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

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Chapter 1



General introduction



Hepatic steatosis: definitions, stages, prevalence and association with insulin resistance

Hepatic steatosis refers to the, nowadays common, condition of ectopic accumulation of triglycerides (TG) in parenchymal cells (hepatocytes) of the liver. This condition is therefore also termed “fatty liver”. Several forms of hepatic steatosis are generally distinguished, depending on underlying cause of the condition and the progression of the disease. Excessive and chronic alcohol consumption has been known for decades to be associated with alcoholic fatty liver, a condition that is beyond the scope of this thesis. Interested readers are therefore referred to recently published, excellent reviews on this topic (1-4).

In 1980, Ludwig *et al.* (5) were the first to describe the appearance of hepatic steatosis without excessive alcohol usage, a condition they termed nonalcoholic steatohepatitis (NASH). Nowadays, non-alcoholic fatty liver (NAFL) is considered the precursor of NASH and both conditions are non-alcoholic fatty liver diseases (NAFLDs) (6). Table 1.1 shows the major causes of NAFLD, as recently published by Adams *et al.* (7). Especially the association of hepatic steatosis with features of the metabolic syndrome is of interest for this thesis and will be discussed in more detail later in this introduction.

Studies suggest that circa 20% of NASH will finally result in cirrhosis (8). A recent editorial in Gastroenterology (9), however, summarized that ‘simple steatosis’ itself might be relatively benign: over a 15-20 years period, only 1-2% of the patients with this condition actually developed cirrhosis. In patients with fibrosis and NASH, this percentage might be up to 12% in 8 years. The largest natural history study performed so far in patients with NAFLD (10) showed that mortality in these patients is higher than in patients without NAFLD and associated not only with cirrhosis, but also with age and impaired fasting glucose. When cirrhotic patients were excluded, the overall mortality was not increased. Thus, these human data stress the importance of staging the hepatic steatosis found.

In NAFLD, the liver contains more than normal amounts of TG, the most energy-dense molecules in mammalian physiology. TG molecules consist of three fatty acids esterified to a glycerol backbone (figure 1.1). The fatty acids can either be saturated, monounsaturated, or polyunsaturated, reflecting the amount of double bonds. In NASH, the increased TG content is accompanied by hepatocellular ballooning and lobular inflammation (11). NASH can be graded and staged depending on which hepatocytes are inflamed, *i.e.*, perivenously or periportally located hepatocytes, and whether ballooning of cells is minimal, present or marked (12). The progression of simple hepatic steatosis (NAFL) towards NASH and finally cirrhosis, is thought to involve two “hits”, a model first proposed by Day and James in 1998 (13). In this model, the first “hit” is the development of the hepatic steatosis: accumulation of TG in hepatocytes. The second “hit” induces the transition of NAFL to NASH and is the result of the actions of inflammatory molecules, *i.e.*, reactive oxygen species (ROS). The second “hit” might be the result of the increased liver TG levels itself because fatty acids are cytotoxic: fatty acid oxidation in mitochondria and peroxisomes might generate ROS (14). However, one must keep in mind that inflammation itself is the major factor in the transition of NAFL to NASH (15).

It is alarming that the prevalence of NAFLD in the adult population in Western societies is estimated at about 20% (11) and it is therefore suggested that 9.1 million individuals in the United States have NAFLD (16). The risk of NAFLD is almost 5-fold higher in persons with body mass index (BMI) ≥ 30 kg/m² (17). BMI is considered a good index for obesity, but waist-to-hip circumference ratio (WHR) is probably a better marker for fat distribution (18). The World Health Organisation (WHO) defines overweight as BMI ≥ 25 kg/m², individuals with BMI ≥ 30 kg/m² are obese (19). In fact, NAFLD is associated with the metabolic syndrome, obesity and diabetes (20). These conditions all share insulin resistance as a diagnostic criterion. Insulin resistance is defined as a decreased biological

response to normal concentrations of circulating insulin. The role of insulin and the consequences of insulin resistance for (hepatic) metabolism will be discussed later.

According to diagnostic criteria for the metabolic syndrome defined by the National Cholesterol Education Program (NCEP) panel (21), a person suffers from the metabolic syndrome when he/she shows at least three of the criteria listed in table 1.2. The WHO stresses even more the importance of insulin resistance in the diagnosis of the metabolic syndrome (22), as mentioned in table 1.3.

Numerous clinical studies have shown that insulin resistance is a predisposing factor for NASH (20,23-27). The question that is still unanswered is whether hepatic steatosis is caused by insulin resistance, as suggested in the “two hit” hypothesis (13), or that hepatic steatosis predisposes to insulin resistance. Another possibility is that a common factor, such as the adipocyte-derived hormones leptin and adiponectin, contributes to development of both hepatic steatosis and insulin resistance. In the remaining part of this introduction, attention will be focussed on the first two possibilities. Considering the third possibility, recent studies show that people with low plasma leptin concentrations, *e.g.* subjects with congenital lipodystrophy, develop both NASH and insulin resistance (28). Leptin replacement therapies in lipodystrophic patients reverses their NASH (29). Leptin-deficient *ob/ob* mice develop hepatic steatosis, but not NASH, in combination with insulin resistance, hyperglycemia and hypertriglyceridemia (30). Low plasma adiponectin levels are associated with the development of insulin resistance (31) and administration of adiponectin to mice with alcohol-induced hepatic steatosis resulted in decreased liver TG concentrations (32). In *ob/ob* mice, adiponectin administration increased insulin sensitivity and reduced hepatic TG levels (32).

Table 1.1. Major causes or associations of nonalcoholic fatty livers (7).

Cause	Association
Primary	Features of the metabolic syndrome
Secondary	
Nutritional	Total parental nutrition Rapid Weight loss Starvation Intestinal bypass surgery
Drugs	Glucocorticoids Estrogens Tamoxifen Amiodaron Methotrexate Aminosalicilate
Toxins	
Metabolic	Lipodystrophy Dysbetalipoproteinemia Acute fatty liver of pregnancy Reye’s syndrome
Other	Inflammatory bowel disease HIV infection

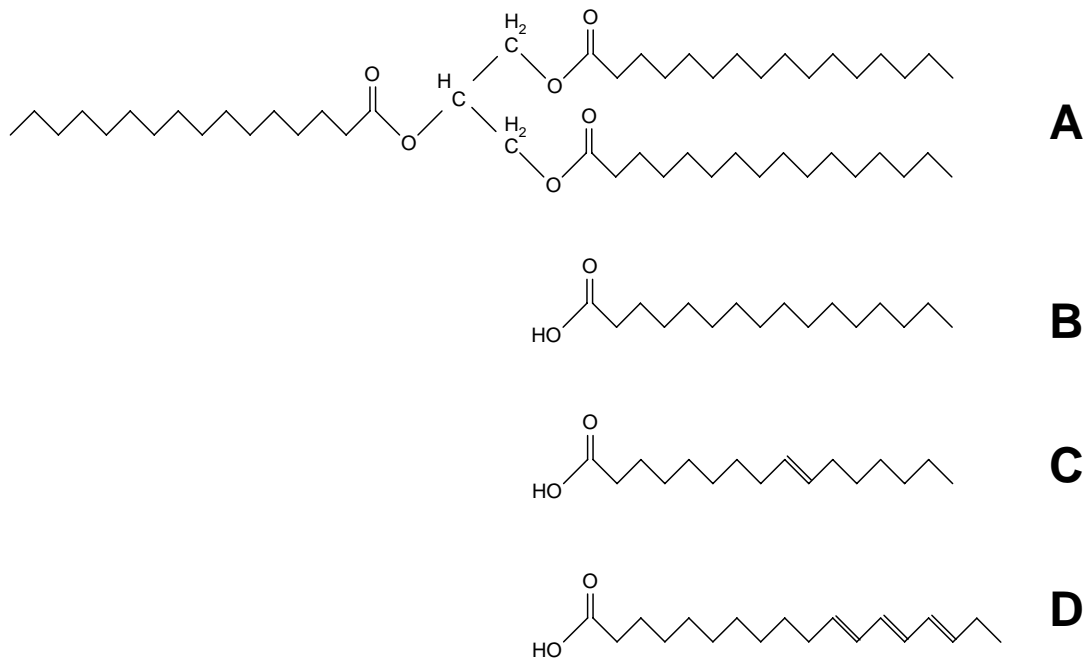


Figure 1.1. Structure of A, triglyceride (tripalmitic acid); B, saturated fatty acid (palmitic acid, C16:0); C, monounsaturated fatty acid (palmitoleic acid, C16:1n7); D, polyunsaturated fatty acid (α -linolenic acid, C18:3n3).

Table 1.2. NCEP diagnosis criteria for the metabolic syndrome (21).

Risk factor	Abnormal level
Waist circumference	> 102 cm (men) or > 88 cm (women)
Fasting blood glucose	≥ 6.1 mM
Serum triglycerides	≥ 1.7 mM
Serum HDL cholesterol	< 1.0 mM (men) or < 1.3 mM (women)
Arterial blood pressure	$\geq 130/85$ mm Hg

Table 1.3 WHO diagnosis criteria for the metabolic syndrome (22).

Required	Insulin	in top 25%
	Fasting blood glucose	≥ 6.1 mM
	2-hour glucose	≥ 7.8 mM
And ≥ 2 of:	Waist-to-hip circumference ratio	> 0.9 (men) or >0.85 (women)
	Body mass index	≥ 30 mg/kg ²
	Serum triglycerides	≥ 1.7 mM
	Serum HDL cholesterol	< 0.9 mM (men) or < 1.0 mM (women)
	Arterial blood pressure	$\geq 140/90$ mm Hg

Insulin: discovery, diabetes and intracellular actions

The hormone insulin was discovered by Best and Banting in 1921 (33) when they investigated the role of the pancreas in diabetes mellitus. Because the first known feature of this disease was the high urinary glucose concentration, it was named diabetes mellitus. “Diabetes” is derived from the Greek word for “pipe-line” to stress the fact that nutrients begin to pass through the system rather than being utilised. “Mellitus” is Latin for “honey” to stress the sweet taste of the urine produced in this condition. Later on, it was discovered that diabetic patients also have increased blood glucose levels.

Insulin is secreted by the B-cells located in the islets of Langerhans of the pancreas when blood glucose levels increase, for instance after a meal. Insulin has energy-saving properties: it enhances storage of glucose in the liver, inhibits hepatic glucose production (HGP) and stimulates uptake of glucose by the peripheral tissues, *i.e.*, muscle and adipose tissue. Insulin also influences whole-body lipid metabolism by inducing hepatic fatty acid and TG synthesis (*de novo* lipogenesis) and inhibiting peripheral breakdown of TG into fatty acids (lipolysis).

Two types of diabetes mellitus are generally distinguished. In diabetes mellitus type 1, high blood glucose levels are the result of the failure of the pancreas to produce sufficient amounts of insulin. Various auto-immune factors have been implicated herein (34) and genetic predisposition is thought to play a major role. Diabetes mellitus type 1 is usually diagnosed in childhood whereas diabetes mellitus type 2 has been associated with elderly but its prevalence in young people and even children is rapidly increasing. Diabetes mellitus type 2 is preceded by insulin resistance. As a result of insulin resistance, the pancreas starts to produce more insulin, a process that in the end will exhaust the B-cells and result in an inability of these cells to produce sufficient insulin. As a result, plain diabetes will develop over time.

Insulin actions are carried out via binding of the hormone to its receptor (a member of the tyrosine kinase receptor family) (figure 1.2). Upon binding, the receptor autophosphorylates and activates two distinct down-stream signaling pathways: the phosphatidylinositol-3-kinase (PI3K) pathway and the MAP kinase/ERK kinase (MEK) pathway. Protein tyrosine phosphatase 1B (PTP-1B) is an enzyme that dephosphorylates the insulin receptor and acts to terminate the signal. In mice, increased levels and activity of PTP-1B result in insulin resistance (35,36). Insulin-mediated effects on glucose and lipid metabolism, most relevant for the work described in this thesis, are thought to be predominantly carried out via the PI3K-pathway but the MEK pathway will also be discussed briefly.

Down-stream insulin receptor substrates (IRSs) are phosphorylated upon autophosphorylation of the insulin receptor. IRS1 and IRS2 are the two isoforms predominantly found in the liver. Recent studies with IRS1- and IRS2-specific knockout mice have shown that they control distinct metabolic pathways (37): IRS1 mainly controls the effects on glucose metabolism whereas IRS2 predominantly mediates the effects on lipid metabolism. In the MEK pathway, IRS phosphorylation results in phosphorylation of the SH2 domain-containing adaptor protein Grb2. As a result, this protein dimerizes with the guanine exchange factor “son-of-sevenless” (SOS) and the subsequent exchange of GTP for GDP activates Ras. Next, GTP-bound Ras binds Raf, the first kinase in the Raf-MAPK route, that will subsequently activate MEK. MEK activates the extracellular signal-related kinase (ERK) 1 and ERK2 that can activate various downstream targets involved in, for instance, gene expression and kinase activity (38).

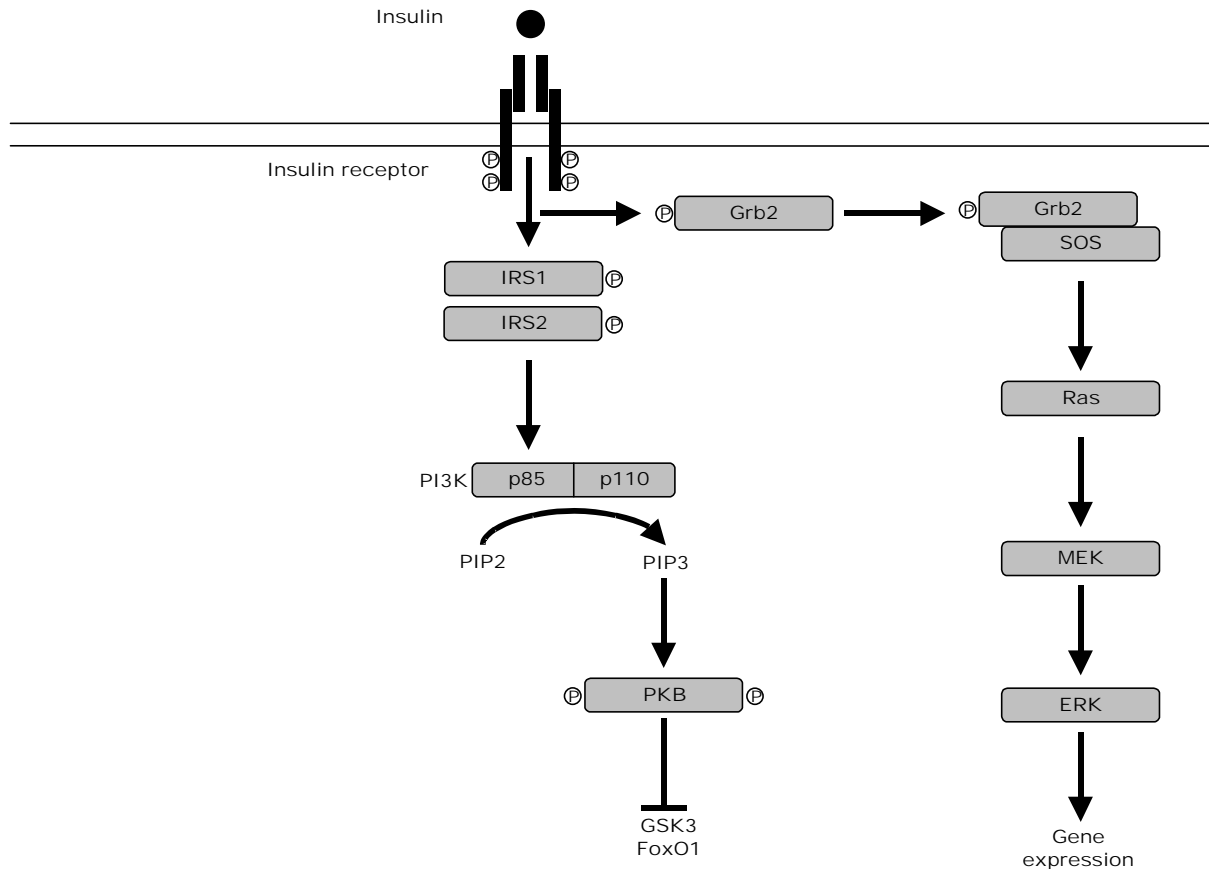


Figure 1.2. Schematic representation of the intracellular insulin signaling cascade. IRS, insulin receptor substrate; ERK, extracellular signal-related kinase; FoxO1, forkhead box “Other”-1; GSK3 β , glycogen synthase kinase-3 β ; MEK, MAP kinase/ERK kinase; PI3K, phosphatidylinositol-4-kinase; PIP2, phosphatidylinositol-4,5-bisphosphate; PIP3, phosphatidylinositol-2,4,5-trisphosphate; PKB, protein kinase B; SOS, son-of-sevenless.

The PI3K pathway also starts after IRS phosphorylation. Phosphorylated IRS has an increased affinity for the p85 subunit of PI3K. Activated PI3K will start the production of the second messenger phosphatidylinositol 2,4,5-trisphosphate (PIP3) from the phospholipid PI4,5P2 (PIP2). This second messenger activates, amongst others, protein kinase B (PKB) via phosphorylation at two sites (Thr308 and Ser473). PKB is a key regulatory protein involved in many steps of insulin-mediated actions on hepatic glucose and lipid metabolism, as will be discussed in more detail in other paragraphs.

Although this topic will not further be discussed in detail, insulin also stimulates peripheral glucose uptake via PKB-mediated translocation of vesicles containing the glucose transporter-4 (GLUT4) to the membrane (39,40). Exactly how PKB influences this translocation is currently not known.

The increasing world-wide prevalence of diabetes mellitus type 2

As mentioned, insulin resistance will normally result in diabetes mellitus type 2, a disease mainly found in adults in their forties or fifties until recently. Nowadays, its prevalence is steeply rising and the condition is increasingly present also in children and adolescents (41,42). In Japanese children, diabetes mellitus type 2 already accounts for 80% of childhood diabetes (43). Not only is childhood prevalence for diabetes mellitus type 2 increasing rapidly, the total number of people world-wide with this condition is thought to rise from 189 million (2003 situation) to 221 million in 2010 and to more than 300 million in 2025 (44-46). Remarkably, the 1995 to 2025 increase in diabetes is estimated to be stronger in developing countries than in developed countries (46). In developing countries, the increase will be 170%, from 84 to 228 million; in developed countries, this increase will be 42%, from 51 to 72 million. This marked difference can, in part, be explained by the “thrifty genotype hypothesis” (47). In ancient times, people in areas prone to famine, *e.g.*, the developing countries, developed a biological mechanism to store more fat to survive prolonged periods of food deprivation. Nowadays, these people, especially in countries like India and China, have more easy access to food and as a result, they accumulate excess fat more easily. When this is also accompanied by a reduction in physical activity (i.e, a sedentary lifestyle), these factors result in obesity, attraction of the metabolic syndrome and diabetes mellitus type 2 (47). The data from Ogden *et al.* (48) show that, in the US, the incidence of obesity is markedly increasing in children. In 1999-2000, the prevalence of overweight (defined as at or above the 95th percentile of the sex-specific BMI) was 15.5 % in 12- through 19-year-olds, 15.3% in the 6- through 11-year-olds, and 10.4% in the 2- through 5-year-olds. In 1988-1994, these percentages were 10.5%, 11.3%, and 7.2%, respectively.

Hepatic glucose metabolism and the actions of insulin

In fed conditions, glucose from the intestine is transported via blood and taken up by tissues by a process regulated, in part, by insulin. Liver and muscle tissue can store excess glucose as glycogen, a highly branched, efficient storage form of glucose (49). The direct pathway from glucose to glycogen consists of two distinct steps (see figure 1.3 for an overview of the intrahepatic glucose fluxes). The first step involves conversion of glucose into glucose-6-phosphate (G6P), a process mediated by hexokinase. The liver-type hexokinase is called glucokinase (GK). The second step involves production of glycogen via uridine diphosphate (UDP)-glucose. Glycogen synthase (GS) is thought to be rate-controlling in the latter process. Although production of glycogen is an efficient way for the liver to store glucose, the glycogen storage capacity is limited. A certain amount of glucose is therefore also broken down in the glycolytic pathway into carbon-3 compounds, *i.e.*, pyruvate and lactate. This process is mediated by enzymes such as phosphofructo-1-kinase and pyruvate kinase (PK). As will be discussed later, the carbon-3 compounds can be the source of acetyl-CoA, that can be used for production of fatty acids (and subsequently TGs) in a process called *de novo* lipogenesis.

In fasted conditions, in contrast, the body largely depends on the glucose produced by the liver. The liver and, to a minor extent, the kidney are the sole organs capable of glucose production and secretion (50). First, the glycogen stores are used to yield glucose, a process that can be considered as a three-step process. The first step is the cleaving of a single glucose-1-phosphate from glycogen, a process controlled by glycogen phosphatase (GP). The second step involves the action of a debranching enzyme that converts glucose-1-phosphate to G6P. The final step is the dephosphorylation of G6P to glucose, mediated by glucose-6-

phosphatase (G6Pase), an enzyme-complex only found in the liver (and kidneys), the reason why only the liver (and the kidney) can produce glucose. Apart from breakdown of glycogen, the liver can produce glucose from other substrates, such as the carbon-3 compounds, in a process called gluconeogenesis (GNG). When fasting exceeds 8 hours in humans, GNG progressively replaces breakdown of glycogen to preserve complete breakdown of this storage form of glucose (51). This gluconeogenic process is controlled by phosphoenolpyruvate carboxykinase (PEPCK) that catalyses conversion of pyruvate to oxaloacetate to phosphoenolpyruvate, by fructose-1,6-bisphosphatase (F1,6BP) that catalyses conversion of fructose-1,6-bisphosphate to fructose-6-phosphate, and by a number of other enzymes. The final step in GNG is the conversion of G6P to glucose by G6Pase.

As mentioned, insulin lowers blood glucose via stimulation of glucose uptake by muscle and adipose tissue as well as by inhibition of HGP. In addition, insulin stimulates conversion of glucose into glycogen and into carbon-3 compounds that are subsequently converted acetyl-CoA, fatty acids, and TG. Studies demonstrate that, in humans, half maximal suppression of HGP occurs at insulin levels of 25 mU/ml, whereas half maximal stimulation of peripheral glucose uptake occurs at 50 mU/ml (52). Thus, HGP is more insulin sensitive than peripheral glucose clearance. Of particular interest are the studies with stable isotope techniques showing that, under basal conditions, HGP in type 2 diabetic individuals was unchanged or only modestly elevated (53-55). Under hyperinsulinemic or hyperglycemic conditions, however, insulin failed to suppress HGP, suggesting abnormal regulation of HGP in diabetes mellitus type 2 (56).

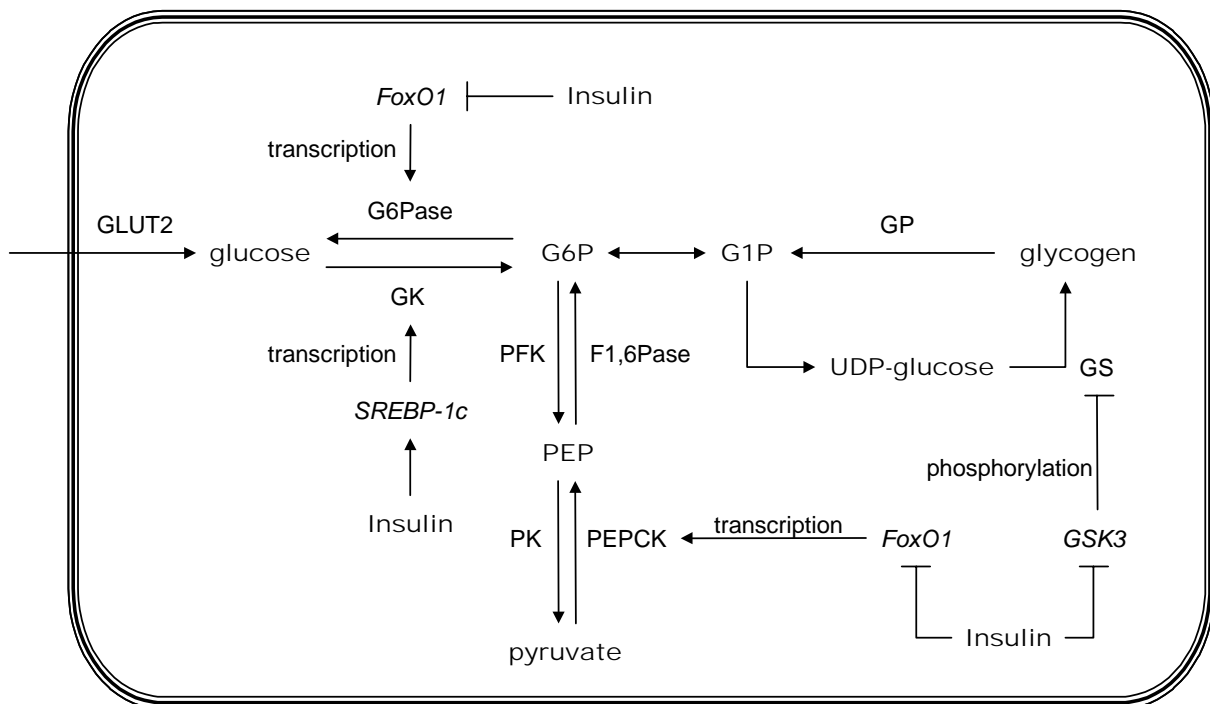


Figure 1.3. Schematic representation of the intrahepatic glucose fluxes and the regulation of these fluxes by insulin. F1,6BP, fructose-1,-bisphosphatase; FoxO1, forkhead box “Other”-1; G1P, glucose-1-phosphate; G6P, glucose-6-phosphate; G6Pase, glucose-6-phosphatase; GK, glucokinase; GLUT2, glucose transporter-2; GP, glycogen phosphorylase; GS, glycogen synthase; GSK3 β , glycogen synthase kinase-3 β ; PEP, phosphoenolpyruvate; PEPCK, phosphoenolpyruvate carboxykinase; PFK, phosphofructo-1-kinase; PK, pyruvate kinase; SREBP-1c, sterol-regulatory element-binding protein-1c.

Via PI3K-induced PKB phosphorylation, insulin enhances phosphorylation of GS kinase-3 β (GSK3 β) (38), a protein whose activity is inhibited by phosphorylation. As a result, GS phosphorylation will be reduced and this enzyme will thus be more active. The final result is an increased storage of glucose in the form of glycogen. GK is also critically involved in insulin-mediated hepatic glucose metabolism. In insulin receptor-deficient mice, overexpression of GK improved glucose tolerance, emphasizing the importance of GK activity (57). Moreover, transcription of the GK gene is regulated by sterol-regulatory element-binding protein-1c (SREBP-1c) (58,59), a key transcription factor in the control of hepatic lipid metabolism (60) whose transcription and activation are regulated by insulin (61). SREBP-1c stimulates transcription of almost all genes involved in fatty acid and TG synthesis (60).

For long time, it was known that insulin suppresses GNG. Recently, the forkhead box “Other”-1 (FoxO1) has been identified as a transcription factor with an important role in mediating the effects of insulin on GNG (62). FoxO1 appears to be a negative modulator of insulin action, as it binds to insulin response sequences found in promoter regions of genes encoding G6Pase (63,64) and PEPCK (65). The actions of FoxO1 on gene transcription are mediated by PKB. PKB can phosphorylate FoxO1 at three sites (Thr24, Ser256, and Ser319 in human FoxO1), resulting in activation and nuclear exclusion of FoxO1 (66-72). Moreover, phosphorylation and cytoplasmic localization of FoxO1 promotes its degradation (73). In vivo evidence for the role of FoxO1 comes from the heterozygous FoxO1 knockout mouse (74,75) that showed reduced G6Pase gene expression and lower plasma insulin levels. Moreover, these heterozygous mice are, on a insulin receptor-knockout background, less insulin resistant than control mice.

In conclusion, via its effects on GS (via GSK3 β), on GK (via SREBP-1c), and on GNG (via FoxO1), insulin stimulates hepatic glucose utilisation and inhibits HGP.

Triglyceride-rich lipoproteins and free fatty acids

Chapter 8 of this thesis discusses hepatic lipid metabolism, very low density lipoprotein (VLDL) assembly and secretion, and the roles of the lipogenic transcription factors SREBP-1c, liver X receptor (LXR) and carbohydrate responsive element binding protein (ChREBP) herein. This introduction will therefore only briefly discuss these issues.

A surplus of (dietary) energy is incorporated into TG and stored in adipose tissue. In fasted conditions, the adipose TG store is used to deliver energy in the form of free fatty acids (FFA) and glycerol to liver and muscle to maintain whole-body energy homeostasis. To maintain this homeostasis, transport of lipids between various tissues (intestine, liver, adipose tissue) plays a crucial role. TGs are very hydrophobic and therefore need to be transported in association with lipoproteins together with cholesterol, phospholipids and proteins. The core of a lipoprotein contains TG and esterified cholesterol while the surface consists of phospholipids and free cholesterol. Embedded in the lipoprotein surface are apolipoproteins. These proteins are needed for stabilisation of the particle and solubility of the core lipids (76). Moreover, apolipoproteins act as ligands for specific receptors and are needed for the actions of enzymes (76).

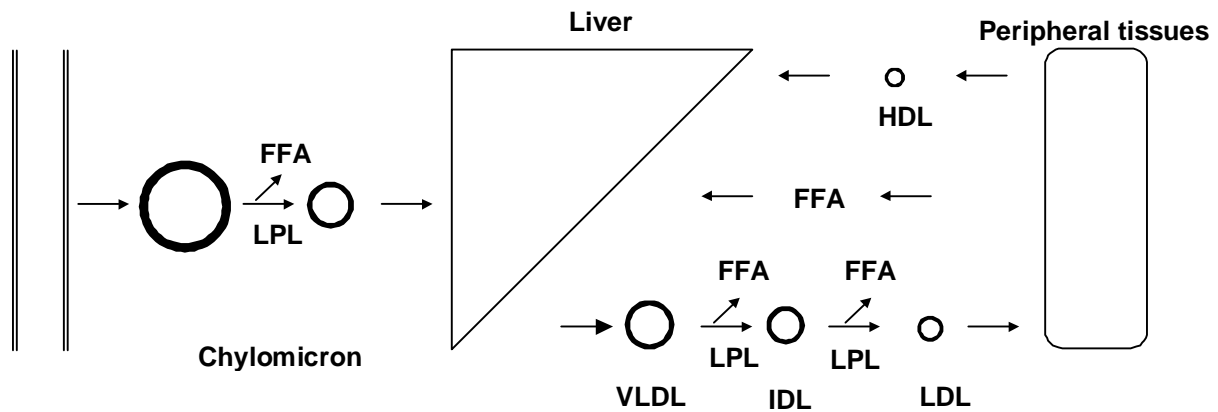


Figure 1.4. Schematic representation of lipoprotein and fatty acid fluxes. FFA, free fatty acid; HDL, high density lipoprotein; IDL, intermediate density lipoprotein; LDL, low density lipoprotein; LPL, lipoprotein lipase; VLDL, very low density lipoprotein.

Table 1.4. Characteristics of lipoproteins

Lipoprotein	Density (g/ml)	Diameter (nm)	Apolipoproteins	Percentage lipids		
				TG	Cholesterol	PL
Chylomicron	0.95	75 – 1200	AI, AII, AIV, B48, CI, CII, CIII, E	80 – 95	2 – 7	3 – 9
VLDL	0.95 – 1.006	30 – 80	B100, CI, CII, CIII, E	55 – 80	5 – 15	10 – 20
IDL	1.006 – 1.019	25 – 35	B100, CI, CII, CIII, E	20 – 50	20 – 40	15 – 25
LDL	1.019 – 1.063	18 – 25	B100	5 – 15	40 – 50	20 – 25
HDL	1.063 – 1.21	5 – 12	AI, AII, AIV, AV, CI, CII, CIII, E	5 – 10	12 – 25	20 – 30

HDL, high density lipoprotein; IDL, intermediate density lipoprotein; LDL, low density lipoprotein; PL, phospholipid; TG, triglyceride; VLDL, very low density lipoprotein.

Figure 1.4 shows a schematic overview of the lipoprotein fluxes within the body, table 1.4 summarizes the properties of the lipoproteins that constitute these fluxes. In the enterocytes, dietary TGs are incorporated into lipoproteins called chylomicrons. Apolipoprotein B (apoB) is the main protein of TG containing lipoproteins (chylomicrons and VLDL). In the surface of chylomicrons, a truncated form of apoB is present, consisting only of 48% of the N-terminal part of the protein and therefore called apoB48. Editing of the apoB100 into apoB48 is regulated by the apoB editing complex-1 (apobec1) (77). The TG content of these particles, when present in the circulation, is lipolysed by the action of lipoprotein lipase (LPL) secreted by muscle and adipose tissue. ApoCIII inhibits the actions of LPL and apoCII enhances these lipolytic actions. The released fatty acids can be taken up and are subsequently reesterified into TGs (*e.g.*, in adipocytes) or used as an energy source (*e.g.*, in muscle). When taken up by the liver, the fatty acids are re-esterified to form TG and first stored in an intracellular TG pool (78). Later on, the TGs stored in this pool can be released to be subsequently secreted in VLDL particles. As a result of lipolysis, chylomicrons are depleted of TGs, become smaller and are referred to as chylomicron remnants. Both chylomicrons and chylomicron remnants are cleared by the liver upon binding to the low density lipoprotein (LDL) receptor, the LDL receptor related protein (LRP) or hepatic lipase (HL) (79).

For transport from the liver to peripheral tissues, TGs need to be incorporated into VLDL particles. VLDL-TGs are lipolyzed by LPL in a similar way as chylomicron-TGs and the fatty acids are taken up by the peripheral tissues. Upon depletion of the TG content, the VLDL particle size decreases and the relative cholesterol concentration increases. The cholesterol-dense VLDL remnant particles are called intermediate density lipoprotein (IDL) or LDL particles, depending on their size and density. Insulin inhibits assembly and secretion of VLDL particles by the liver, as will be discussed later.

In fasted conditions, FFAs are released from the adipose stores after lipolysis of stored TG by two enzymes called triglyceride hydrolase (TGH) or adipose TG lipase (ATGL) and hormone sensitive lipase (HSL) (80) and carried by serum albumin to the liver. Insulin inhibits lipolysis in peripheral tissues. Thus, upon fasting, when insulin levels are low, the insulin-mediated inhibition of lipolysis is absent and FFAs are released into the circulation, taken up by the liver and muscle and used in the β -oxidation process. Part of the FFA not directly used for oxidative purposes is stored in the liver, causing the well-established hepatic steatosis associated with fasting (81).

Hepatic lipid metabolism and the actions of insulin

As discussed, dietary glucose taken up by the liver is stored as glycogen or broken down in the glycolytic pathway to yield carbon-3 compounds such as pyruvate. Pyruvate can be converted to citrate in the tricarboxylic acid (TCA) cycle and citrate can be converted to acetyl-CoA by ATP citrate lyase (ACL) (figure 1.5). Acetyl-CoA is the moiety from which fatty acids are synthesized. Two acetyl-CoA can be covalently linked to each other to form malonyl-CoA, a process controlled by acetyl-CoA carboxylase (ACC). Subsequently, via the actions of fatty acid synthase (FAS), malonyl-CoA condense to form fatty acids. Fatty acids can be incorporated into TG via the actions of acyl-CoA synthase (ACS) and glycerol-3-phosphate acyltransferase (GPAT). The expression of genes encoding ACL, ACC, FAS, ACS, and GPAT is regulated by both SREBP-1c (60) and ChREBP (82,83), whereas expression *Pk* of is regulated by ChREBP but not by SREBP-1c (84). Of notice, the SREBP-1c protein activity and gene transcription are regulated by insulin (61); the activity of ChREBP is regulated by glucose (83,85). More about these transcription factors can be found in chapter 8 of this thesis.

The liver is very well capable to synthesize fatty acids and TG from glucose and thus contributes to control of whole-body energy homeostasis. Insulin plays a key role herein, mainly via its effects on SREBP-1c expression (61). In the liver, insulin not only stimulates *de novo* lipogenesis but also inhibits VLDL assembly and secretion. How insulin influences VLDL assembly and secretion is not exactly known. Insulin might (i) decrease lipidation of the pre-VLDL particle via inhibition of microsomal TG transporting protein (MTTP) (86,87), (ii) stimulate degradation of the VLDL apolipoprotein apoB (88-90), and/or (iii) inhibit supply of substrates needed for VLDL assembly (78), *i.e.*, TG, cholesterol and phospholipids. More details about VLDL assembly and secretion and the role of insulin herein are discussed in chapter 8 of this thesis.

Comparable insulin-mediated, energy-conserving effects are found in peripheral tissues, especially in adipose tissues where insulin inhibits lipolysis. Of course, both hepatic and peripheral lipid-related effects of insulin are consistent with its role in fed conditions. In this condition, the liver does not need to add lipids to the total lipoprotein pool via secretion of VLDL and adipocytes do not need to deliver FFA to the periphery and the liver.

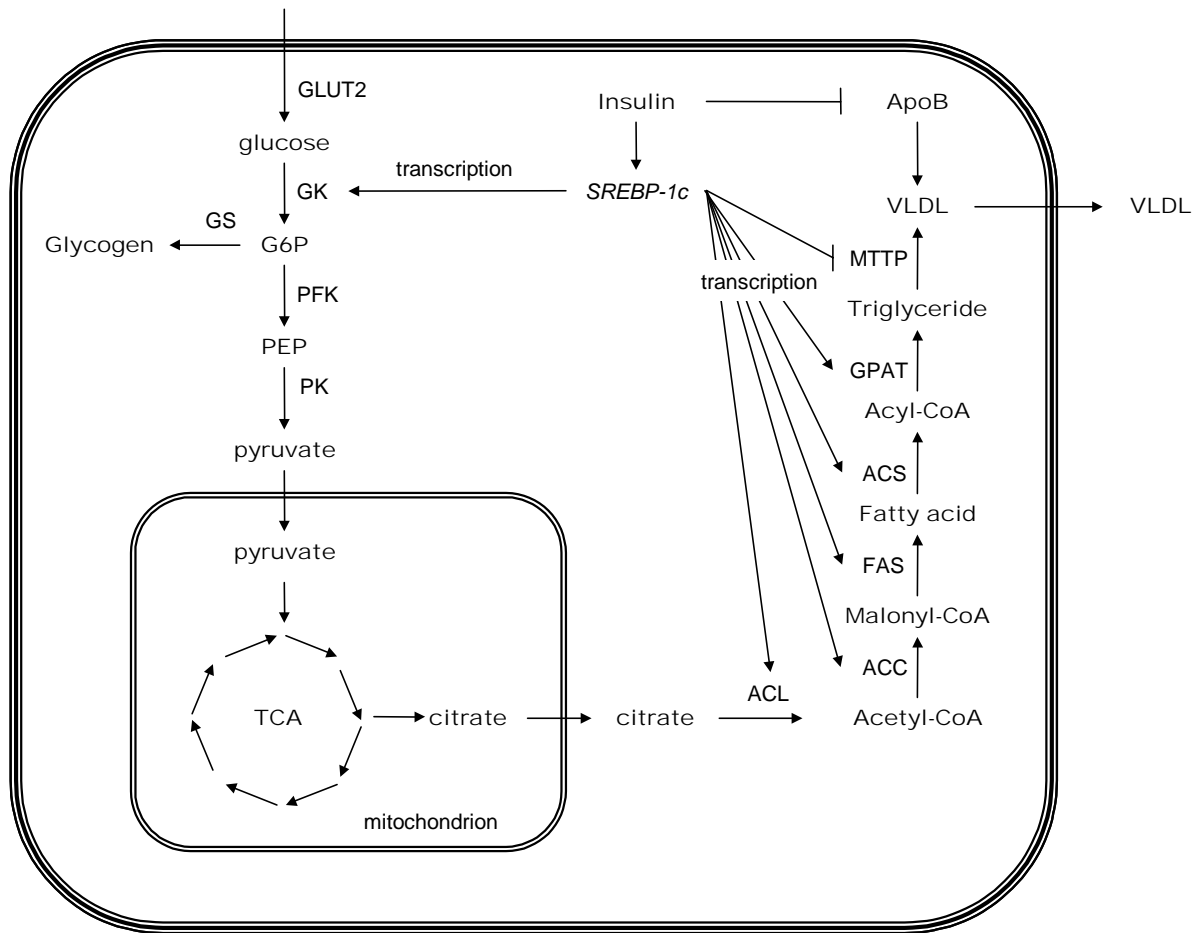


Figure 1.5. Schematic representation of the link between hepatic glucose and lipid metabolism and the role of insulin herein. ACC, acetyl-CoA carboxylase; ACL, ATP citrate lyase; ACS, acetyl-CoA synthase; apoB, apolipoprotein B; FAS, fatty acid synthase; G6P, glucose-6-phosphate; GLUT2, glucose transporter-2; GK, glucokinase; GS, glycogen synthase; GPAT, glycerol-phosphate acyltransferase; MTP, microsomal triglyceride transport protein; PEP, phosphoenolpyruvate; PFK, phosphofructo-1-kinase; PK, pyruvate kinase; SREBP-1c, sterol-regulatory element-binding protein-1c; TCA, tricarboxylic acid cycle; VLDL, very low density lipoprotein.

Hepatic steatosis and insulin resistance: beyond triglycerides

It is important to realize that TGs are inert molecules and therefore not cytotoxic themselves. Fatty acids, however, might display direct (adverse) cellular effects. Increased plasma FFA concentrations have for long been associated with enhanced hepatic glucose production and insulin resistance (reviewed by Boden (91)). Fatty acids are the substrates for hepatic β -oxidation, a process in which fatty acids are broken down to generate ketone bodies and energy for GNG (92). Interestingly, fatty acids stimulate transcription of genes encoding proteins involved in β -oxidation via binding to the nuclear peroxisome proliferator activated receptor- α (PPAR α) (93). Thus, fatty acids might continuously stimulate β -oxidation, resulting in elevated HGP, even under hyperinsulinemic conditions. The latter is a feature of hepatic insulin resistance. On the other hand, because insulin inhibits lipolysis of TG in adipose tissue, increased plasma FFA levels could also be the result of insulin resistance and not the cause of insulin resistance.

Various studies report other direct effects of fatty acids on insulin resistance. For instance, it was shown that PPAR α induces transcription of TRB3 (94), a protein that prevents PKB phosphorylation and activation (95). In mice, TRB3 seems to promote hyperglycemia by increasing glucose production by the liver due to decreased PKB-mediated GSK-3 β and FoxO1 phosphorylation.

Apart from their effects via PPAR α , fatty acids are also source for other molecules suggested to interfere with insulin signaling, for instance ceramide and glycosphingolipids (figure 1.6). The major mammalian fatty acid palmitate is a precursor in ceramide synthesis. Ceramide is a precursor for numerous different glycosphingolipids (figure 1.6) (96). In skeletal muscle from obese insulin resistant individuals, the ceramide concentrations were 2-fold increased (97). Moreover, *in vitro* studies showed that excessive ceramide concentrations disturb insulin signaling via inhibitory effects on PKB phosphorylation (98). Ceramide is the precursor for glycosphingolipids (GSLs), thus the possibility exists that the latter molecules might also play an important role in the development of insulin resistance (99). Glycosphingolipids are important components of the protein-enriched membrane domains called rafts and caveolae (100) and insulin receptors are predominantly found in these membrane structures, at least in adipocytes (101). It is therefore speculated that the close contact interactions between the insulin receptor and glycosphingolipids result in reduced receptor integrity (99). As a consequence, the tissue in which the receptor is located is less insulin sensitive. Indeed, it was reported that addition of the glycosphingolipid GM3 to cultured adipocytes suppressed phosphorylation of the insulin receptor and IRS1, resulting in reduced glucose uptake (102). Compared to their wild-type littermates, mice that are deficient for GM3 synthase, an important enzyme in glycosphingolipid synthesis, showed decreased glycosphingolipid levels in combination with enhanced peripheral insulin signaling (103). In addition, the GM3-synthase deficient mice were protected from high-fat diet induced insulin resistance (103).

In conclusion, fatty acids themselves might induce insulin resistance via continuously enhanced GNG due to elevated β -oxidation, via PPAR α -induced TRB3 transcription resulting in PKB blockade, via ceramide-mediated PKB inactivation, glycosphingolipids interfering with insulin receptor integrity, or combinations of these factors.

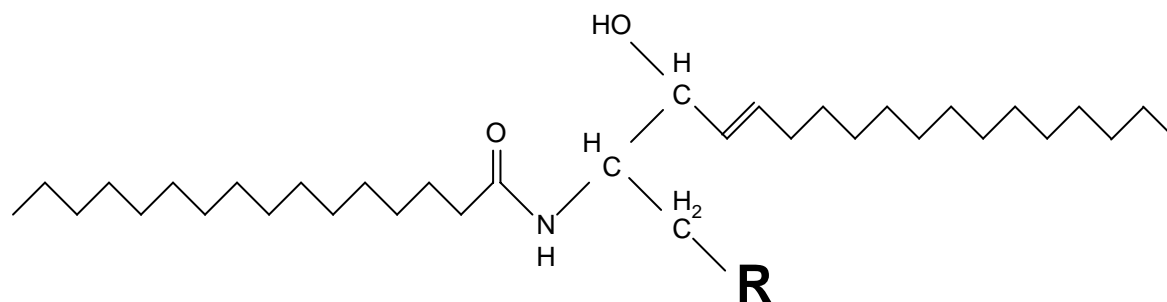


Figure 1.6. Basic structure of sphingolipids. For ceramide, R is H; for glycosphingolipids, R consists of series of glucose, galactose, N-acetyl galactosamine, and M-acetyl neuramic moieties.

Animal models of hepatic steatosis

Several animal models have proven to be useful tools in metabolic studies, because whole-body metabolism is a complex network of various different factors that influence each other. Such interplay is necessary to ensure proper regulation of energy homeostasis needed to maintain life in under varying conditions.. For instance, one can argue that the main goal of whole-body glucose metabolism is to provide glucose to the brain, because this organ needs glucose to function. The brain is, however, not capable to synthesise its alternate energy sources, ketone bodies, by oxidation of amino acids or fatty acids without undergoing adverse structural and functional damage (104). Thus, the liver is the organ that produces glucose and ketone bodies, and secretes VLDL, depending on the overall metabolic condition of the body, simply to maintain whole-body energy homeostasis. Factors derived from peripheral tissues, such as leptin, adiponectin and fatty acids, and those derived from the liver, such as glucose, TGs and ketone bodies, all act together to help maintaining this homeostasis.

Knowing this, it is easy to imagine that, to study the effects of hepatic steatosis on insulin sensitivity, experiments with animals are preferred above those with isolated cells. Recently, Den Boer *et al.* (105) published an overview of the role of animal models of hepatic steatosis for research on insulin sensitivity. The authors concluded that complex interactions between endocrine, metabolic, and transcriptional pathways are involved in TG-induced hepatic insulin resistance. Thus, the liver seems be passively and actively involved in insulin resistance associated with hepatic steatosis. More recently, studies from Rossetti's group (106-108) emphasised the role of the brain in the regulation of the actions of insulin on the liver. The overall conclusion from these studies is that, in extreme conditions, the insulin-mediated control of HGP is controlled by the brain. So far, no studies were performed on the role of the brain in the other aspects of hepatic insulin sensitivity, *e.g.* control of VLDL production.

Objectives and outline of this thesis

From the studies discussed in this introduction, it is clear that animal models of hepatic steatose are useful tools. We therefore performed *in vivo* experiments to study the effect of hepatic steatosis on insulin sensitivity in various animal models. In chapters 2, 4 and 5, the leptin-deficient *ob/ob* mice were used. These mice show enhanced *de novo* lipogenesis (30) that is considered a major cause of their hepatic steatosis. In chapter 2, we questioned whether the hyperglycemia seen in *ob/ob* mice was the result of reduced peripheral glucose clearance or enhanced HGP. Chapter 4 describes studies in which both hepatic and peripheral insulin sensitivity was investigated in more detail. For this, we used the "golden standard" to determine insulin sensitivity: the hyperinsulinemic euglycemic clamp technique. In the studies described in chapter 5 we used the *ob/ob* mice to study the effects of a novel inhibitor of the glycosphingolipid synthesis, the iminosugar derivative N-(5'-adamantane-1'-yl-methoxy)-pentyl-1-deoxynojirimycin (AMP-DNM). In these studies, insulin sensitivity was tested using the hyperinsulinemic euglycemic clamp technique described in chapter 4.

It was shown in previous studies that pharmacological LXR activation results in severe hepatic steatosis (109) and we used this model in the studies described in chapters 3 and 4. In chapter 3, we questioned whether LXR-induced hepatic steatosis resulted in affected VLDL-TG production. In chapter 4 we investigated whether hepatic and peripheral insulin sensitivity of glucose metabolism was affected due to this hepatic steatosis. In the studies described in chapter 6, we studied whether hepatic steatosis due to pharmacological inhibition of the rate-controlling enzyme in β -oxidation, carnitine palmitoyl transferase-1 (CPT1), affected insulin sensitivity of VLDL production. In the studies described in chapter 7, we questioned which lipogenic transcription factor (SREBP-1c, LXR α or ChREBP) was responsible for enhanced lipogenic gene expression upon pharmacological inhibition of glucose-6-phosphatase.

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