinal lipid absorption. Instead, CD36 deficiency caused hypertriglyceridemia by

decreased LPL-mediated hydrolysis of TG-rich lipoproteins resulting from FA-induced product inhibition.

Increased plasma FA levels are commonly associated with insulin resistance.[12] In the *cd36*$^{-/-}$ mice despite increased plasma FA (and TG) levels, the periphery is even more sensitive to insulin-stimulated glucose uptake compared to controls.[11] It appears that tissue-specific uptake of FA is more important than plasma FA levels *per se*. The *cd36*$^{-/-}$ mice may be more sensitive to insulin-stimulation of glucose uptake, because in the periphery there is no possibility to use FA as an energy source. This is in accordance with the Randle hypothesis, which states that the availability of FA for oxidation determines insulin sensitivity and the rate of glucose oxidation.[13,14]

Ritonavir-treated mice

The introduction of highly active antiviral therapy (HAART) has led to a considerable reduction in the morbidity and mortality that was associated with HIV-infection. Unfortunately, these drugs are associated with severe adverse metabolic effects, such as the lipodystrophy syndrome. In this syndrome subcutaneous wasting of fat is observed (lipoatrophy) with or without accumulation of fat in the dorso-cervical region ("buffalo hump") or in the abdomen (lipodystrophy). Several metabolic disturbances such as hyperlipidemia, hyperglycemia and insulin resistance are observed in subjects with the lipodystrophy syndrome. Hepatic steatosis is also observed frequently.[15] Few studies have shown a direct mechanism involved in the emergence of this syndrome. Several studies indicated that the hyperlipidemia induced by HIV protease inhibitors such as RTV is due to an increase in hepatic VLDL-TG production. A study in HIV-infected patients hypothesized that excessive FA mobilization occurred due to insulin resistance of adipose tissue resulting in increased hepatic VLDL-TG production.[16] Studies in C57Bl/6 and AKR/J mice showed increased VLDL-TG production after RTV treatment.[17,18] Evidence also existed that HIV protease inhibitors do not reduce the clearance of VLDL-TG particles[17-19] providing additional support for a mechanism based on increased production of TG-rich particles. However, other studies indicated that impaired lipoprotein clearance may contribute to protease inhibitor-induced hyperlipidemia. Baril *et al*. found that both LPL and hepatic lipase (HL) were decreased in HIV-infected patients treated with protease inhibitors such as RTV.[20] TG-rich lipoprotein

clearance was reduced in HIV-patients after a high fat meal.[21] Obviously, many contradictory hypotheses with regard to the mechanism underlying protease inhibitor-induced hyperlipidemia existed. In Chapter 6 we conclusively elucidated the mechanism behind RTV-induced hypertriglyceridemia. RTV decreases plasma LPL activity, either by decreasing expression levels of LPL but most probably also via inhibition of the activity of the LPL enzyme that is present. With respect to the underlying mechanism of lipodystrophy, we found that the adipose tissue of RTV-treated mice takes up less FA derived from the plasma free FA pool and from VLDL-TG particles. Therefore, long-term inhibition of FA uptake by adipose tissue may eventually lead to decreased adipose tissue mass. In addition, we found in unpublished observations that RTV-treated mice showed hepatic steatosis and hepatic insulin resistance (Figure 1A and 1B). It may be that the excess FA that cannot be taken up into the adipose tissue are taken up by the liver, although this was not evident from the data of our study on tissue-specific FA uptake. There is an intriguing resemblance between the *cd36*$^{-/-}$ mice described in Chapter 4 and RTV treated mice (Chapter 6). Apparently, in both mouse models hypertriglyceridemia is present and FA uptake from plasma is decreased. RTV-treated and CD36 deficient mice show hepatic steatosis and severe hepatic insulin resistance as is shown in Figure 1. However, in *cd36*$^{-/-}$ mice this is associated with increased peripheral insulin sensitivity, whereas in RTV-treated mice peripheral insulin sensitivity was not changed. Most likely, this discrepancy indicates that there are different tissue specific alterations between both models, which were not addressed directly in our study design. For instance, muscle TG content was increased in RTV-treated mice compared to control mice, whereas it remained unchanged in *cd36*$^{-/-}$ mice compared to littermates.

Interestingly, in presence of excess adipose tissue (obesity) and in the absence of adipose tissue (lipoatrophy), similar metabolic disturbances are observed: hyperglycemia, hyperinsulinemia and hyperlipidemia. Disturbances in adipose tissue metabolism affect hepatic FA/TG metabolism, and *vice versa.* Several important questions remain, however. At present, it remains unclear to what extent the results obtained in the RTV-treated APOE*3-Leiden transgenic mice can be extended to the action of protease inhibitors in HIV-infected patients. In addition, it is important to understand the actual biochemical mechanism(s) behind the RTV-induced decrease in adipose tissue FA uptake. For instance, the selectivity for adipose tissue suggests

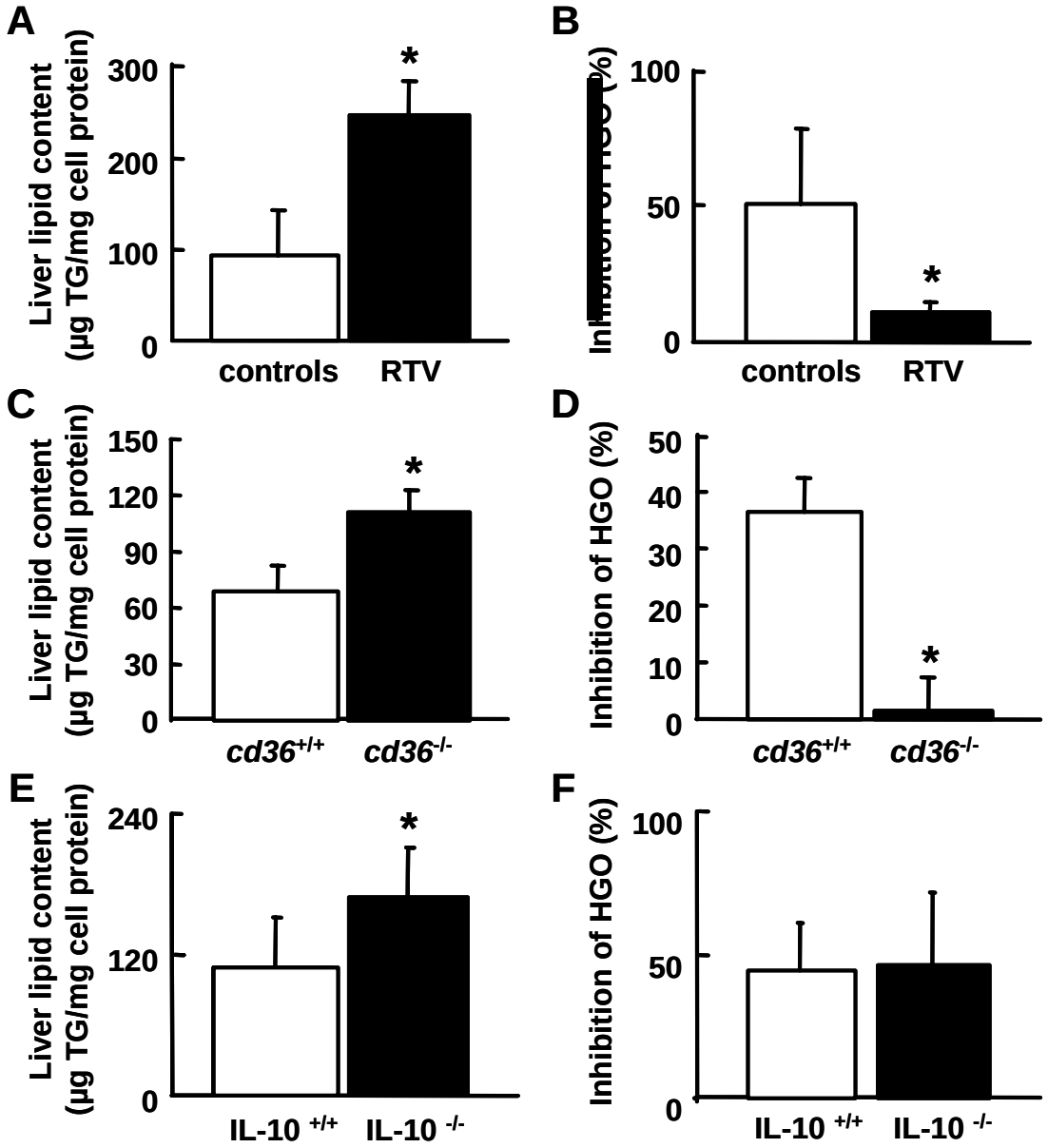

Figure 1. Hepatic TG content and insulin sensitivity in the 3 described models. Using high performance thin layer chromatography hepatic TG content was determined in RTV-treated mice (**A**), *cd36*$^{-/-}$ mice (**C**) and IL-10$^{-/-}$ mice (**E**) and their appropriate controls. Hepatic insulin sensitivity was determined using the hyperinsulinemic euglycemic clamp analysis. RTV-treated mice (**B**) and *cd36*$^{-/-}$ mice (**D**) showed a significantly decreased insulin-mediated inhibition of hepatic glucose output whereas in IL-10$^{-/-}$ mice (**F**) hepatic insulin sensitivity remained unchanged. * $P < 0.05$

the possible involvement of factors like peroxisome proliferator-activated receptor γ (PPARγ) which is also important in the regulation of CD36. Further studies are needed to investigate the molecular mechanism behind the lipodystrophy syndrome.
It could be speculated that HIV-infected patients have a high risk of developing hepatic steatosis. Multiple factors have been hypothesized to be necessary for the development and progression of this condition.[22] Potential risk factors in HIV-infected individuals include disturbances in glucose and lipid metabolism, chronic inflammation, hepatitis co-infection, and treatment with antiretroviral drugs such as protease inhibitors. Hepatic steatosis, which is often observed in HIV-infected subjects, is associated with increased plasma glucose, FA and TG levels which are traditional cardiovascular risk factors. However, studies on steatosis in HIV-infected patients are still rare. Nevertheless, while waiting for prospective studies in HIV-infected patients, improved recognition, diagnosis and management of steatosis are required in these patients.

Hepatic steatosis and atherosclerotic risk

Human cohort studies showed that HAART-treated patients are at greater risk of developing premature atherosclerosis.[23] This group however has increased cardiovascular risk factors which may overshadow the beneficial inhibitory effects of RTV on atherosclerosis.[24] HIV-infected patients with CHD are older than patients without CHD.[23] Patients may already have some atherosclerotic lesion formation due to their age, whereas our mice started treatment while they were “young adults”. Several opportunistic infections may play a role in the pathophysiology of CHD. It has been suggested that cytomegalovirus and *Chlamydiae pneumoniae* may promote atherosclerosis.[25] Furthermore, before treatment is started patients have been exposed to chronic systemic inflammation due to HIV-infection for sometimes up to 10 years. The prevalence of the traditional risk factor cigarette smoking is high among HIV-infected patients with CHD (69 %).[23] It may be of interest to follow HIV-infected children on HAART, and follow the development of atherosclerosis in these subjects. The problem here is that it will probably take up to 50 years before conclusive results can be drawn from such a study. Therefore, in this thesis we studied the development of atherosclerosis in RTV treated mice, which developed hepatic steatosis and hepatic insulin resistance, in addition to an atherogenic lipoprotein profile. From these adverse effects of RTV on cardiovascular risk factors,

we expected that RTV would induce or accelerate atherosclerosis. However, in contrast to our expectations, RTV protects against the development of atherosclerosis in the APOE*3-Leiden transgenic mice (Chapter 7). The important question is to what extent we can extrapolate this remarkable observation in (APOE*3-Leiden transgenic) mice to RTV-treated HIV-infected humans, treated with other HAART drugs as well. Nonetheless, at present, our mouse model is the most appropriate substitute, in which we can study the effects of drugs such as RTV without the many complicating genetic and environmental factors that can influence results in human studies.

In the literature discussion exists whether hepatic steatosis should be added to a cluster of cardiovascular risk factors (metabolic syndrome) important in determining cardiovascular risk. Since a fatty liver is involved the production of cardiovascular risk factors, it may be important to take this condition into consideration when establishing individual cardiovascular risk. However, the fact that the relationship between hepatic steatosis and metabolic disturbances leading to increased cardiovascular risk is apparently not straightforward has to be taken into account.

Hepatic steatosis without hepatic insulin resistance

The association between increased hepatic TG content and hepatic insulin resistance does not always hold. In Chapter 2 we already discussed some dissociations in this respect, for example the *ob/ob* mouse treated with rosiglitazone[26] or wild type mice treated with LXR-agonists.[27] These models show increased hepatic TG content with paradoxically increased or unchanged hepatic insulin sensitivity compared to their respective controls. Another mouse model with increased hepatic TG content without a change in hepatic insulin sensitivity is the interleukin-10-(IL-10-) deficient mouse.

IL-10 deficient mice

In epidemiological studies insulin resistance is associated with chronic low-grade inflammation.[28] This is reflected in associations between the degree of insulin sensitivity and plasma levels of several cytokines, such as tumor necrosis factor (TNF)α and interleukin-(IL)6.[29,30] IL-10 is a potent anti-inflammatory cytokine, which is produced by T-cells, B-cells, monocytes and macrophages and plays a crucial role in the innate immune system.[31,32] IL-10 potently inhibits the production of pro-inflammatory cytokines, including TNFα and IL-6.[33] Previous studies in humans have

shown an association between the production capacity of IL-10 by blood cells and cardiovascular risk factors.[34] To evaluate a causal relationship between IL-10 production and metabolic dysregulation, we assessed in Chapter 5 the direct consequences of IL-10 deficiency on hepatic and peripheral insulin sensitivity. Our data showed, that basal IL-10 production protects against hepatic steatosis during high fat feeding (Figure 1E). However, endogenous IL-10 production did not improve hepatic or whole-body insulin sensitivity during high fat feeding as assessed by the hyperinsulinemic euglycemic clamp technique (Figure 1F). This finding is in contrast to the strong association that is found between liver TG content and insulin resistance in several other models (Chapter 2). Strikingly, the IL-10$^{-/-}$ mice showed decreased plasma insulin levels compared to control mice while infusing similar insulin concentrations. Although this complicates the interpretation of the clamp results, we can still conclude, that basal IL-10 expression does not improve hepatic insulin sensitivity. It would be interesting to perform insulin clearance studies in these mice to gain a better insight into the mechanism behind this difference in plasma insulin levels.

The cause of the increased liver TG content may be the increased plasma FA levels after overnight fasting. The increased plasma FA levels are most probably due to the increased visceral fat mass in the IL-10$^{-/-}$ mice compared to their wild type counterparts. Interleukins have been shown to affect adipose tissue metabolism in other murine models. The IL-1 receptor antagonist knockout (IL-1Ra$^{-/-}$) mice have a defect in lipid accumulation in adipose tissue, exhibiting leanness, which could be expected from their catabolic state.[35] IL-6-deficient mice developed obesity and obesity-related disorders which could be partly reversed by replacement with the pro-inflammatory IL-6.[36] The absence of the anti-inflammatory IL-10 was also expected to lead to a higher inflammatory (catabolic) state, and consequently, to a decreased amount of adipose tissue. However, plasma levels of fibrinogen and serum amyloid A, which reflect liver and systemic inflammation, respectively, were not changed in the IL-10$^{-/-}$ mice compared to the wild type controls. In contrast to our expectations, we found an increased amount of visceral adipose tissue in the IL-10$^{-/-}$ mice compared to the wild type controls. We currently do not know why the adipose tissue mass is increased in the IL-10-deficient mice. A factor that largely determines the uptake of FA by the adipose tissue is LPL-activity. An oral fat load experiment in which plasma TG and FA appearance in time are measured after an oral olive oil

bolus may give an indication of LPL-activity in the IL-10$^{-/-}$ mice. It is also interesting to measure the uptake of FA by the adipose tissue in these mice to determine whether there is an increased FA uptake from VLDL-TG or the albumin-bound FA pool leading to increased adipose tissue mass.[37] In our clamp study we did not determine adipose tissue-specific insulin sensitivity. The ~40% decrease in plasma FA during hyperinsulinemia in both the IL-10$^{-/-}$ mice and the control mice suggests no change in adipose tissue insulin sensitivity. To exclude an effect of IL-10 deficiency on adipose tissue insulin sensitivity more specific *in vivo* and *in vitro* experiments investigating adipose tissue lipolysis are required.

Our observations in the IL-10-deficient mice argue against a simple protective role of endogenous IL-10 secretion in insulin resistant states. Nonetheless, our data also indicate that endogenous IL-10 secretion is not metabolically inert, since we documented clear effects of IL-10 deficiency on hepatic and peripheral lipid metabolism. However, our study did not support a causal role of IL-10 in the protection against diet-induced hepatic insulin resistance and other metabolic disturbances. IL-10 is a locally acting cytokine, and therefore plasma levels may not be causally involved in insulin resistance. The results from epidemiological studies investigating similar plasma parameters should therefore be interpreted with caution with respect to underlying causal mechanisms.

Hepatic steatosis: Cause or consequence of metabolic disturbances?

The different models used in this thesis clearly show, that not every form of hepatic steatosis has the same metabolic causes and consequences. Different causes of steatosis may have different metabolic effects. Human studies investigating causes of fatty liver and consequent metabolic disturbances showed that etiology can make a difference.[38] Like in several mouse models, the causes and effects of hepatic steatosis in humans probably also depend on the genetic and environmental background. This remains difficult to investigate this since the liver is not easily accessible in humans. Therefore, we decided to study the causes and consequences of hepatic steatosis in several mouse models.

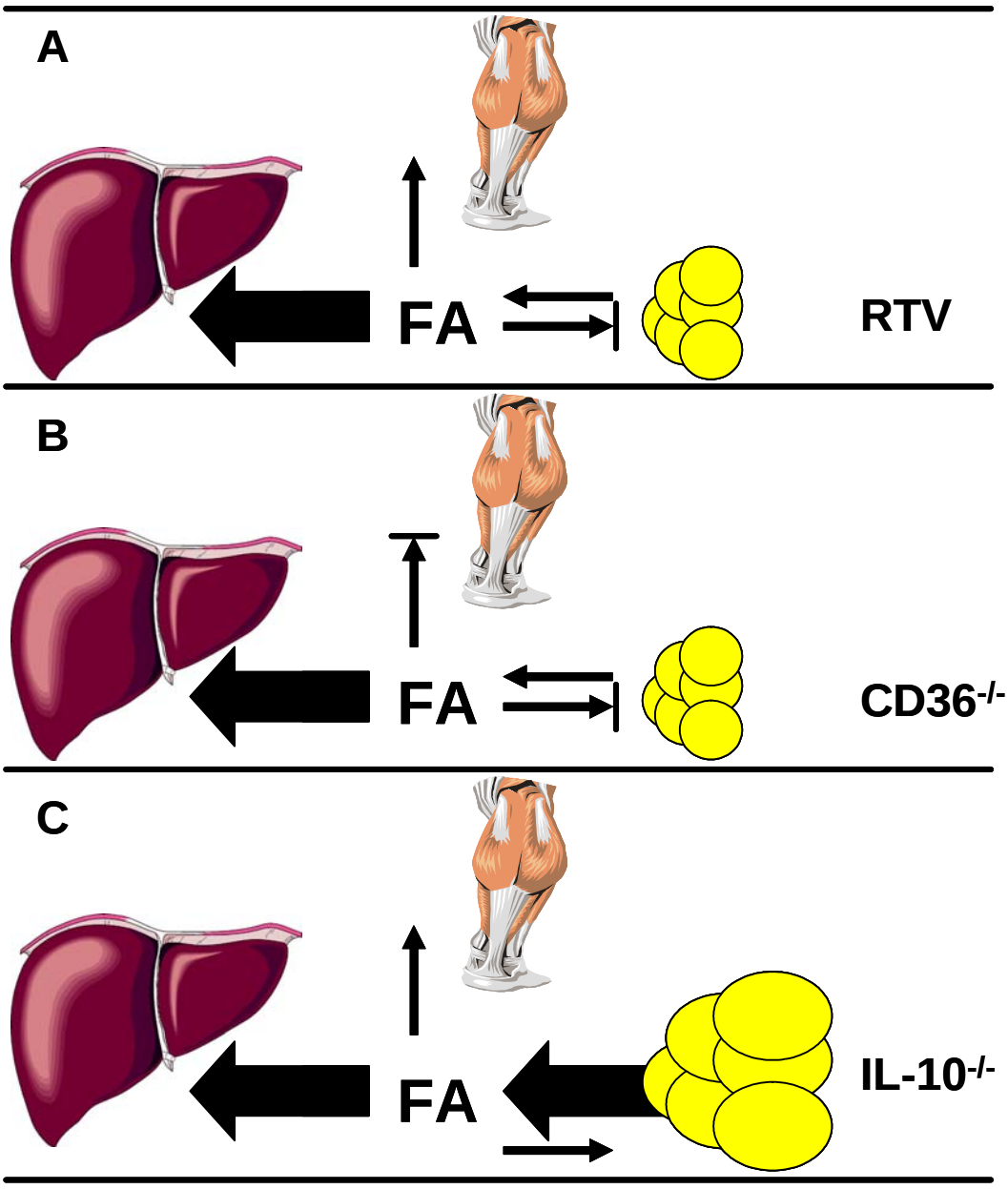

Figure 2. Increased plasma FA fluxes cause hepatic steatosis. The causes and consequences of hepatic steatosis differ between the three models described in this thesis, but in all models an increased flux of FA is most probably involved. **A.** RTV-treated mice show increased plasma FA levels which are due to decreased FA uptake by adipose tissue and an increased postprandial FA response. **B.** CD36-deficient mice have increased plasma FA levels due to decreased uptake of FA in peripheral tissues such as adipose tissue and muscle. **C.** IL-10-deficient mice show increased plasma FA levels after overnight fasting which are most probably due to the increased visceral adipose tissue mass observed in these mice.

Plasma FA flux appears to be important in the emergence of a fatty liver. The 3 models studied in this thesis all show increased plasma FA levels which are due to decreased FA uptake and/or decreased LPL-mediated TG hydrolysis or increased FA release from adipose tissue as is shown in Figure 2. The *cd36*$^{-/-}$ mice and the IL-10$^{-/-}$ mice both show hepatic steatosis, most probably due to increased plasma FA levels. This induces hepatic insulin resistance in the *cd36*$^{-/-}$ mice, but not in the IL-10$^{-/-}$ mice. Both mouse models show increased plasma FA levels after overnight fasting. In the *cd36*$^{-/-}$ mice this is due to decreased peripheral FA uptake.[8] In the IL-10$^{-/-}$ mice this is probably due to an increased release of FA from the increased visceral adipose tissue mass (Chapter 5). The important difference between these two models is the exposure time to the increased plasma FA. The *cd36*$^{-/-}$ mice have increased plasma FA levels from birth, or even *in utero*, while the IL-10$^{-/-}$ mice only displayed increased plasma FA after overnight fasting. The RTV-treated mice show hepatic steatosis and hepatic insulin resistance (Figure 1A and B), but here the cause is unclear. It has been hypothesized that RTV induces accumulation of activated forms of sterol regulatory binding protein (SREBP)-1 and -2 in the nucleus of liver and adipose tissue, resulting in elevated expression of lipid metabolism genes.[39] We observed that postprandially these mice show significantly increased plasma TG and FA, but the plasma FA and TG levels were also increased after 4 h fasting. Similar to the *cd36*$^{-/-}$ mice, the RTV-treated mice may also be continuously exposed to increased plasma FA levels which may be involved in the emergence of steatosis and insulin resistance.

We have not investigated the distribution of the TG in the hepatic lobules by histology. This may also be of importance in the different metabolic causes and consequences of hepatic steatosis since metabolic pathways are not uniformly distributed in the liver.[40-42] Diabetes-associated steatosis is predominantly present in the perivenous zones of the liver.

In recent studies from the group of Rossetti the role of the brain in the regulation of insulin action on the liver was investigated.[43-45] The overall conclusion from those studies was that insulin-mediated control of HGO is controlled by the brain, and more specifically, by the hypothalamus. No studies have yet been performed on the role of the brain in other aspects of hepatic insulin sensitivity such as in the control of hepatic VLDL-TG production. It would be interesting to investigate this aspect of insulin action on the liver in a model without hypothalamic control. The hepatic

glucose production is under parasympathetic and sympathetic neuronal control, which can be eliminated in an experimental setting by transsection of the hepatic parasympathetic or sympathetic nerves.[46] With these studies of Rossetti in mind, it would be interesting to determine to what extent hypothalamic control is involved in different consequences of hepatic steatosis. *Cd36*$^{-/-}$ mice especially would lend themselves as good models to investigate this aspect of insulin sensitivity.

In this thesis we considered the causes and consequences of hepatic steatosis. The liver is an essential organ involved in the integrative physiology of whole-body glucose and FA metabolism. It is very difficult to dissect the causes and consequences of hepatic steatosis in the intact individual, due to the complex interactions between different organs. These interactions include multiple metabolic and endocrine factors transported by the blood between organs and also tissue-specific activity of the autonomous nervous system. The hierarchy between these different factors in modulating hepatic insulin sensitivity remains at present unclear. In general, experimental conditions are usually focused on a single factor. Therefore, the relative contribution of each of these individual factors on the metabolic causes and effects of liver steatosis is difficult to estimate. Because the prevalence of metabolic syndrome is reaching endemic proportions, it is important to investigate the causes and consequences of hepatic steatosis both in human and in animal studies.

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Chapter 9

Summary

In this thesis we focused on the causes and consequences of hepatic steatosis. Epidemiological studies in humans, as well as experimental studies in animal models, have shown an association between visceral obesity and dyslipidemia, insulin resistance and type 2 diabetes mellitus. The mechanism underlying this association remains unclear. Recently, attention has focused on the role of excessive accumulation of triglycerides (TG) in the liver (hepatic steatosis) in this association. Hepatic steatosis was considered a benign condition until it was discovered that a nonalcoholic fatty liver is associated with many cardiovascular risk factors. Subsequently, many studies have shown a strong association between hepatic TG content and hepatic insulin resistance. However, it remains unclear to what extent hepatic steatosis is actively or passively involved in the metabolic derangements of the glucose and lipid metabolism.

In Chapter 2 we summarize important principles of the pathophysiological involvement of the liver in disturbances in the glucose and lipid metabolism obtained in rodent models. We observed that in some models the strong association between hepatic TG content and hepatic insulin resistance does not hold. From this review we concluded that the liver is both actively and passively involved in the disturbances of the glucose and lipid metabolism.

The effect of insulin in normal livers with regard to the hepatic glucose output (HGO) has been studied extensively. In Chapter 3 we have compared the dose-dependent effects of insulin on HGO and VLDL production in the liver under hyperinsulinemic euglycemic conditions with different insulin concentrations. Interestingly, while the liver plays a central role in glucose and lipid metabolism, HGO and hepatic VLDL-TG production are differentially regulated by insulin. We found that hepatic glucose output is much more sensitive to insulin-mediated inhibition than hepatic VLDL-TG production.

CD36, or fatty acid translocase (FAT), is involved in the high affinity uptake of FA in the periphery. Mice lacking CD36 have considerably impaired FA uptake in muscle and in adipose tissue. These mice exhibit increased plasma FA and TG levels and show decreased plasma glucose levels. Furthermore these mice have an increased hepatic TG content and have severely insulin resistant livers. We showed in Chapter

4 that the increased plasma TG levels in CD36 deficiency were not due to a previously hypothesized enhancing effect on hepatic VLDL-TG production or an effect on intestinal lipid absorption. Instead, CD36 deficiency resulted in hypertriglyceridemia caused by decreased LPL-mediated hydrolysis of TG-rich lipoproteins resulting from FA-induced product inhibition.

In epidemiological studies insulin resistance is associated with chronic low-grade inflammation. This is reflected in associations between the degree of insulin sensitivity and plasma levels of several cytokines, such as tumor necrosis factor (TNF)α and interleukin(IL)-6. IL-10 is a potent anti-inflammatory cytokine, which is produced by T-cells, B-cells, monocytes and macrophages and plays a crucial role in the innate immune system. IL-10 potently inhibits the production of pro-inflammatory cytokines, including TNFα and IL-6. In Chapter 5 we established the direct consequences of IL-10 deficiency on hepatic and peripheral insulin sensitivity. Our data showed, that basal IL-10 production protects against hepatic steatosis during high fat feeding. However, endogenous IL-10 did not improve hepatic or whole-body insulin sensitivity during high fat feeding as assessed by the hyperinsulinemic euglycemic clamp technique.

The introduction of highly active antiviral therapy (HAART) has led to a significant reduction in the morbidity and mortality that was associated with HIV-infection. Unfortunately, these drugs are associated with severe adverse metabolic effects, such as the lipodystrophy syndrome. In this syndrome subcutaneous wasting of fat is observed (lipoatrophy) with or without accumulation of fat in the dorso-cervical region (“buffalo hump”) or in the abdomen (lipodystrophy). Hepatic steatosis is often observed in these patients. Several metabolic disturbances such as hyperlipidemia, hyperglycemia and insulin resistance are observed in subjects with the lipodystrophy syndrome. Few studies have shown a direct mechanism involved in the emergence of this syndrome. In Chapter 6 we conclusively elucidated the mechanism behind RTV-induced hypertriglyceridemia. RTV decreases plasma LPL activity, either by decreasing expression levels of LPL but most probably also via inhibition of the activity of the LPL enzyme that is present. With regard to the mechanism underlying lipodystrophy we found that the adipose tissue of RTV-treated mice takes up less FA derived from the plasma free FA pool and from VLDL-TG particles, compared to

untreated mice. In contrast to our expectations, although RTV induces an atherogenic lipoprotein profile, it protects against the development of atherosclerosis in the APOE*3-Leiden transgenic mice (Chapter 7).

In conclusion, the studies in this thesis show that hepatic steatosis is actively and passively involved in the metabolic disturbances in the glucose and lipid metabolism. The prevalence of hepatic steatosis in western countries is high and will certainly increase with the epidemics of obesity and diabetes. This will put an increasing number of subjects at risk for disturbances in the glucose and lipid metabolism and concomitantly for cardiovascular disease.

Samenvatting

In dit proefschrift hebben we studies uitgevoerd om de oorzaken en gevolgen van leversteatose te bestuderen. Epidemiologische studies in mensen en experimentele studies in diermodellen hebben een associatie laten zien tussen viscerale obesitas en dyslipidemie, insuline resistentie en type 2 diabetes mellitus. Het mechanisme achter deze associatie is nog onduidelijk. Recent is de aandacht gevestigd op de rol van overmatige triglyceriden (TG) accumulatie in de lever in deze associatie. Leversteatose werd vroeger beschouwd als een goedaardige conditie, totdat in epidemiologische studies ontdekt werd dat een vette lever is geassocieerd met vele cardiovasculaire risicofactoren. Veel studies hebben een sterke associatie tussen het lever TG gehalte en hepatische insuline resistentie laten zien. Het blijft echter onduidelijk in hoeverre leversteatose actief of passief betrokken is bij de metabole verstoringen van het glucose en lipidenmetabolisme.

In Hoofdstuk 2 hebben we de resultaten van een aantal belangrijke studies in diermodellen samengevat, die inzicht hebben gegeven in de pathofysiologische rol van de lever in de metabole veranderingen en van het glucose en lipidenmetabolisme. In sommige modellen bleek de sterke associatie tussen lever TG inhoud en hepatische insuline resistentie echter niet stand te houden. Uit dit review hebben we geconcludeerd dat de lever zowel actief als passief betrokken is bij de verstoringen van het glucose- en lipidenmetabolisme.

Het effect van insuline op de hepatische glucose productie in normale levers is uitgebreid bestudeerd. In Hoofdstuk 3 hebben we de dosis-afhankelijke effecten van insuline op zowel de hepatische glucose productie als de very-low density lipoproteïnen (VLDL) productie door de lever vergeleken. Hoewel de lever in zowel het glucose- als het lipidenmetabolisme een centrale rol speelt, worden de hepatische glucose productie en VLDL productie verschillend gereguleerd. Uit onze studie hebben we geconcludeerd dat de hepatische glucose productie veel gevoeliger is voor remming door insuline dan de VLDL productie.

CD36, ofwel fatty acid translocase (FAT), is betrokken bij de opname van vetzuren in de perifere weefsels. In muizen zonder CD36 (*cd36* $^{-/-}$) is de opname van vetzuren in de spieren en in het vetweefsel grotendeels verhinderd. Deze muizen hebben hoge plasma vetzuren en TG en lage plasma glucose spiegels. De *cd36* $^{-/-}$ muizen hebben

een verhoogde TG inhoud in de lever en hebben zeer insuline resistente levers. In Hoofdstuk 4 laten we zien dat de verhoogde plasma TG spiegels niet werden veroorzaakt door een verhoogde hepatische VLDL productie of een veranderde darmopname van vetten, zoals eerder was gepostuleerd. Wij concluderen dat in de afwezigheid van CD36 hypertriglyceridemie ontstaat doordat de lipoproteïne lipase (LPL)-gemedieerde hydrolyse van TG-rijke lipoproteïnen wordt geremd via de verhoogde plasma vetzuren (product inhibitie).

In epidemiologische studies wordt insuline resistentie geassocieerd met chronische sub-klinische inflammatie. Dit blijkt ook uit de associaties tussen de mate van insuline gevoeligheid en de plasma levels van verschillende cytokinen zoals tumor necrose factor α (TNFα) en interleukine(IL)-6. IL-10 is een anti-inflammatoir cytokine dat geproduceerd wordt door T-cellen, B-cellen, monocyten en macrofagen. Het speelt een belangrijke rol in het aangeboren immuunsysteem. IL-10 remt zeer krachtig de productie van pro-inflammatoire cytokinen zoals IL-6 en TNFα. In Hoofdstuk 5 hebben we de directe consequenties van IL-10 deficiëntie op lever-specifieke en perifere insuline gevoeligheid bestudeerd. Onze resultaten laten zien dat basale IL-10 productie beschermt tegen leversteatose tijdens een hoog vet dieet. Uit het hyperinsulinemische euglycemische clamp experiment bleek echter dat endogeen IL-10 niet de insuline gevoeligheid tijdens een hoog vet dieet verbetert.

De introductie van highly active antiretroviral therapy (HAART) heeft tot een enorme reductie in de met HIV-infectie geassocieerde morbiditeit en mortaliteit geleid. Helaas zijn deze medicijnen geassocieerd met ongewenste metabole bijwerkingen zoals het lipodystrophie syndroom. Dit syndroom wordt gekarakteriseerd door het verdwijnen van subcutaan vet (lipoatrophie) met of zonder accumulatie van vet in de dorso-cervicale regio (“buffalo hump”) of in de buikholte. Tevens wordt vaak leversteatose gevonden. Patiënten met het lipodystrophie syndroom hebben bovendien vaak verschillende metabole bijwerkingen zoals hyperlipidemie, hyperglykemie en insuline resistentie. Slechts weinig studies hebben een mechanisme laten zien dat dit syndroom zou kunnen verklaren. In Hoofdstuk 6 hebben wij het mechanisme dat de door ritonavir (RTV) geïnduceerde hypertriglyceridemie veroorzaakt opgehelderd. RTV verlaagt de plasma LPL activiteit, waarschijnlijk via verminderde mRNA en/of eiwit expressie levels, maar waarschijnlijk ook via de remming van het LPL enzym

zelf dat in het plasma aanwezig is. We hebben tevens gevonden dat het vetweefsel van RTV-behandelde muizen minder vetzuren opneemt uit de vrije vetzuur pool en uit VLDL-deeltjes vergeleken met controle muizen. Hoewel RTV een atherogeen lipoproteïnen profiel veroorzaakt, beschermt het tegen de ontwikkeling van atherosclerose in de APOE*3-Leiden transgene muizen (Hoofdstuk 7).

De resultaten van de studies beschreven in dit proefschrift laten zien dat leversteatose zowel actief als passief betrokken is bij de metabole verstoringen van het glucose- en lipidenmetabolisme. De prevalentie van leversteatose in de westerse landen is hoog en zal zeker stijgen met de stijging van de prevalentie van obesitas en diabetes. Hierdoor zal voor een groot aantal mensen het risico op verstoringen van het glucose- en lipiden metabolisme stijgen en zo ook het risico op cardiovasculaire ziekten.

List of Publications

1. Annalise M. Martin, Emma Hammond, David Nolan, Craig Pace, **Marion den Boer**, Louise Taylor, Henry Moore, Olivia P. Martinez, Frank T Christiansen, Simon Mallal. Accumulation of Mitochondrial DNA Mutations in Human Immunodeficiency Virus-infected Patients Treated with Nucleoside-analogue Reverse-transcriptase Inhibitors. ***Am J Hum Genet*** **2003 Mar; 72(3):549-60**

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Curriculum Vitae

Marion den Boer werd op 22 september 1979 geboren te Middelharnis. In deze plaats voltooide zij in 1997 het VWO aan de Chr. Scholengemeenschap Prins Maurits. In datzelfde jaar begon ze aan de studie Biomedische Wetenschappen aan de Universiteit Leiden waar zij in 1998 haar propedeuse diploma behaalde.

In 2000 liep Marion stage in het Royal Perth Hospital te Perth in Australië onder begeleiding van Dr. A. Martin en Dr. S. Mallal. Hier deed zij onderzoek naar het ontstaan van mitochondrionale DNA mutaties in HIV-patiënten die behandeld werden met bepaalde medicijnen. Teruggekeerd in Nederland begon zij in 2001 aan haar afstudeerstage, waarin zij de metabole bijwerkingen van antiretrovirale middelen bestudeerde onder begeleiding van Prof. Dr. L.M. Havekes en Prof. Dr. J.A. Romijn. Daarnaast schreef zij een afstudeerscriptie over lever steatose met betrekking tot de metabole implicaties van triglyceriden stapeling. Na haar afstuderen in 2002 begon zij haar promotie onderzoek bij de afdeling Endocrinologie in het Leids Universitair Medisch Centrum en TNO-Kwaliteit van Leven onder begeleiding van Prof. Dr. J.A. Romijn en Prof. Dr. L.M. Havekes. De resultaten van dit promotie onderzoek (onderdeel van het NWO project 903-39-291) zijn beschreven in dit proefschrift.

In september 2006 begint Marion aan het derde jaar van de studie Geneeskunde aan de Universiteit Leiden.