Chapter 8

General Discussion
Insulin resistance is a key feature in the development of type 2 diabetes mellitus and is already present 10-20 years before the onset of clinically overt diabetes (1). The etiology of insulin resistance and type 2 diabetes is still under debate but it is clear that development of both is influenced by genetic and environmental factors. Metabolic consequences of the insulin resistant condition affect both hepatic glucose and fat metabolism. In insulin resistance, insulin-stimulated glucose disposal and suppression of glucose production is less effective. Hepatic fat metabolism is also affected resulting in hepatic steatosis, increased VLDL production and hyperlipidemia. Hyperlipidemia is partly due to a defective insulin action and partly a consequence of altered glucose metabolism. Increased hepatic VLDL production, in particular overproduction of large triglyceride-rich VLDL particles, is one of the hallmarks of insulin resistant conditions (2). Processing of these large particles leads to the formation of small, dense LDL particles (3-5), which are more susceptible to oxidation and constitute, therefore, an increased risk for development of premature atherosclerosis (6-8). Simultaneous reduction of HDL cholesterol, as a result of ongoing intravascular lipid exchange, adds to the risk. It has been estimated that up to 80% of diabetes-related mortality is due to cardiovascular diseases a result form an increased mortality rate in diabetes patients (9,10).

The studies described in this thesis are focused on the disturbances in hepatic glucose and fat metabolism that occur in insulin resistant conditions that may contribute to the progress to frank diabetes and development of the characteristic pattern of hyperlipidemia. In particular, emphasis has been put on quantitative assessment of relevant metabolic fluxes in high-fat feeding and genetically-based animal models of insulin resistance. Metabolic flux rates were related to hepatic and/or intestinal gene expression profiles and to enzyme activities of key enzymes involved in these pathways. In this way, an integrated view of the metabolic consequences of insulin resistance could be obtained. The animal models used in this thesis include the high-fat diet fed rats, as a model for diet-induced insulin resistance, the diabetic and obese ob/ob mouse model and the gene knockout Fabp1-/- mouse model. To investigate interactions between hepatic glucose and fat metabolism in detail, independent of the presence of insulin resistance, rats were infused with a chlorogenic acid derivative (S4048) which blocks glucose-6-phosphatase (G6Pase) enzyme activity and thereby acutely impairs hepatic glucose production.

1. **High-fat feeding in rats.** The high-fat diet used in feeding experiments resembled a Western-type diet, high in calories and enriched with saturated fats, and was compared to a low-fat diet of similar fatty acid composition. Diets of similar composition were also used to study the effects on insulin sensitivity and on the contribution of gluconeogenesis and glycogenolysis to postabsorptive glucose production in human subjects within the framework of the same research program (11,12). The aim to the program was to unravel the role of nutrition, paracrine factors and their interrelationship in the regulation of hepatic glucose and fat metabolism in insulin resistant states.

   High-fat feeding in human subjects resulted in a decreased postabsorptive glucose production compared to low-fat feeding while, at the same time, the contribution of gluconeogenesis and glycogenolysis to the postabsorptive glucose production were altered. The relative contribution of gluconeogenesis was increased at the expense of glycogenolysis (12). During hyperinsulinemic euglycemic clamping, insulin decreased the endogenous glucose production in low-fat and high-fat fed subjects but insulin was clearly less effective during high-fat feeding (11). Furthermore, high-fat feeding resulted in a decreased insulin-induced glucose oxidation and an increased nonoxidative glucose disposal (11). These studies
showed that high-fat feeding in humans rapidly results in characteristic metabolic alterations that occur in the early stages during the development of insulin resistance.

Rats fed the high-fat diet for only three weeks developed hepatic insulin resistance, became glucose intolerant and also showed a phenotype of early diabetes (Chapter 2 and 3). The hepatic insulin signal transduction pathway was impaired: components of the phosphatidylinositol 3-kinase (PI3K) pathway were not activated to a similar extent as observed in low-fat diet fed controls. This was associated with a decreased hepatic sensitivity of both glucose and fat metabolism. Under basal conditions, several parameters of hepatic glucose metabolism were not affected by high-fat feeding. However, during hyperinsulinemic euglycemic clamping, sensitivity of hepatic glucose production, de novo glucose-6-phosphate (G6P) synthesis and glucose disposal for insulin were all significantly decreased. This resulted in an elevated hepatic glucose production rate and a decreased glucose disposal rate in rats fed the high-fat diet (Chapter 2). High-fat feeding also resulted in increased basal VLDL-TG and -apoB100 production rates that were not effectively suppressed by insulin (Chapter 3). Thus, high-fat feeding in rats results in the development of characteristic metabolic defects associated with insulin resistance, probably as a consequence of decreased hepatic insulin signaling.

The PI3K pathway is probably the major pathway involved in insulin-mediated control on glucose homeostasis as well as on VLDL production (13,14). In other animal models of diabetes, like the obese ob/ob mouse (15,16), db/db mouse (17) and ZDF rat (18), insulin signaling is impaired at the level of the insulin receptor substrate (IRS) and/or PI3K. The serine/threonine protein kinases B and C (PKB or Akt and PKC) are both activated downstream of PI3K and might modulate the insulin signal transduction pathway and thereby influence hepatic glucose and fat metabolism. PKB might be involved in the feedback regulation of IRS activation since it has been shown to stabilize IRS activity by phosphorylation of specific serine residues, thereby prolonging insulin-signaling (19). Different PKC isoforms have been associated with impaired insulin signaling (20,21). In a fatty liver, therefore, over-activation of PKC and/or inhibition of PKB signaling may contribute to impaired insulin signaling (19,22) and hence to insulin resistance.

2. The ob/ob mouse model. The obese and diabetic ob/ob mouse model has been studied extensively for many years. In this model, the absence of adipose-derived hormone leptin is the primary factor responsible for the obese and diabetic phenotype. An increased FA flux from the intestine (directly from diet) and adipose tissue, apparent as elevated plasma FFA levels, together with increased hepatic de novo lipogenesis (DNL) results in hepatic steatosis. In contrast to expectations, ob/ob mice did not show increased hepatic VLDL production under basal conditions (Chapter 4). However, insulin did not reduce VLDL production in ob/ob mice, indicating a decreased sensitivity of the process for the effects of insulin. Ob/ob mice did show features consistent with a decreased hepatic insulin signaling. Hepatic gene expression and protein phosphorylation of the insulin receptor and insulin receptor substrate-isoforms were reduced in ob/ob mice. Hepatic triglyceride accumulation was predominantly present in perivenous areas of the liver whereas apoB synthesis was more associated with the perportal areas. Hepatic triglyceride might therefore be less available for VLDL production in ob/ob mice. Alternatively, the availability of newly synthesized cholesterol may also influence VLDL formation. Total hepatic cholesterol content in ob/ob mouse liver was increased but the absolute cholesterol synthesis rate was not affected (Chapter 4). Limited availability of newly synthesized cholesterol may be rate-controlling for VLDL production in ob/ob mice.

A possible explanation for the increased DNL in this animal model is directly related to the absence of leptin. This hormone has been shown to be able to downregulate SREBP-1c
expression and protein levels together with the expression of its target gene (Fas) in ob/ob adipocytes (23) and in wildtype mouse liver (24). Furthermore, treatment of ob/ob mice with daily leptin injections for a week reduced total body weight, plasma glucose and insulin concentration and hepatic SREBP-1c expression levels (own unpublished observation). The same has been shown in IRS2+/− mice. Leptin treatment in these mice normalizes the elevated hepatic SREBP-1 levels (25). The absence of leptin in ob/ob mice may directly influence hepatic fat metabolism resulting in elevated SREBP-1c expression and activity and therefore increased fatty acid synthesis, increased fat deposition (fatty liver) and the development of hepatic insulin resistance.

3. The Fabp1−/− mouse model. The Fabp1−/− mouse model is a relative new model and the physiological basis is for the development of the insulin resistant condition is not yet understood. A polymorphism in the human equivalent of the Fabp1 gene (FABP2) is associated with an increased incidence of insulin resistance and type 2 diabetes in Pima Indians (26) and Japanese men (27). Fabp1−/− mice were insulin resistant but hepatic gene expression levels of the insulin receptor (Ir) and the insulin receptor substrates (Irs-1 and Irs-2) were not different between Fabp1−/− and Fabp1+/+ mice, indicating that the insulin resistance is not caused by impaired hepatic expression of these genes. Fabp1−/− mice showed, despite the insulin resistant condition and in contrast to the other animal models described in this thesis, a decreased VLDL production (Chapter 5). Increased hepatic ketogenesis is associated with decreased hepatic VLDL production (28). Fabp1−/− mice showed slightly increased hepatic expression levels of genes involved in this pathway and clearly increased plasma β-OH-butyric acid levels, i.e., indications for increased ketogenesis. However, (28) it seems unlikely that increased fatty acid oxidation/ketogenesis is the sole factor responsible for the observed effects on VLDL formation in Fabp1−/− mice: the precise mechanism remains speculative.

The observed phenotype in Fabp1−/− mice was similar to that previously reported in rats that were infused with the long chain fatty acid oleic acid via the portal vein (29). In this experiment, the induction of Hypothalamus-Pituitary-Adrenal (HPA)-axis activity and increased sympathoadrenal activity were observed, as reflected by elevated levels of corticosterol and epinephrine/norepinephrine, respectively. Portal oleic acid infusion in rats was also accompanied by acute development of insulin resistance and a decreased VLDL-triglyceride production (29), similar to what was observed in Fabp1−/− mice. The underlying disturbances in Fabp1−/− mice and oleic acid-infused rats might therefore be the same. Some evidence suggests a possible role for this system in hepatic fat metabolism. The adrenocorticotropic hormone (ACTH) is a mediator of HPA-axis function and is secreted from the anterior pituitary (or neurohypophysis) gland. ACTH stimulates glucocorticoid and cortisol production from the adrenal glands. Glucocorticosteroids are able to increase hepatic FA oxidation and may, therefore, decrease VLDL production. ACTH itself is able to lower LDL and VLDL production in rat hepatocytes (30) and their plasma levels in healthy volunteers (31,32). In the human hepatoma HepG2 cell line, ACTH decreases apoB mRNA expression level and secretion of the apoB protein (33). The precise role of the HPA-axis in hepatic fat metabolism and its association with insulin resistance clearly requires further investigation.

4. Interactions between hepatic glucose and fat metabolism in rats. Glucose-6-phosphatase (G6Pase) and glucokinase (GK) are two enzymes critically involved in controlling the rate and direction of the hepatic glucose flux. G6Pase catalyses the terminal step of both the gluconeogenic and glycolytic pathways (34). A class of chlorogenic acid derivatives has been developed that inhibit G6Pase activity by blocking glucose-6-phosphate translocase (G6PT) (35) and inhibit hepatic glucose production and lower blood glucose concentration in
a dose-dependent way (36,37). Inhibition of G6PT, by the chlorogenic acid derivative S4048 in rats, resulted in a marked increase in hepatic G6P and glycogen content (Chapter 6 and 7) resulting from a redirection of newly synthesized G6P (gluconeogenesis) towards glycogen synthesis rather than from reduction of gluconeogenesis. Hepatic gene expression level of glycogen synthase was increased in accordance with the increased flux towards glycogen. In addition, inhibition of the G6PT resulted in an increased DNL and a massive hepatic steatosis (Chapter 7). Cholesterogenesis was not affected, implying a dissociated regulation of cholesterol and fatty acid synthesis under this condition. This was also seen at the levels of hepatic gene expression levels. Genes involved in DNL, i.e., fatty acid synthase and acylCoA carboxylase, were elevated in livers of rats receiving S4048. However, genes involved in cholesterogenesis, i.e., HMGCoA synthase and reductase were not affected. The increased DNL and hepatic lipid accumulation alone was not associated with increased VLDL-triglyceride secretion (Chapter 7). Studies described in Chapter 6 and 7 delineate that altered G6Pase activity affects metabolic pathways in hepatic glucose as well as fat metabolism demonstrating the strong interactions that exist between these metabolic pathways.

Insulin resistance in humans is associated with increased production of VLDL-triglyceride and -apolipoprotein B by the liver (38-41) resulting in an atherogenic lipoprotein profile with hypertriglyceridemia, low HDL and a preponderance of small, dense LDL particles (3). Basal VLDL-triglyceride production was increased in high-fat diet-induced insulin resistant rats but not in ob/ob mice. However, in both models this process was clearly less sensitive for the suppressive effects of insulin. Insulin resistant Fabp1-/- mice showed a decreased basal VLDL-triglyceride production rate, probably induced by (a) yet unknown factor(s), overcoming the effects of insulin resistance.

Increased lipid availability from intracellular stores and/or de novo lipogenesis per se did not result in increased VLDL production. In rats fed the high-fat diet DNL was likely to be decreased since the hepatic expression levels of genes involved in DNL, i.e., fatty acid synthase and acylCoA carboxylase, were strongly decreased. In ob/ob mice DNL was 10-fold increased but this did not result in increased hepatic VLDL production. A similar phenomenon was seen in rats receiving the chlorogenic acid derivative S4048. A more than 10-fold increase in DNL was not associated with increased VLDL production. It seems that, at least in these animal models, increased DNL-derived FAs are not a rate-controlling factor for VLDL production. However, in both the ob/ob mouse model and S4048-receiving rats, cholesterol synthesis rates were not affected. This suggests that newly synthesized cholesterol could be a rate-controlling step of VLDL production in these models.

Interactions between glucose and fat metabolism are complex and affected by the insulin resistant condition. Changes in glucose metabolism induced by inhibition of the glucose-6-phosphatase translocase resulted in a blockade in glucose production and redirection of newly synthesis G6P toward glycogenolysis. An increased glycolytic flux may have resulted in an increased availability of acetylCoA moieties derived from glucose, as is the case in ob/ob mice (42) that can be used for fatty acid synthesis. In both models increased DNL was found in association with hepatic steatosis.

The development of hepatic insulin resistance is a consequence of adaptations in the liver that disturbs the adequate response to specific metabolic changes, e.g., increased lipid storage and oxidation when FA supply is in excess. When excessive FA supply to the liver is permanent, this may result in persistent metabolic changes in glucose and fat metabolism, including a decreased ability of insulin to suppress glucose and VLDL production, which may lead to further deterioration of insulin sensitivity. All the models studied showed an increased
FA supply to the liver, originating either from diet and/or from adipose tissue or intestine. Overeating (ob/ob mouse), high-fat feeding (high-fat diet fed rats) and a genetic defect resulting a redirection of diet-derived FA (Fabp1<sup>−/−</sup> mice), all lead to an altered FA supply to the liver. The liver has to respond to this either by storing, oxidizing or secretion the additional amounts of FAs. In doing so, intracellular adaptations arise at the level of transcription factors and the control of their target genes. These adaptations are temporary necessities in order to restore the normal situation, however, when persistent, these adaptations may become harmful. The increased FA flux toward the liver will activate the peroxisomal proliferator activated receptors (PPAR<sub>α</sub>) and thereby increase FA oxidation. Increased β-oxidation is associated with decreased VLDL production rates and this may explain, at least in part, the observed effects on VLDL production in ob/ob and Fabp1<sup>−/−</sup> mice. It was found that the expression of PPAR<sub>α</sub> is permanently reduced in fatty liver (Chapter 4).

An important transcription factor involved in control of hepatic effects of insulin on the expression levels of genes involved in fatty acid synthesis and glucose metabolism is sterol regulatory element binding protein (SREBP)-1c (43-46). Insulin induces the expression of this transcription factor. Due to elevated plasma insulin levels, SREBP-1c may become constantly highly expressed or may become more active in insulin resistant states, since insulin-mediated phosphorylation of SREBP-1c enhances the activity of this protein (45,47-49). SREBP-1c not only affects genes involved in fatty acid synthesis but also affects genes involved in the gluconeogenetic pathway and might therefore also directly affect genes involved in hepatic glucose metabolism (44-46).

Increased FA flux may exceed FA usage, in which case compensatory upregulation of FA oxidation is required to maintain intracellular FA homeostasis. FA themselves are ligands for PPAR<sub>α</sub> and are able to upregulate FA oxidation. PPAR<sub>γ</sub> is also associated with increased FA metabolism, although this isoform is normally hardly expressed in non-adipocytes. Yet, its expression is increased in steatotic livers (Chapter 4). PPAR<sub>γ</sub> and SREBP-1c are able to increase lipogenesis by increasing the expression levels of genes encoding enzymes in this pathway, i.e., acylCoA carboxylase and fatty acid synthase (50,51). Overnutrition, therefore, may lead to increased activation of PPAR<sub>γ</sub> (FA induced) and SREBP-1c (insulin induced) resulting in TG deposition and the development of hepatic steatosis.

In Western societies in which the diet is high in fat calories and essentially unlimited, the incidence of insulin resistance and type 2 diabetes mellitus will increase due to persistently increased fat loads exposed on the liver that has severe metabolic consequences. Hyperinsulinemia, hyperglycemia, hypertriglyceridemia, hypertension, low HDL and the presence of small, dense highly atherogenic LDL particles result in an increased incidence of coronary artery disease, the number one mortality cause of type 2 diabetes patients.

REFERENCES


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