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Lipid metabolism in the pancreatic beta-cell. Implications for insulin secretion.

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2006

Link to publication

Citation for published version (APA):
Fex, M. (2006). *Lipid metabolism in the pancreatic beta-cell. Implications for insulin secretion.* [Doctoral Thesis (compilation), Department of Experimental Medical Science]. Experimental Medical Science, Lund University.

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Lipid metabolism in the pancreatic β-cell

Implications for insulin secretion

By

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Lund 2006

Nothing shocks me. I'm a scientist.
Harrison Ford (as Indiana Jones)

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I. Fex M, Dekker Nitert M, Wierup N, Ling C, Sundler F, Mulder H (2006) ***Enhanced mitochondrial metabolism may account for the adaptation to insulin resistance in islets from high fat diet-fed C57BL/6J mice.*** Diabetologia (In press 2006)

II. Mulder H, Sörhede-Winzell M, Contreras JA, Fex M, Ström K, Ploug T, Galbo H, Arner P, Lundberg C, Sundler F, Ahrén B, Holm C (2003) ***Hormone-sensitive lipase null mice exhibit signs of impaired insulin sensitivity whereas insulin secretion is intact.*** J Biol Chem 278: 36380-3638

III. Fex M, Olofsson CS, Fransson U, Bacos K, Lindvall H, Sörhede-Winzell M, Rorsman P, Holm C, Mulder H (2004) ***Hormone-sensitive lipase deficiency in mouse islets abolishes neutral cholesterol ester hydrolase activity but leaves lipolysis, acylglycerides, fat oxidation, and insulin secretion intact.*** Endocrinology 145: 3746-3753

IV. Fex M, Haemmerle G, Wierup N, Dekker Nitert M, Rehn M, Ristow M, Eliasson L, Sundler F, Holm C, Mulder H (2006***) A β-cell-specific Knock out of Hormone-Sensitive Lipase results in hyperglycemia and disruption of phasic insulin secretion.***
Manuscript

Abbreviations

Acyl-CoA	**Acyl-Coenzyme A**
ADP	**Adenosine diphosphate**
ATGL	**Adipocyte triglyceride lipase**
ATP	**Adenosine triphosphate**
BAY	**5-(2H)-isoxazolonyl urea**
Bcl2	**B-cell leukemia/lymphoma 2**
cAMP	**Cyclic adenosine monophosphate**
CPT-1	**Carnitine palmitoyl transferase 1**
Cre	**Cre recombinase**
DAG	**Diacyl glycerol**
DMP	**3,5-Dimethylpyrazole**
DNA	**Deoxyribonucleic acid**
FAD+	**Flavin adenine dinucleotide**
FFAs	**Free fatty acids**
GDH	**Glutamate dehydrogenase**
GLP-1	**Glucagon-like peptide 1**
GLUT	**Glucose transporter**
HF	**High fat**
HSL	**Hormone sensitive lipase**
HSL KO	**Hormone sensitive lipase knock out**
IAPP	**Islet amyloid polypeptide**
iNOS	**Inducible nitric oxide synthase**
IRS	**Insulin receptor substrate**
K_{ATP} channel	**ATP-sensitive K^+ channel**
LADA	**Latent autoimmune diabetes in the adult**
LC-CoA	**Long chain acyl-Coenzyme A**
Malonyl-CoA	**Malonyl-Coenzyme A**
NAD+	**Nicotinamide adenine dinucleotide**
NADP+	**Nicotinamide adenine dinucleotide phosphate**
NCEH	**Neutral Cholesterolester hydrolase**
NFkb	**Nuclear factor κβ**
Nnt	**Nicotinamide nucleotide transdehydrogenase**
NO	**Nitric oxide**
PDE3B	**Phosphodiesterase 3B**
PDH	**Pyruvate dehydrogenase**
PKA	**Protein kinase A**
PKC	**Protein kinase C**
PP	**Pancreatic polypeptide**
PPARγ	**Peroxisome proliferator activated receptor γ**
PYY	**Peptide YY**
RIA	**Radioimmuno assay**
RNA	**Ribonucleic acid**
RRP	**Readily releasable pool**
SUR1	**Sulfonylurea receptor 1**
TCA cycle	**Tricarboxylic acid cycle**
Tfam	**Mitochondrial transcription factor**
TGs	**Triglycerides**
α-KIC	**α-ketoisocaproic acid**
β-HSL KO	**β-cell specific knock out of HSL**

General Introduction

Type 2 Diabetes

Two major classes of diabetes exist, Type 1 and Type 2 diabetes. While Type 1 diabetes is a T-cell mediated autoimmune process destroying the insulin-producing β-cells, Type 2 diabetes depends on genetic and environmental factors, resulting in insulin resistance glucose intolerance and ultimately overt hyperglycemia. Typically, Type 1 diabetes affects young individuals, while Type 2 diabetes occurs in elderly people. However, autoimmune responses may occur also in older patients and is termed Latent Autoimmune Diabetes in the Adult (LADA). Conversely, insulin resistance and Type 2 diabetes are now also encountered in younger individuals.

The incidence of Type 2 diabetes is increasing tremendously and has now reached epidemic proportions, affecting more than 170 million people worldwide [1]. It has been estimated that by the year 2010, the prevalence of this disease will increase by 50%, most cases occurring in developing countries, such as Africa, Asia and South America [2]. The large economic burden of Type 2 diabetes is a consequence of its associated complications, mostly vascular disorders. For instance, cardiovascular morbidity is 2 to 4 times higher in Type 2 diabetic patients than in non-diabetic people [1].

A sedentary lifestyle, with accelerated food intake and obesity as a result, is a strong risk factor for the development of Type 2 diabetes. In fact, 65% of adult Americans are categorized as overweight (body mass index > 25 kg/m^2) [3].

However, equally important in the development of Type 2 diabetes are genetics. Positive family history for Type 2 diabetes confers a 2-4 fold increased risk of developing the disease. In addition, 15-20% of first degree relatives develop impaired glucose tolerance or Type 2 diabetes [4]. Native American Indians tribes, with a high degree of inbreeding (e.g., Pima Indians), exhibit a high prevalence of obesity and Type 2 diabetes [5]. In summary, the pathophysiology of Type 2 diabetes appears to depend on many different factors. At this point, it is of the utmost importance to find new preventive and therapeutic strategies to deal with the great socioeconomic and personal burdens that follow this disease.

Glycemic control (glucose intolerance)—To understand the cellular and molecular mechanisms behind Type 2 diabetes it is important to elucidate the physiological mechanisms which control glucose homeostasis. Under normal conditions, insulin is secreted from the pancreatic β-cells in response to a rise in blood glucose, typically after a meal. As a consequence of insulin action, glucose is taken up by peripheral tissues (muscle and adipose tissue), where it is used as fuel or stored either as glycogen or triglycerides (TGs). Euglycemia is maintained when secretion of insulin and glucose disposal in the periphery are in balance. The pancreatic β-cell adapts to fluctuations in blood glucose, i.e., a decrease in insulin action in the periphery is accompanied by an increase in insulin secretion. When the sensitivity to insulin decreases, insulin action is impaired. This may occur at old age or in obesity, and consequently there will be a

tendency for blood glucose levels to rise. However, healthy β-cells compensate for this with enhanced insulin secretion, thereby maintaining euglycemia. In contrast, if the β-cells are unable to compensate adequately, blood glucose levels will increase over time leading to glucose intolerance and ultimately frank diabetes [6]. In fact, a reduction in insulin sensitivity, however small, may have a detrimental impact on β-cells in predisposed individuals, and cause cellular dysfunction, which results in disturbed insulin secretion over time and finally Type 2 diabetes [7].

Insulin resistance—Insulin resistance a hallmark of Type 2 diabetes and is frequently observed in obesity. Insulin resistance is usually accompanied by an adaptive increase in insulin secretion from the β-cells. However, in certain individuals, perhaps genetically predisposed, it may not be possible for β-cells to fully compensate insulin resistance in the long run and, as a consequence, Type 2 diabetes will evolve [8].

Insulin resistance implies that the action of insulin to properly exert its effect in peripheral tissues is somehow compromised. When insulin binds to its receptor, its tyrosine kinase activity is increased, which leads to autophosphorylation as well as phosphorylation of various downstream substrates. Among these substrates are insulin receptor substrates 1 and 2 (IRS-1 and IRS-2). The IRS substrates have both been implicated in the development of insulin resistance and Type 2 diabetes [9]. In humans, rare mutations in the IRS-1 gene are associated with insulin resistance [10,11]. Moreover, disruption of the IRS-1 gene in mice results in insulin resistance in mainly muscle and fat tissue. Gene targeting of IRS-2 in mice however, results in insulin resistance, β-cell failure and frank diabetes [12].

Recently, focus has been placed on the adipose tissue as a major player in the development of insulin resistance [13]. Adipose tissue regulates whole body glucose metabolism by increasing or decreasing the levels of circulating free fatty acids (FFAs) and also by secreting a number of adipokines, which influence insulin sensitivity and food intake. Thus, adipose tissue has emerged as an endocrine organ [14]. Insulin normally restrains lipolysis in adipose tissue [15]. Therefore, in insulin resistance, there is an increase in circulating free fatty acids (FFAs), reflecting dysregulated lipolysis. As a consequence, other tissues are exposed to elevated levels of FFAs, which may become inappropriately stored. The incorporation of FFAs into TGs has been proposed to be involved both in the insulin-desensitizing process and insulin secretion from the β-cell as well as cellular dysfunction in multiple tissues [16-19]. For instance, the glucose transporter 4 (GLUT4) in skeletal muscle is downregulated in insulin resistant states. It has been shown in humans that this downregulation is mediated by increased plasma FFAs which interfere with the activation of IRS-1 by insulin and its associated PI-3 kinase activity [20]. In addition, the distribution of glucose transporter 2 (GLUT2) in β-cells has also been shown to be negatively affected by high fat feeding, possibly due to increased lipid flux in high fat fed C57BL76J mice [21]. Recently, it was postulated that an inverse relationship exists between cytotoxicity and triglyceride incorporation in pancreatic β-cells, suggesting that toxic effects arise in β-cells when the esterification process is disturbed [22], perhaps leading to increased intracellular flux of lipids.

However, the exact nature of these processes in β-cells and the development of insulin resistance appears to be multifactorial and is not fully understood at this point.

Lipotoxicity—Lipotoxicity refers to a condition where excess lipids are incorporated into tissues other than adipose tissue, such as muscle, liver and both endocrine and exocrine pancreas, causing a toxic environment in these tissues. One theory is that this is due to the malfunction of the adipose tissue, resulting in its inability to store TGs [14], and as a consequence FFA spillover into non-adipose tissues. In Type 2 diabetes and obesity, levels of FFAs are frequently elevated. It has been shown in rodents that elevated levels of FFAs result in defective insulin secretion from pancreatic islets. For example, after a 48 hour lipid infusion in the rat pancreas, glucose-stimulated insulin secretion was severely blunted [23]. Subsequent studies have, in addition to a blunted insulin response, shown suppression of proinsulin biosynthesis and depletion of insulin stores [18,19]. In human islets it has been shown that exposure to increased FFA concentrations induce apoptosis of β-cells and a marked decrease in insulin secretion. These effects were associated with decreased expression of B-cell leukemia/lymphoma 2 (Bcl2), an anti-apoptotic protein [24]. Clearly, chronic exposure of FFAs may negatively impact β-cells and other tissues. It is believed that these problems may be due to the inability of these tissues to store large amounts of TGs. In that scenario, TGs could enter harmful pathways resulting in, e.g. ceramide formation. Ceramides induce apoptosis by activation of Nuclear Factor (NF)κB [25], which up regulates the expression of inducible nitric oxide synthase (iNOS). The increased formation of nitric oxide (NO) may produce peroxy-nitrate, which has been implicated in apoptosis [26,27].
In diet-induced obesity, non-adipose tissue is protected from excess TG storage in the early stages by increased secretion of leptin. Leptin is a hormone released from the adipose tissue (adipokine) and is believed to regulate food intake via the hypothalamus. Leptin has been implicated in protection of non-adipose tissue from over-accumulation of stored TGs [13]. TGs are normally stored in small amounts within non-adipocytes, serving to maintain membrane integrity and providing anchors for membrane proteins and lipid-derived signaling molecules. In 1999 Unger et al. formulated an attractive hypothesis: if the housekeeping TG stores in non-adipose tissue, for example the muscle, liver or pancreatic β-cell, were diverted to use as fuel, cell function and viability in those cells would be compromised. This hypothesis is based on both *in vivo* and *in vitro* findings in the ob/ob mice and Zucker diabetic fatty rat (fa/fa; fa/fa – mutation in leptin receptor gene; ob/ob – deficiency in the leptin gene), showing that accumulation of TGs in islets of these animal models is much greater than in wild type counterparts [13]. This was attributed to leptin deficiency. Accordingly, transfer of the wild type leptin receptor gene (OB-R) to fa/fa islets corrects the lipotoxic abnormalities [28-30]. Moreover, overexpression of the leptin gene in normal rats depletes non-adipose tissue of stored triglyceride. The fat-depleted islets are morphologically normal but unresponsive to glucose or arginine stimulation [31]. Thus, while intracellular TG stores are needed for normal function, TGs in excess cause cellular toxicity. These examples imply that leptin and resistance to leptin action may be involved in the lipotoxic events seen in many cases

of Type 2 diabetes and obesity. Yet another theory about causes of β-cell dysfunction is that elevated levels of glucose, observed in insulin resistant states, causes a toxic environment for the β-cell, i.e., glucotoxicity. This may lead to increased β-cell apoptosis by several mechanisms, with enhanced oxidative stress playing a major role [32,33]. Moreover, if this is accompanied by elevated levels of FFAs there will be a negative synergy, exaggerating the harmful effects on β-cells and insulin-sensitive tissues, i.e., glucolipotoxicity [7], causing a toxic environment for the β-cell. Under these circumstances, glucose metabolism could generate reactive oxygen species that together with an accumulation of metabolites from fatty acid esterification would inhibit glucose-stimulated insulin secretion and insulin gene expression [7,34].

Therapy in Type 2 diabetes—Lifestyle and dietary measures are extremely important in managing Type 2 diabetes. However, a large number of patients will still require drug treatment to maintain glycemic control. At this time, several different drugs exist to reduce plasma glucose. They act by different mechanisms, such as increasing insulin secretion, reducing insulin resistance, and decreasing intestinal breakdown of complex carbohydrates [35]. The sulphonylureas have been used for quite some time. They act by increasing insulin secretion, even in the absence of high glucose via a block of the ATP sensitive K^+ (K_{ATP}) channels in the β-cells [36]. Metformin, another drug also used to lower plasma glucose, directly reduces glucose output from the liver [37]. Peroxisome proliferator activated receptor γ agonists (thiazolidinediones) enhance insulin action in the periphery, and α-glucosidase inhibitors interfere with gut glucose absorption. Insulin is of course also used, and suppresses glucose production and augments glucose utilization. However, a particular concern regarding some of these conventional drugs is that they may have side effects. One problem with for instance the sulphonylureas is that patients over time become unresponsive to the treatment [35]; they are also known to cause hypoglycemia, since their action is independent of the plasma glucose concentration.

Recently, several new principles of pharmacological treatment in Type 2 diabetes have evolved. Glucagon-like Peptide (GLP)-1 receptor agonists (Exenatide), act through G-coupled receptors on β-cells, increasing insulin secretion, and possibly β-cell mass [38]. The drug potentiates glucose-stimulated insulin secretion, and therefore, in contrast to sulphonylureas, is not associated with hypoglycemia. In addition, GLP-1 could also exert other beneficial anti-diabetic effects, such as delaying gastric emptying, restraining glucagon secretion and have potential anorexic effects [38]. Since obesity has been shown to be a major cause of the development of insulin resistance that ultimately can result in Type 2 diabetes, a relatively new approach is to target obesity and appetite regulation. One example is the neuroendocrine pathway of melanocortin, that reportedly exerts positive effects on peripheral metabolism [39]. Other potential targets include leptin, which has been shown to prevent diet-induced obesity in rodents by reducing fat accumulation in the peripheral tissues [40]. Yet another approach is to centrally inhibit the enzyme fatty acid synthase and thereby reduce appetite [41]. In pursuit of new drug

therapies, science is constantly trying to identify potential new genetic and molecular targets to overcome the massive problems of Type 2 diabetes worldwide.

The pancreatic β-cell

β-cell morphology—The pancreas consists of exocrine tissue and islets of denser tissue: the endocrine pancreas, also called islets of Langerhans. These islets were first described by the German pathologist and anatomist Paul Langerhans in 1869 in his doctoral thesis. A healthy human pancreas contains about 1 million islets, which constitute about 1-2% of the whole pancreatic volume. Several important hormones are secreted from the islet, which consists of several different cell types. The insulin-producing β-cell makes up 65-80 % of the whole islet, the glucagon-secreting α-cells 15-20%, somatostatin-secreting δ-cells 3-10%, and pancreatic polypeptide (PP)-secreting cells ~1%. Secretion of islet hormones is highly regulated by innervations of both sympathetic, parasympathetic sensory nerve fibers as well as concentrations of different metabolites and hormones in the blood stream. Islet cells also control each other through paracrine and autocrine mechanisms. Apart from the classical hormones, several other peptides are secreted from the islet. For instance, it was found that islet amyloid polypeptide (IAPP) is expressed in β- and δ-cells [42], and peptide YY (PYY) is secreted from α-cells [43]. Ghrelin, a newly discovered peptide predominantly produced in the stomach, has recently been reported to be secreted from islet cells [44]; apparently, these cells do not belong to the classical islet cell types described here.

Glucose-stimulated biphasic insulin secretion—In the 1960s, the study of stimulus-secretion coupling evolved. This research led to three central discoveries. First, it was discovered that glucose had to be metabolized to induce insulin secretion. This was based on the fact that the effect of glucose on insulin release was abolished by pharmacological agents that interfere with cellular metabolism [45,46]. Second, Ca^{2+} ions were found to play a critical role in insulin secretion. This was recognized by experiments performed in the absence of extracellular Ca^{2+} [47,48]. Third, pancreatic β-cells were found to be electrically excitable. This was established by recordings of action potentials in glucose-stimulated β-cells [49]. It was further established that the changes in membrane potential upon glucose metabolism reflect alterations in K^+ permeability [49-51]. In patch clamp studies, the channel responsible for the changes in K^+ permeability was recognized as the K_{ATP} channel. This channel is in fact a key component of stimulus-secretion coupling in the β-cell [52,53].

The process of insulin secretion comprises two major cellular events: the triggering and the amplifying pathway [54]. The triggering pathway starts by metabolism of glucose in the β-cell and as a consequence the ATP/ADP ratio increases. This increase closes the K_{ATP} channels and the plasma membrane depolarizes. When depolarization reaches the threshold for voltage-gated Ca^{2+} channels, an influx of Ca^{2+} from the extra cellular space occurs, augmented by release of Ca^{2+} from intracellular stores, such as the endoplasmatic

reticulum. The intracellular increase in Ca^{2+} ($[Ca^{2+}]_i$) is the main trigger of exocytosis, resulting in a rapid release of insulin granules from the β-cell (**Figure. 1**).
Glucose-stimulated insulin secretion, however, involves more than triggering of insulin release, because insulin secretion is not sustained by elevated $[Ca^{2+}]_i$ alone [55]. In addition, an amplifying pathway exists, which has not yet been fully elucidated. Part of the amplifying pathway of insulin secretion was unraveled in the 1990s, using pharmacological agents targeting the K_{ATP} channel (diazoxide, which opens K_{ATP} channels, and Tolbutamide which closes the same channel) in the presence of high extra-cellular $[K^+]$, which confers depolarization of the membrane and thereby Ca^{2+} influx [56-58]. Under conditions where the K_{ATP} channel is kept open by diazoxide and the membrane depolarized by high extracellular $[K^+]$, glucose is still able to induce insulin secretion in the absence of an additional rise in $[Ca^{2+}]_i$. This indicates that metabolism of glucose *per se* serves an important role in sustaining insulin secretion. In fact, it was later shown that the amplifying pathway is not dependent on glucose metabolism alone, but operates upon metabolism of several other nutrients, such as amino acids and FFAs [59,60].
Insulin secretion is often referred to as being biphasic, although this pattern may be less apparent in some species. It has been suggested that the triggering pathway accounts for the first phase and the amplifying pathway for the second phase [61]. *In vivo*, the first phase of insulin secretion is characterized by a large increase in insulin secretion shortly after β-cells are exposed to elevated levels of glucose. This initial peak is followed by a drop in insulin levels and then gradually the second phase evolves [62]. The second phase has been shown to be dependent upon mitochondrial metabolism, suggesting that this phase is more dependent on energy, perhaps due to reactions involved in mobilization and docking of granules, which require ATP [63-65].
It has been implied that the differences seen in the first phase and second phase of insulin secretion reflect the distribution of insulin granules within the β-cells [62,66]. A specific pool of granules (about 1-5% of all granules) are always ready to be released, and contains granules docked to or in close vicinity of the plasma membrane [67]. These are termed the readily releasable pool (RRP), and are believed to be released during the first phase of insulin release [62]. Yet another pool of granules, termed the reserve pools (95-99% of granules), is situated further into the cell cytoplasm and needs to be translocated to the plasma membrane by reactions requiring ATP, Ca^{2+} and interactions with several proteins involved in the exocytotic machinery. This process is collectively called mobilization and priming of granules. The reserve pool is believed to supply granules for the second phase of insulin secretion [68].
The possible contribution of coupling factors involved in the biphasic insulin release has been extensively studied. The first phase is dependent upon a rise in the ATP/ADP ratio causing closure of the K_{ATP} channels, and a large increase in $[Ca^{2+}]_i$ via voltage-gated Ca^{2+} channels, as previously described [54]. However, finding a coupling signal for the second phase has proved to be more difficult. ATP was early on a strong candidate, since gradual lowering of the ATP/ADP ratio by azide, which inhibits oxidative phosphorylation at the level of cytochrome oxidase, inhibits insulin secretion even when

$[Ca^{2+}]_i$ levels remains high [60]. Glucose metabolism in the β-cell is accompanied by anaplerosis, a process whereby the tricarboxylic acid (TCA) cycle intermediates are replenished by reactions other than that catalyzed by pyruvate dehydrogenase (PDH). Because input and output from the TCA cycle are in equilibrium, anaplerosis is balanced by similar exit of intermediates (cataplerosis). Thus, if citrate leaves the TCA cycle, malonyl-CoA will accumulate and inhibit carnitine palmitoyl transferase 1 (CPT1; a transporter of long-chain acyl-CoAs (LC-CoA) into the mitochondria). Thereby fatty acid oxidation is diminished and, consequently, LC-CoA levels should rise in the cytoplasm and could account for the second phase of insulin secretion [69,70]. Another proposed coupling factor is mitochondrially derived glutamate. Glucose-stimulated insulin secretion induces the production of glutamate [71], which is then exported to the cytoplasm. Cytoplasmic glutamate is believed to sensitize the exocytotic machinery to Ca^{2+}, possibly via a direct interaction with the insulin-containing granules [71-73]. Whether this holds true or not is controversial [74,75]. Normally, the amino acid glutamine does not evoke insulin secretion on its own. However, glutamine and other aminoacids may be transaminated to glutamate, providing a substrate for the glutamate dehydrogenase (GDH) reaction. In the presence of leucine, an allosteric activator of GDH this pathway is anaplerotic and stimulates insulin secretion [76]. Recently, observations in mice deficient in the sulfonylurea receptor 1 (SUR1), which exhibit only K_{ATP}-independent insulin secretion, suggest that cellular glutamine derived from glutamate is involved in control of insulin secretion [77]. Here, several complex metabolic steps, including an anaplerotic contribution to the TCA cycle, result in a stimulation of insulin secretion. However, this effect is only apparent when ATP and $[Ca^{2+}]_i$ are elevated. NADPH is another potential signaling molecule from mitochondrial metabolism [78,79]. Watkins and Moore [80] showed that NADPH is taken up by insulin granules in the toadfish islet and stimulates insulin release. Recently, it was discovered that by increasing the $NADPH/NADP^+$ ratio in β-cells, the inactivation phase of K_{ATP} channels is prolonged and hence insulin secretion is potentiated [81]. Moreover, the $NADPH/NADP^+$ ratio rises in β-cells upon stimulation by glucose [82], further implicating this nucleotide as a metabolic coupling factor. cAMP is known to modulate Ca^{2+}-induced exocytosis. cAMP levels rise in the β-cell upon stimulation with, e.g., glucagon [83] and GLP-1 [84]. GLP-1 and glucagon exert their effects on β-cells by activation of G-coupled receptors, which increase cAMP levels within the cell, thereby potentiating insulin secretion. cAMP is known to activate protein kinase A (PKA), and it has been shown that inhibition of PKA reduces insulin secretion [85]. Renström et al. proposed in 1997 [86] a mechanism in single mouse β-cells by which cAMP/PKA stimulated insulin secretion via two different mechanisms: one involving cAMP and PKA, the PKA-dependent pathway, and one involving cAMP alone, the PKA-independent pathway. The latter process appears to take place in a late stage of insulin secretion, and operates by increasing the release probability of granules in the RRP pool. The PKA-dependent pathway, however, promotes refilling of the RRP pool by stimulating granule mobilization to the RRP. Nevertheless, the precise nature of all these

modulators and coupling signals has remained elusive, and it may very well be that all, or many of them, work in concert to control insulin secretion.

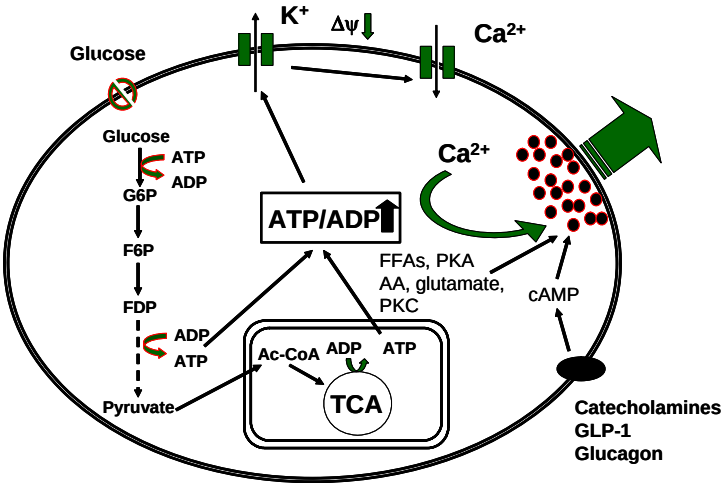

Figure 1. As glucose enters the β-cell via GLUT2 it is metabolized in glycolysis to yield pyruvate. Pyruvate is converted to Ac-CoA upon entering the mitochondria. Metabolism in the TCA cycle increases the ADP/ATP ratio. Hereby, the K_{ATP} channels close and the cell membrane depolarizes. An opening of the Ca^{2+} channels occurs and causes an influx of Ca^{2+} and ultimately exocytosis. Insulin secretion is sustained and facilitated in the amplifying pathway by several other putative factors such as FFAs, PKA, PKC, metabolism of amino acids and glutamate. Catecholamines, GLP-1 and glucagon all affect the β-cell via G-coupled receptors controlling the cAMP level in the cell. An increase in cAMP activates PKA, which can generate numerous lipid signaling molecules via phopsphorylation and activation of lipases.

In type 2 diabetes, *in vivo* disturbances in the release pattern of insulin are often observed [87]. The first phase of insulin release appears to be more frequently affected than the second phase. The exact underlying mechanisms are not clear at this time. However, since several important mechanisms are controlling movement, priming and docking of granules and exocytosis of insulin, all or some of these factors could potentially be perturbed and cause Type 2 diabetes. Given the essential role of metabolism in β-cell stimulus-secretion coupling, it is not unlikely that a metabolic disturbance accounts for the secretory deficiency in Type 2 diabetes.

β-cell mitochondria and metabolism—Since both the first and second phase of insulin secretion appear to depend on generation of ATP and mitochondrial metabolism, mitochondria play a pivotal role in control of insulin secretion. Mitochondria are small organelles within the cell of bacterial origin, containing their own DNA [88]. Metabolism of sugars, fatty acids and amino acids take place in mitochondria. Pyruvate is the end product of glycolysis, and the main substrate for mitochondrial metabolism. As pyruvate enters mitochondria it is converted to Acetyl-CoA and enters the TCA cycle (**Figure. 2**). From the complete oxidation of Acetyl-CoA in the TCA cycle, CO_2, ATP and the reducing equivalents NADH and $FADH_2$ are produced. NADH and $FADH_2$ supply electrons to the respiratory chain, where an electrochemical gradient of protons is built up, driving the generation of ATP by ATP synthase. The energetic compounds derived from mitochondrial metabolism are transported to different sites in the cell, mediating other molecular reactions as energy substrates or signaling molecules.

It was established early on that mutations in several mitochondrial genes cause diabetes [89,90]. The homozygous knock out mouse for nuclear encoded mitochondrial transcriptional factor (Tfam) is lethal, however, the heterozygote form is viable but severely diabetic [91]. The conditional knock out of Tfam in β-cells exhibits a similar phenotype, thus supporting the crucial role of mitochondrial function in this disease [92]. Moreover, deletion of the mitochondrial protein Nicotinamide nucleotide transdehydro-

genase (Nnt) in mice results in glucose intolerance and impaired insulin secretion *in vivo.* Knock down of the same protein with siRNA in MIN-6 cells abolishes glucose-dependent increases in both $[Ca^{2+}]_i$ and insulin secretion [93]. This is possibly due to impaired ATP production. In addition, both functional and morphological alterations of mitochondria have been observed in β-cells in Type 2 diabetic patients [94]. Consequently, mitochondrial function is crucial for normal β-cell function and viability.

Several potential coupling factors for the amplifying pathway of insulin secretion, such as ATP, LC-CoAs, glutamate and NADPH, all derive from/or have an impact on mitochondrial metabolism. However, another putative signal is Ca^{2+}, well-accepted as the main trigger of exocytosis. It has been shown that enzymes in mitochondrial metabolism, such as PDH, isocitrate dehydrogenase and α-ketoglutarate dehydrogenase are all activated by Ca^{2+} [95-99]. Still, the mechanism of the second phase of insulin secretion appears not only to be dependent upon Ca^{2+} [56,100]. The activity in the TCA cycle increases the intracellular ATP/ADP ratio, raising $[Ca^{2+}]_i$ due to the closure of K_{ATP} channels, as previously described. However, Maechler at al. showed that glucose increases the level of cytoplasmic ATP in β-cells [101], and that this increase precedes the rise in $[Ca^{2+}]_i$. In addition, the influx of Ca^{2+} is associated with an intra-mitochondrial increase in Ca^{2+}, believed to activate mitochondrial metabolism [102], presumably via activation of the dehydrogenases related above. The increase in $[Ca^{2+}]_i$ could then together with coupling factors from the mitochondria [103] provoke insulin secretion.

The putative coupling factors of the second phase of insulin secretion, discussed in the previous section have one aspect in common: anaplerotic pathways [104]. The anaplerotic enzyme pyruvate carboxylase is highly expressed and active in β-cells [104]. About 40% of pyruvate entering the TCA cycle upon glucose stimulation is carboxylated by this enzyme, indicating an important role in insulin secretion [105]. It has been shown that inhibition of this enzyme decreases insulin secretion in INS-1 cells, 823/13 cells and rat islets [106-108]. Furthermore, this inhibition was previously thought to affect the second phase more than the first phase of insulin secretion. However, studies by Fransson et al. [109] show that inhibition of pyruvate carboxylase affects both phases, indicating that anaplerosis *per se* is involved in both the first and second phase of insulin secretion. Other anaplerotic pathways also exist in the β-cell. GDH converts glutamate to α-ketoglutarate, a TCA cycle intermediate [110]. In fact, it has been shown that over-expression of GDH in rat islets enhances the secretory response of insulin to glucose [111].

In sum, several factors in mitochondrial metabolism appear to be involved in both the first and second phase of insulin secretion.

Lipids in insulin secretion—Lipids are believed to play an important role in insulin secretion both under normal and pathological conditions [14,22,112,113]. One view that has emerged is that lipids may play a primary role in the evolution of metabolic abnormalities in Type 2 diabetes. This notion includes the concept of lipotoxicity, in which lipids are suggested to exert negative effects on β-cells as well as interfere with the peripheral actions of the hormone [114]. Therefore, increased levels of circulating

FFAs and triglycerides, could perturb cellular function directly or indirectly via accumulation in β-cells, liver and skeletal muscle.

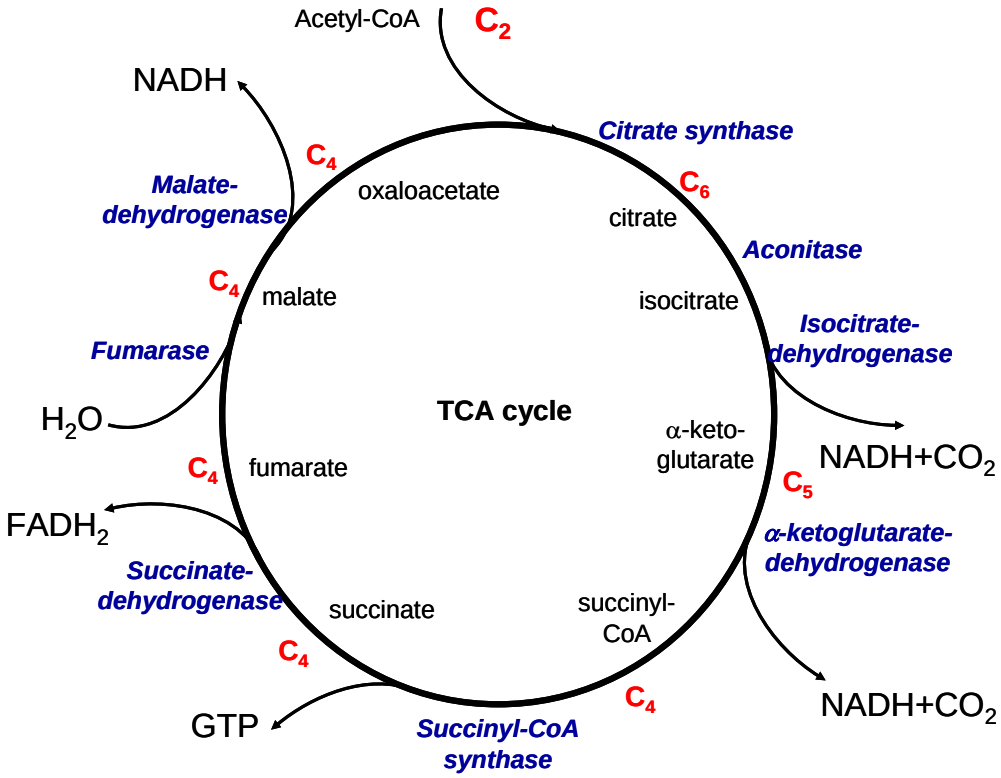

Figure 2. When Pyruvate enters the mitochodrion it is converted to Acetyl-CoA, which is readily condensed with oxaloacetate to form citrate. Citrate is metabolized by numerous enzymatic reactions in the TCA cycle to yield ATP, GTP and the reducing equivalents NADH and $FADH_2$, which donate electrons to the respiratory chain, creating an electrochemical gradient across the inner mitochondrial membrane. The flow of protons back along this gradient through ATP synthase ultimately generates ATP.

However, the lipids are not only harmful. It has also become clear that lipids are required by β-cells for proper function. Corkey and Prentki have proposed a model in which glucose metabolism in the β-cell is linked to lipid metabolism [115]. Glucose metabolism will eventually cause a rise in malonyl-CoA levels. This metabolite, which in fat-synthesizing cells is the starting point for fatty acid synthesis, will block the transport of LC-CoA into the mitochondrion, via inhibition of CPT-1, as previously mentioned. As a result, levels of LC-CoAs in the cytoplasm should rise, and could potentially mediate the glucose stimulus to secretion. At that point, an unexplored area was how LC-CoAs were provided to stimulus-secretion coupling in the β-cell. If a lipid signal is involved in regulating insulin secretion, one or several lipases would be required to release them from intracellular TG stores. Islets do contain triglycerides, an accumulation that may increase during the pathogenesis of diabetes [116], but the molecular machinery surrounding the TG stores within the β-cell are at this point unknown. Furthermore, hydrolysis of these intracellular stores may drive the compensatory hyperinsulinemic response from β-cells observed in insulin resistance. This may be a beneficial process, since it prevents hyperglycemia in the insulin resistant state.

The important role for lipids in glucose homeostasis has been further implied by studies in rats made hyperleptinemic by recombinant adenoviruses containing leptin cDNA. The rats display pancreatic islets depleted of lipids, which are unresponsive to glucose [31], but glucose responsiveness is restored by adding exogenous FFA. It has also been shown that glucose responsiveness in fasted rats both *in vivo* and vitro is dependent on the presence of FFA [112], an observation also made in humans [113]. Thus, it can be concluded that a fatty acid-derived factor may play an important role in insulin secretion; this factor was abrogated in the referred experiments either by leptin treatment or by fasting. Again, how such a factor is produced in cells remains unknown, but the action of one or several lipases would be required.

Hormone sensitive Lipase

Structure/function of Hormone sensitive lipase (HSL)—Hormone sensitive lipase was first described in the 1960s as a lipase active in the adipose tissue [117-119], where it is believed to be a key player in lipid metabolism as well as in overall energy homeostasis. HSL is acutely regulated by hormones, as implied by the name. It is activated by catecholamines via adrenergic G-coupled receptors and inactivated by insulin.

HSL has a broad substrate specificity and readily hydrolyses several different substrates, such as triacyl-, diacyl- and monoacylglycerols as well as retinyl esters, cholesteryl esters and steroid fatty acid esters [120-124]. Upon lipolytic activation by catecholamines, intracellular levels of cAMP increase. cAMP activates PKA which has two main targets in the adipocyte, both involved in regulation of lipolyis: HSL and perilipin [125]. Perilipin is a protein coating the lipid droplet, protecting triglycerides from constitutive hydrolysis in the basal, unstimulated state [126,127]. Phosphorylation of perilipin has been shown to be essential for a full lipolytic response [128,129].

When the adipocyte is stimulated with catecholamines, PKA phosphorylates HSL and perilipin. HSL then translocates from the cytoplasm to the lipid droplet and lipolysis ensues. The counter-regulatory mechanism is induced by insulin, causing a dephosphorylation of HSL, and involves phosphodiesterase 3B (PDE3B), a protein which hydrolyzes cAMP in the cell, thereby reducing PKA activity. It has been shown that selective inhibition of PDE3 results in a deactivation of HSL and thereby reduced lipolysis [130,131].

When HSL was cloned from adipose tissue it became evident that HSL shared virtually no sequence homology with any other mammalian protein [132-134]. Nevertheless, it was subsequently reported that HSL does share some sequence homology with a few bacterial proteins [135]. In 1996, Contreras et al. found that HSL and enzymes from a superfamily of esterases and lipases, inclucing acetylcholinesterase and bile salt stimulated-lipase, were structurally related by secondary structure formation [136]. Furthermore, it was shown that the adipocyte form of HSL consists of two structural domains [120] (**Figure. 3**). The N-terminal region of HSL is a part of the protein with a high degree of sequence homology between humans and rodents (rat and mouse). This part is believed to be involved in protein/protein interaction [137]. After the N-terminus

follows a short sequence of 18 amino acids believed to serve as a connective region between the N- and the C-terminus. The C-terminal domain includes two distinct parts: the catalytic domain harboring the catalytic triad (Ser-423, Asp-703 and His-733), which is involved in the catalytic cleavage of substrates, and the regulatory module containing the four known phosphorylation sites of HSL (Ser-563, -565, -659 and -660) (**Figure. 3**) [120,136,138].

At least two other proteins have been shown to interact with HSL [137,139]. Adipocyte lipid binding protein (ALBP), a protein acting as an intracellular fatty acid carrier [137], and lipotransin [139]. ALBP interacts with the N-terminal region. The interaction between HSL and lipotransin is not yet fully understood.

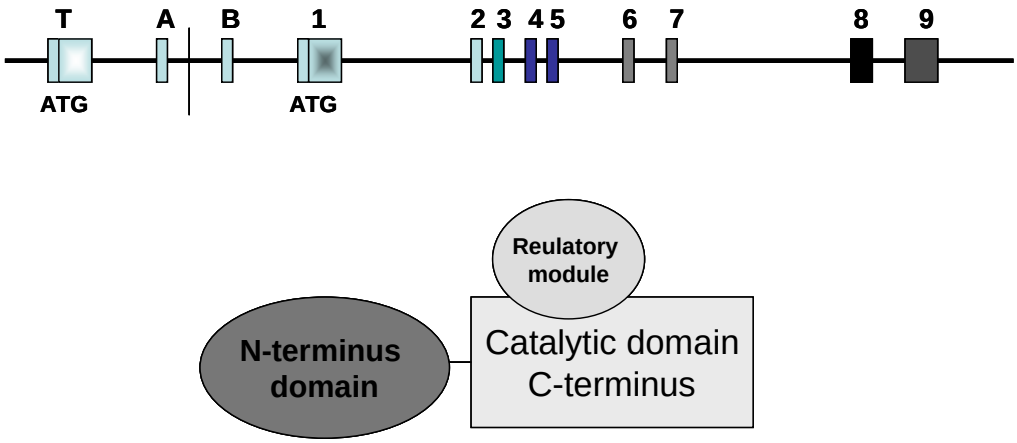

Figure 3. Organisation of the human HSL gene and domain structure of HSL $_{adi}$ protein. Exon and intron organisation of the HSL gene with the two translational start sites for testicular HSL (T) and HSL $_{adi}$. The gene comprises 9 exons in total (top figure). Bottom figure shows the proposed domain structure of HSL_{adi}, with the N- and C- terminal domains and the regulatory module. The C-terminus is the catalytic domain containing the catalytic triad. The regulatory module contains the four phosphorylation sites for HSL.

HSL in β-cells—HSL is also expressed in several tissues other than the adipose, such as muscle, heart, adrenals, testes, ovaries, macrophages, mammary glands, hypothalamus and β-cells [140-145]. Depending on the tissue where HSL is expressed, several functions have been suggested, including regulation of substrate for export in adipocytes, release of oxidative fuels in high energy demand tissue, such as muscle and heart, substrates for steroidogenesis in adrenals, testes and ovaries, and as a source for cholesterol in macrophages [146].

In the β-cell, two distinct isoforms of HSL are expressed: the adipocyte form (85 kD; HSL_{adi}) protein, and a slightly larger isoform (89 kD) encoded by exons 1-9 including exon A [147]. Exon A is spliced to exon 1, hereby adding 43 amino acids to the N-terminal end of the protein, producing the slightly larger protein. In the adipocyte, HSL is a cytosolic protein. However, in the β-cell, the enzyme appears to be located in close proximity to the insulin granules [147].

HSL has been proposed to be involved in the generation of metabolic coupling signals in the β-cell, such as LC-CoAs and diacylglycerols (DAG) [69,115,148-150]. LC-CoAs are

known to stimulate insulin secretion in clonal β-cells, possibly via the activation of protein kinase C (PKC) [151], or via direct activation of the exocytotic machinery [152]. Since it is believed that lipid signaling plays a crucial role in the regulation of insulin secretion in response to nutrient fuel [115] lipolysis has been studied in relation to insulin secretion. Masiello et al. showed that 3,5-Dimethylpyrazole (DMP), an inhibitor of adipose tissue triacylglycerol lipase [153], inhibits insulin release from isolated rat islets in response to both glucose and cAMP agonists known to stimulate lipolysis [154]. Later, Mulder et al. showed that lipolysis does take place in rat islets and that lipolysis is essential for normal glucose-stimulated insulin secretion [155]. In addition, a positive correlation between lipolysis and insulin secretion was observed in INS-1 cells and mouse islets [156]. Therefore, in the β-cell, a loss of HSL could cause a block of mobilization of lipids from stored triglycerides and subsequently an accumulation of triglycerides within the pancreatic β-cell. The effect of this could be dual. First, the harmful effect of an excess of stored lipid in the β-cell could lead to cellular dysfunction and ultimately Type 2 diabetes [22,114]. Second, since lipids are important for adequate insulin secretion, the disruption of HSL could result in the failure of generating the lipid signals necessary for a full secretory response [115]. Recently, it was observed that a specific inhibitor of HSL (5-(2H)-isoxazolonyl urea; BAY) inhibits insulin secretion in rat islets stimulated by either carbacholine or GLP-1[157]. Taken together, these data imply an important role for HSL in insulin secretion.

HSL knock out mice—To elucidate the role of HSL in overall glucose homeostasis and insulin secretion from the pancreatic β-cell, several lines of HSL knock out (HSL KO) mice have been created. At this time, four different lines exist and have been evaluated [158-162]. All lines share male infertility due to oligospermia. However, several discrepancies between the different lines exist. For instance, the line from Zechner's laboratory exhibits an increase in fasting plasma glucose [158], whereas the Mitchell line displays no change in this parameter [162]. In contrast, the line from the Ishibashi laboratory shows a decrease in fasting plasma glucose [160]. In the Mitchell line insulin resistance and an *in vitro* β-cell secretory deficiency have been reported [162]. However, neither β-cell physiology nor insulin secretion has been extensively studied in either the Zechner line or the Ishibashi line. Therefore it is difficult to evaluate the results from glucose homeostasis derived from these mouse lines. Nevertheless the discrepancies may be attributed to differences in genetic background, genetic redundancy and/or the compensatory actions of other lipases. In fact, a novel lipase, adipocyte triglyceride lipase (ATGL), was recently discovered in HSL KO mice [163]. Since the results from the different HSL KO lines are not conclusive, and a clear role for HSL in insulin secretion is yet to be elucidated.

Material and methods

Animals and animal models

Model for insulin resistance (C57BL/6J mice fed high fat diet)—To study the effect of β-cell adaptation to insulin resistance, we used a well-characterized model of high fat feeding of female C57BL/6J mice [164] **(paper I)**. This model was originally introduced by Surwit et al. in 1988 [165] and characterized by insulin resistance and insufficient islet compensation [166]. Later, it was shown that this model does not develop overt diabetes, but remains insulin resistant and glucose intolerant with compensatory hyperinsulinemia [167]. Mice were given a high fat diet containing 60% fat (on a caloric basis) or a control diet containing 10% fat, for 12 weeks.

Global KO of Hormone sensitive lipase—HSL KO mice were generated by targeted disruption of the HSL gene in 129SV-derived embryonic stem cells by standard procedures [168]. In short, the cDNA encoding the *Aequorea victoria* green fluorescent protein was inserted in-frame into exon 5 of the HSL gene, followed by a neomycin resistance gene, hereby disrupting the catalytic domain. The herpes simplex thymidine kinase gene was inserted at the 3'-end of the construct. After electroporation of embryonic stem cells, 96 colonies resistant to both G418 and ganciclovir were isolated, 10 of which showed homologous recombination as determined by Southern blot analysis. Two of these colonies were used for generation of two independent HSL KO mouse lines. Preliminary experiments showed that glucose homeostasis was similar in both HSL KO lines and that diglyceride hydrolysis in white adipocytes was virtually absent; therefore, mice from both HSL KO lines were pooled for the experiments. **(Paper II-III)**.

β-cell specific KO of HSL—The β-cell specific KO (β-HSL KO) mouse was created by loxP/Cre mediated recombination. The mouse line [SV129/C57BL6J] carrying a mutated HSL allele, in which exons 2-7 were flanked by lox p sites as previously described [158] was crossed with a line [C57BL/6J] expressing the Cre recombinase under control of the rat insulin 2 promoter (RIP2) [169,170]. Three consecutive steps of breeding were performed. First, to produce the breeding pairs, $Hsl^{+/-}$ mice were crossed with $Cre^{+/-}$ mice. This generated the $Hsl^{+/-}/Cre^{+/-}$ mouse. Second, to produce the $Hsl^{+/+}$ animals, $Hsl^{+/-}$ were bred on to each other. Third, $Hsl^{+/-}/Cre^{+/-}$ and $Hsl^{+/+}$ from the two first breeding steps were used to produce the conditional β-cell KO ($Hsl^{+/+}/Cre^{+/-}$) and the other offspring were: $Hsl^{+/+}/Cre^{-/-}$, $Hsl^{+/-}/Cre^{-/-}$, and $Hsl^{+/-}/Cre^{+/-}$. The $Hsl^{+/+}/Cre^{-/-}$ from the same litters were used as controls and is referred to as WT/lox in the experiments **(Paper IV)**.

Genotyping KO animals—Tail tips and/or ear fragments were used as a source of DNA for genotyping both the global HSL KO and the β-HSL KO. After collecting tissue samples, digestion was performed over night in a mechanical shaker. The crude DNA preparations were diluted in water. DNA was mixed with primers accordingly; for the global HSL KO; KO/WT fwd 5´-ACTCAACAGCCTGGCAAAAT-3´, KO rev 5´-GCT-

GAACTTGTGGCCGTTTA-3′ and WT rev 5′-AGG TCACAGTGCTTGACAGC-3′. For the β-HSL KO mouse, the following primers were used: HSL I 5′-CATGCACCTA-GTGCCATCCTTC-3′ and HSL II 5′-CTCACTGAGGCCTGTCTCGTTG-3′. Since the genotype of the β-HSL KO also includes the expression of Cre recombinase, this PCR was performed in a separate reaction using the following primers; Cre I 5′-ATCTCAC-GTACTGACGGTGG-3′ and Cre II 5′-ACCACGTTGCATGATCTCC-3′. Primers and DNA were mixed with Dynazyme (Finnzymes, Finland) buffer and water to a 25 ml PCR reaction. Products from touch down PCR were separated on a 1% agarose gel, enabling determination of different genotypes **(Papers II-IV)**.

Tissue isolation and reverse transcriptase (RT) PCR—Islets and adipose tissue were collected from 5 week old female β-HSL KO and WT/lox ($Hsl^{+/+}$) mice. Islets were isolated by standard collagenase digestion and mRNA was extracted immediately from the freshly isolated islets. Adipose tissue was promptly frozen (-80°C), and mRNA was extracted from thawed samples. cDNA was obtained by reverse transcription. Then PCR with the following primers was performed: I, 5'-CATGCACCTAGTGCCATCCTTC-3'; II, 5'-CTCACTGAGGCCTGTCTCGT TG-3'; and III, 5'-TACCGAGCCATCTTCCT-AGTCC-3'. Primers I and II were used to detect the full length HSL construct (390 bp) and primers I and III to detect the truncated HSL construct (250 bp) **(Paper IV)**.

Glucose homeostasis in vivo *and* in vitro

Tolerance tests—Blood was collected from anesthetized mice by retro-orbital sampling. Glucose was determined in plasma either by Glucose Trinder Assay (Sigma Diagnostics, Sigma Aldrich, Dorset, United Kingdom (Papers I-III)) or Infinity (Glucose Ox, TR 1521-125; Thermo Electron Corporation. Melbourne, Australia (Paper IV)). Insulin, glucagon, leptin and adiponectin in plasma were measured by radioimmuno assay (RIA). For the intravenous glucose tolerance test (IVGTT), D-glucose (1g/kg) was injected into the tail vein of anesthetized mice. In an additional experiment, 5 mg of arginine were injected intravenously to elicit insulin and glucagon secretion. In the insulin tolerance test (ITT), 0.75 mU/g human insulin were given intraperitoneally to fasted, anesthetized mice. For fasting samples, mice were deprived of food at 11.00 p.m. and retro-orbital blood was drawn from anesthetized mice at 7:00 am **(Papers I-IV)**.

Islet isolation and insulin secretion in vitro—Islets were isolated by standard collagenase digestion, and handpicked under a stereomicroscope. Batches (n=8) of 3 islets for each condition were kept in HEPES balanced salt solution containing 3 mM glucose for 60 minutes in an incubator at 37°C. Then, 3 islets were transferred to a 96-well plate kept on ice and containing 200 µl per well of the same buffer but with the addition of the respective secretagogue. Following transfer of all islets, the plate was again placed in an incubator at 37°C. At 60 minutes, a sample from the buffer was removed for measurement of insulin by either RIA or Insulin ELISA **(Paper IV)**. As a complement, insulin secretion was also determined upon perifusion of ~20 islets sandwiched between

two layers of gel in a column **(Paper II)**; the flow-rate of HBSS was 0.5 ml/min and fractions for determination of insulin by ELISA were collected every minute.

Fuel oxidation—After isolation, islets were kept in HBSS containing 2.8 mM glucose for 60 min at 37°C. Batches of islets (n=20) were transferred to a rubber cup suspended from a rubber sleeve stopper, inserted into a glass scintillation vial. For glucose oxidation, a reaction mixture containing radiolabelled D-[^{14}C]-glucose was added to low and high glucose concentrations. The reaction was terminated after 1 h by injection of perchloric acid into the suspended cup. For glutamine oxidation, the incubation medium contained radiolabelled L-[^{14}C]-glutamine mixed with 10 mM glutamine and 10 mM 2-Amino-2-norbornane-carboxylic acid (BCH) for 1 h. Oxidation of palmitate was determined over 2 h in a reaction mixture consisting of palmitic acid complexed to 1% BSA, with radio labelled [1-^{14}C]-palmitic acid as a tracer, together with L-carnitine to enable the fatty acid to enter mitochondria; glucose was added at low and high concentrations. The oxidation rate of radiolabelled D-[^{14}C]-glucose, L-[^{14}C]-glutamine, or [1-^{14}C]-palmitic acid was measured as released $^{14}CO_2$ trapped by adding benzethonium hydroxide to the bottom of the sealed vials. After discarding the cups and rubber stoppers, trapped $^{14}CO_2$ was determined by scintillation counting **(Papers I-III)**.

Enzyme activities and determination of tissue acylglyceride content—Diglyceride lipase and neutral cholesterol ester hydrolase (NCEH) activities were determined in cellular extracts, using established procedures ([120] **(Paper II-III)**. Lipolytic activity in islets was assessed by measuring release of glycerol from cells, assuming that glycerol kinase is not expressed in islets **(Paper III)**; glycerol was determined with a bioluminescence (luciferase)-based method [171]. Lipolysis in isolated adipocytes was determined as release of both glycerol and fatty acids **(Paper II)**. Acylglycerides were extracted from cells according to the method by Folch [172], and assayed using the action of lipoprotein lipase, and subsequent measurement of glycerol in a reaction coupled to NADH generation **(Paper II-III)**. In **Paper I and IV,** tissue acylglyceride content was assessed by staining of the islets with Oil red O, a dye with specific affinity for neutral lipid.

β-cell mass and morphology

Cavalieri's principle for determination of cell volume—Islet number and β-cell volume were determined in an unbiased procedure, according to the principles of stereological morphometry. Serial sections (*n*=100–120) were prepared and immunostained for insulin, using the peroxidase-anti-peroxidase method as described previously [42]. Islet numbers were determined by dissector analysis [173], employing adjacent sections as reference and look-up sections, respectively. Total β-cell volume was determined in serial sections, using point-counting and Cavalieri's principle [174]. As an index of β-cell mass, mean islet β-cell volume was calculated by dividing total islet β-cell volume by total number of islets in the sections; these numbers were compared statistically. The investigator was unaware of the identity of the sections during analysis **(Paper II)**.

Morphometrical analysis and β-cell mass—Sections of mouse pancreas from β-HSL KO and WT/lox mice were subjected to indirect immunofluorescence, using a proinsulin antibody. The insulin-stained sections from the two genotypes were then analyzed as digitized images. All islets in every section were counted and measured, and the mean area of insulin stained β-cells was calculated. The identity of the different sections was at all times unknown to the investigator **(Paper IV)**.

Electron Microscopy imaging and calculations of mitochondrial mass—Islets were isolated from 3 HF mice and 3 controls, respectively. Fifty islets from each animal were pooled in two different preparations. Specimens were prepared and fixed for electron microscopy analysis and embedded in Epon plastic. Ultrathin sections were cut and placed on copper grids before examination in a Philips CM10 transmission electron microscope. The mitochondrial area was measured in digitized images of 10 randomly chosen β-cells; data were expressed as fractional mitochondrial area/β-cell. The identity of the sections was unknown to the investigator **(Paper I)**.

Aims of the thesis:

- ***To understand β-cell adaptation to insulin resistance.***

- ***To understand the role of hormone sensitive lipase in glucose homeostasis, insulin secretion, insulin action, and lipid metabolism, using a global and β-cell-specific knock out of the lipase.***

Present investigations

β-cell adaptation to insulin resistance (paper I)

In paper I, we investigated the underlying mechanisms of β-cell adaptation to insulin resistance. We used a well-known model for insulin resistance: the female high fat diet fed (HF) C57BL/6J mouse. When fed a high fat diet for 12 weeks, the mice gained significantly more weight (26.2 ± 1.1 vs. 21.4±0.4 $P < 0.001$) than mice fed a control diet. While plasma glucose levels did not rise significantly in the HF mice, insulin levels were markedly elevated. This suggests that the mice have developed insulin resistance, which is adequately compensated by the β-cells. Indeed, IVGTTs confirmed glucose intolerance and insulin resistance. Moreover, we observed that islets isolated from high fat diet fed mice displayed a blunted response towards glucose; basal insulin secretion was elevated with just a small additional increase at stimulatory concentrations of glucose. In contrast, islets from HF mice were clearly more sensitive to other secretagogues, such as dimethyl-glutamate, glutamine, methyl-succinate, α-ketoisocaproic acid (α-KIC) and palmitate, which all elicited a hyperinsulinemic response. In addition, glucose oxidation in islets isolated from HF mice was significantly less increased by an elevation in glucose concentration than in control animals. Previous findings in this model indicate that GLUT2 is dislocated from the plasma membrane [21], and that this is part of the reason why glucose oxidation is impaired. Conceivably, if glucose is unable to enter the β-cell appropriately, glucose-stimulated increases in insulin secretion and oxidation will be perturbed. We were able to replicate this in our experiments and found a similar perturbation of GLUT2 localization. In contrast, we found a significant increase in oxidation of both glutamine and palmitate in islets from HF mice. Interestingly, the shift in metabolism from oxidation of glucose to that of mitochondrial fuels was accompanied by morphological changes in mitochondria. We found a significant increase in mitochondrial surface area. Furthermore, increased staining for neutral lipids, Oil Red O, was observed in islets in pancreatic sections from HF mice, indicating an increase in stored lipids, presumably TGs. Supporting this, electron microscopic imaging of β-cells in HF mice revealed the presence of lipid droplets, which were virtually absent in β-cells in the control mice.

Based on these findings, we propose that β-cells compensate for insulin resistance by increasing mitochondrial mass, hereby increasing mitochondrial metabolism, which in turn underlies the circulating hyperinsulinemia and the exaggerated response to mitochondrial fuels. The increased flux of FFAs that arises in the mice as a consequence of HF feeding may be an important factor driving these events.

Global knock out of Hormone sensitive lipase – Glucose homeostasis and lipid metabolism (paper II)

In paper II and III we characterized the global HSL KO created in the laboratory of Cecilia Holm [140], with regard to glucose homeostasis, insulin secretion and lipid metabolism. In paper II we expected that the ablation of HSL would cause an increase in adipocyte TG storage with obesity and cellular dysfunction as a result. Interestingly,

mice were not obese and only liver displayed an increase in TG stores. However, we observed that white adipocytes displayed a blunted catecholamine-stimulated lipolytic response, as determined by release of glycerol and FFAs. This was accompanied by an accumulation of diglycerides within the adipocytes from the HSL KO mouse, indicating that HSL mainly hydrolyses diglycerides within this tissue.
IVGTTs in the fasted state revealed significantly retarded glucose clearance and increased insulin secretion in response to the sugar, indicating glucose intolerance with compensatory hyperinsulinemia. Fasted plasma glucose levels were significantly higher in HSL knock out mice. Accordingly, islets from HSL KO mice exhibited an increase in insulin content and β-cell mass; insulin secretion, assessed by perifusion of islets, was not different in the two genotypes. Furthermore, overall impaired insulin sensitivity was observed in the periphery (muscle, liver and white adipose tissue). In the liver, glucose production *in vivo* was inadequately inhibited by insulin and glucose uptake *in vitro* in skeletal muscle was inhibited. Insulin-stimulated lipogenesis in white adipose tissue was also significantly impaired. All these processes may, in different ways, contribute to insulin resistance observed in mice lacking HSL. However, when insulin sensitivity was assessed in a euglycemic hyperinsulinemic clamp, no significant differences were observed. This may be due to the fact that glucose disposal in the mouse primarily (~70%) occurs by a non-insulin-dependent mechanism [175]. Our data suggest an important role for HSL in overall glucose homeostasis and mobilization of TG stores in some tissues.

Global knock out of Hormone sensitive lipase – *Insulin secretion and islet metabolism (paper III)*

Next, we assessed the role of HSL in insulin secretion in our global HSL KO. A high NCEH activity of HSL is a unique property of mammalian lipases. Findings in another strain of HSL KO mice suggest that this activity is mainly due to HSL [160]. In islets from HSL KO mice, NCEH activity was almost completely abolished. This is in complete accordance with the ablation of HSL in these islets. In islets from WT mice, NCEH activity was readily detectable. However, when we investigated diglyceride lipase activity and lipid storage, as levels of acylglycerides, in islets from both HSL KO and WT mice, we found no significant differences between the genotypes. Moreover, oxidation of palmitate and glucose in islets from WT and HSL KO mice was not significantly changed. Next, we assessed insulin secretion *in vitro* in isolated islets from HSL KO and WT animals. We stimulated islets with glucose under both normal and K_{ATP}-independent conditions (35 mM K^+ and 250 μM Diazoxide). Furthermore, we stimulated islets *in vitro* with palmitate, α-KIC, GLP-1 and carbacholine. None of these conditions revealed any changes between the genotypes in islets isolated from 4 weeks, 4 months or 7.5 months animals. This was also confirmed with measurements of $[Ca^{2+}]_i$ in intact islets. These experiments would circumvent compensation in insulin secretion induced by enhanced β-cell mass and/or insulin content, which we found in our HSL KO mouse (Paper II). Nevertheless, islets from both WT and HSL KO mice responded similarly with a rise in $[Ca^{2+}]_i$ in response to elevated glucose.

In view of these data, loss of HSL does not affect insulin secretion in our global HSL KO. However, since several other lines of HSL KO mice exist, and differences in the phenotype of these animals with respect to insulin secretion are evident, compensatory action of another lipase is a plausible explanation for the lack of impact on insulin secretion by HSL-deficinecy in our mice.

β-cell specific knock out of Hormone sensitive lipase (paper IV)

In paper IV, we investigated the role of HSL in insulin secretion in a tissue-specific knock out of HSL in β-cells (β-HSL KO). This knock out was created by loxP/Cre mediated recombination, crossing a mouse line [SV129/C57BL6J] carrying a mutated HSL allele, in which exons 2-7 were flanked by lox p sites as previously described [158], with a line [C57BL/6J] expressing the Cre recombinase under control of the rat insulin 2 promoter (RIP2) [169,170]. This approach could in part circumvent the compensatory mechanisms that may have come into play in the global knock out of HSL. We discovered that β-HSL KO mice were significantly hyperglycemic at 12 weeks. Furthermore, an IVGTT at 12 weeks revealed a blunting of the initial insulin response to glucose, and retarded elimination of glucose. Arginine-stimulated insulin secretion was also markedly diminished *in vivo*. However, *in vitro* static (1 hour) incubations revealed no significant differences in insulin secretion when islets were stimulated with various secretagogues, such as high and low glucose, high K^+ together with diazoxide, palmitate, GLP-1, carbacholine and α-KIC. Interestingly, short (10 minute) *in vitro* static incubations in isolated islets revealed a loss of insulin secretion in response to 16.7 mM glucose or 70 mM KCl. Moreover, β-cell mass and islet insulin content were increased by 2.6-fold and 2.3-fold, respectively, further suggesting a compensatory mechanism by which β-cells strive to overcome a secretory deficiency and maintain euglycemia.

We believe that the secretory disturbance observed *in vivo* in β-HSL KO mice during tolerance tests and short (10 minute) static incubation *in vitro*, is mainly due to a loss of the first phase of insulin secretion. Recently, it was published by Lindvall et al. that HSL in β-cells is located in close vicinity of the insulin granules [147]. This localization may make it possible for the lipase to provide an important lipid-derived coupling factor, which is pivotal in the exocytotic process. When the action of HSL is absent, then first phase insulin secretion is lost.

General Discussion

The research presented in this thesis has increased our understanding of the importance and complexity of lipid metabolism in whole body and cellular metabolism, with special reference to β-cell physiology and insulin secretion.

In insulin resistant states, the pancreatic β-cells normally respond by hypersecretion of insulin in order to compensate for the elevated levels of glucose. This compensation has been proposed to be mediated by several different processes, such as enhanced β-cell function in response to increased glucose and lipid metabolism, increased sensitivity to incretins as well as increased parasympathetic activity in islets [107,176,177]. We have found that β-cells adapt to insulin resistance by a shift in substrate oxidation from glucose to other fuels, such as amino acids and FFAs (Paper I). In addition, this is accompanied by an adaptive increase in mitochondrial surface area. Indeed, morphological and structural changes of mitochondria have been observed in Type 2 diabetic patients [94], indicating an important role for mitochondrial adaptation to β-cell dysfunction. Furthermore, ablation of the mitochondrial protein Nnt from either mouse islets or MIN-6 cells perturbs insulin secretion severely, possibly due to defective ATP production [93]. In addition, hypersensitivity towards α-KIC was observed in a study using Brattleboro mice, further indicating the importance of mitochondrial fuels other than glucose when insulin resistance is present [178]. Moreover, it was recently shown in a study of Zucker diabetic fatty rats that increased lipolysis and FFA signaling also may be involved in the adaptation of β-cells to insulin resistance [179]. This agrees with our findings, because increased lipolysis will mobilize FFA from stored triglycerides to serve as possible lipid coupling signals and/or be β-oxidized.

Because lipolysis is known to be essential for adequate insulin secretion in pancreatic β-cells [154-156], HSL has become an interesting protein to study with regard to insulin secretion. Our group showed that HSL is expressed and active in the pancreatic β-cell [144]. The possible role for HSL in generating several different lipid coupling signals [69,115,148-150] makes it an interesting pharmacological target for therapeutic treatment in Type 2 diabetes.

Our results from the two different HSL KO lines produced in our lab (Paper II-IV) have indicated a role for HSL in overall glucose homeostasis. However, some discrepancies regarding the β-cell phenotype of these two different mouse lines are evident. The global HSL KO did not reveal any apparent β-cell phenotype, other than increased β-cell mass and insulin content. These characteristics are most likely adaptive mechanisms to maintain euglycemia, since peripheral tissues in the HSL KO mouse exhibited insulin resistance. However, in another global HSL KO line, created in the Mitchell laboratory, both peripheral insulin resistance and a secretory deficiency in islets are observed [161,162]. Since several lines of global HSL KO mice exist and different results have been obtained from these studies, a clear role for HSL in the context of a global KO has not been determined. However, it should be kept in mind that the inactivation of HSL in our HSL KO was introduced at the gastrula stage of

embryogenesis, giving compensatory mechanisms ample time to evolve. The other global HSL KO lines were created with similar inactivation mechanisms of HSL [158,160,161]. However, these lines were developed from different genetic backgrounds, which may in addition to compensatory mechanisms be a plausible explanation for the different results obtained from these studies.

In our β-HSL KO, the inactivation of the gene was introduced 11 days post conception, when Cre recombinase reportedly becomes active in the pancreatic β-cell [169], thereby possibly circumventing the issues of compensatory mechanisms. Indeed, in this line, a β-cell-specific phenotype was observed, comprising a blunted first phase of insulin secretion and glucose intolerance. This is corroborated by a pharmacological study in rat islets, where specific inhibition of HSL with the agent BAY impaired GLP-1- and carbacholine-stimulated insulin secretion [157]. However, HSL deserves to be more extensively studied in relation to β-cell function and insulin secretion. Nevertheless, our results here, imply that the ablation of HSL from either the whole mouse or specifically from the β-cells perturbs overall glucose homeostasis and the first phase of insulin secretion. With respect to the β-cells, we speculate that the localization of HSL in close proximity of the insulin granules [147] allows the lipase to provide the exocytotic machinery with one or more lipid-derived signals, a function which is perturbed in the β-HSL KO mice. The mechanism for impaired insulin sensitivity is less clear in the global HSL KO. With the exception of liver, no significant increases in tissue TGs were observed, ruling out the possibility of a lipotoxic effect; instead, it is more likely that the lack of a lipid-derived signal in the absence of HSL may play a role. In contrast, β-HSL KO mice were more insulin-sensitive than their WT littermates. This has probably arisen as a compensatory change, maintaining euglycemia. The phenomenon also highlights the fact that there may exist cross talk between β-cells and insulin-sensitive tissues, a very interesting but still unclear process.

Summary

We have examined β-cell adaptation to insulin resistance, where lipids have proved to play a crucial role, perhaps both as a cause of cellular toxicity and as a cue to the β-cell to adapt to insulin resistance by increasing mitochondrial metabolism.

HSL is responsible for the mobilization of TG in the adipocyte and has been shown to serve an important role in several other tissues. Since HSL has the capacity to generate a number of active lipid-derived molecules, the enzyme could be of the utmost importance for generating intracellular signaling molecules.

In the pancreatic β-cell insulin secretion is controlled in part by metabolic events. The nature of all these metabolic events is at this time not clear. Our initial studies on a specific role of HSL in insulin secretion did not yield entirely clear results. However, with our investigation of a β-HSL KO, we are approaching a fuller understanding of the role for HSL in insulin secretion. It is becoming more and more evident that HSL does play a significant role in insulin secretion. If we are successful in our further investigations about the role of this enzyme, we may uncover novel mechanisms for how insulin secretion is controlled. Likely, these mechanisms involve generation of

intracellular signals derived from lipid stores. Such advances may provide new pharmacological targets for treatment of Type 2 Diabetes, thus helping people worldwide in their struggle against a devastating disease.

Major conclusions

- ***β-cells exposed to increased lipid flux in insulin resistance respond by increasing mitochondrial mass. This expansion is associated with enhanced mitochondrial metabolism as means of β-cell compensation through hyperinsulinemia.***

- ***Actions of HSL, as revealed by its absence in the global HSL KO, appear to be critical to maintain insulin sensitivity in peripheral tissues. In islets, however, insulin secretion was unaffected by the loss of HSL, indicating compensatory mechanisms, perhaps of another lipase(s).***

- ***β-cell specific ablation of HSL revealed that actions of the enzyme ensures an adequate first phase of insulin secretion. HSL may serve to generate lipid-derived coupling signals essential for exocytosis.***

Populärvetenskaplig sammanfattning

Introduktion—Åldersdiabetes, typ 2 diabetes, är en folksjukdom, som omfattar en stor del av världens befolkning. Detta beror bland annat på kopplingen mellan denna sjukdom och övervikt. I västvärlden har en alltför hög vikt och de komplikationer som detta medför länge varit ett stort problem. Nu ökar fetma dramatiskt i alla delar av världen och man förutspår att år 2010 kommer typ 2 diabetes att ha ökat med ca 50 %. Detta kommer framförallt att ses i utvecklingsländer, som Afrika, Asien och Sydamerika. Sjukdomen leder till allvarliga problem med hjärt-kärl- och nervsystemet. Den genomsnittliga livslängden minskar med ~10 år. Livskvaliteten försämras också avsevärt då komplikationerna sätter in.

I bukspottskörteln finns de Langerhanska öarna. Dessa öar är kluster av olika celltyper där bland annat β-celler finns. I dessa β-celler produceras insulin som frisätts till blodet. Insulinets uppgift är att se till att socker från maten vi äter kommer cellerna i vår kropp till godo och lagras i muskulatur, fettväv och lever. Upptaget av socker i perifera vävnader gör också att blodsockret hålls inom en normal nivå. Fetma skapar en resistens mot insulinets verkan i lagringsvävnaderna, vilket kan leda till förhöjda blodsockervärden. Kroppen kan känna av förändringar i blodsockret och kompenserar då med en ökad frisättning av insulin från β-cellerna. Hos vissa människor klarar kroppen inte av att kompensera tillräckligt och då stiger blodsockernivån och typ 2 diabetes utvecklas.

Frisättningen av insulin regleras av den mat vi får i oss med vår dagliga kost, såsom socker, fetter och proteiner. Insulincellen verkar som en sensor och känner av nivåerna av dessa ämnen i blodet. Socker, fett och aminosyror bryts ned i β-cellen. Dessa processer ger upphov till molekylära signaler, som i sin tur kontrollerar frisättningen av insulin. Trots att vi idag känner till många av de mekanismer, som kontrollerar frisättningen av insulin, förstår vi fortfarande inte exakt hur det går till och vilka steg som är viktiga för att kontrollera det som sker.

Man fann tidigt att fett och övervikt är kopplade till typ 2 diabetes. Det är också känt att fetter, som lagras utanför fettväven (t.ex. muskel, lever och bukspottskörtelns Langerhanska öar) på något sätt bidrar till resistensen mot insulin. Märkligt nog har man också funnit att cirkulerande fetter och fettdepåer i andra vävnader än fettväven är viktiga för cellernas funktion. Det måste finnas en viss mängd fett att tillgå i β-cellen för att insulinutsöndringen ska fungera normalt. En paradox kan man tycka, att fetter i sig kan verka både positivt och negativt på cellers funktion.

Artikel I—Här har vi undersökt de molekylära mekanismer, som ligger bakom β-cellens kompensation vid insulinresistens. För att studera detta har vi använt möss, som under 12 veckors tid fått mat, som innehåller mycket fett. Dessa möss har sedan jämförts med möss, som fått kost med normal mängd fett. Vidare har vi undersökt de mekanismer, som är viktiga för hur fett lagras i β-celler och hur fettet påverkar cellernas förmåga att frisätta insulin. I mössen, som ätit mycket fett, genomgår β-cellerna en metabolisk förändring. Istället för att spjälka (metabolisera) socker, vilket β-cellerna normalt gör, metaboliseras fetter och aminosyror. Detta tror vi beror på att mycket fett i β-cellen stör

regleringen av viktiga proteiner, som transporterar socker in i cellen; troligen påverkar ökningen av fett också själva nedbrytningsprocessen av socker i β-cellerna. I samma undersökning fann vi att cellens förmåga att bryta ner socker, fetter och aminosyror är kopplad till förändringar i cellens mitokondrier – små cellorgan, som genererar energi åt celler. När mitokondrierna studerades i ett elektronmikroskop kunde man tydligt se att ytan på dessa små organeller var kraftigt förstorad i de djur, som fått en fettrik diet. Vi fann också att det fanns ett större fettlager i β-cellerna på de möss, som fått fet mat. Våra resultat visar således att en fettrik diet ökar inlagringen av fett i β-cellen och gör att denna kompenserar genom att frisätta mer insulin för att bibehålla blodsockernivån. För att uppnå detta sker en förändring i cellernas nedbrytningsprocesser i mitokondrierna.

Artikel II och III—Som tidigare nämnts är fettet viktigt för en normal insulinutsöndring. β-cellerna kan normalt lagra fett i form av så kallade triglycerider. Vi har tidigare visat att enzymet hormonkänsligt lipas (HKL), som i fettceller ansvarar för nedbrytning av triglycerider till fettsyror och glycerol, också är aktivt i β-celler. Det har också visats att HKL ligger nära de grynlika strukturer (granula) i β-cellen, som innehåller insulin. Detta skulle kunna tyda på att HKL har en viktig funktion i regleringen av utsöndringen av insulin. Eventuella rubbningar i HKLs funktion skulle då kunna leda till en ökad lagring av fetter i β-cellen. Detta skulle i sin tur kunna ge upphov till funktionsproblem i cellerna med diabetes som följd. Då skulle β-cellernas signalsystem inte fungera normalt. För att undersöka detta har vi skapat en mus, som saknar HKL i alla kroppens vävnader – en s.k. knock out (HKL KO). Denna mus beskrivs i artikel II och III.
I artikel II har vi funnit att förlusten av HKL i musens alla vävnader påverkar känsligheten för insulin i muskel, fettväv och lever, d.v.s. i de organ som kroppen använder för energiupplagring. När glukosupptaget undersöktes i muskel, lever och vid nybildning av fetter i fettvävnad, fann man förändringar i HKL KO musen, som tyder på att insulinkänsligheten är minskad i denna musmodell. Dessa förändringar följdes av en något förhöjd basal insulinutsöndring i den levande musen. Resultaten tyder på att HKL spelar en viktig roll i upprätthållandet av sockerbalansen i blodet. I artikel III undersöker vi hur β-cellerna reagerar på olika stimuli efter att de plockats ut ur djuret och undersöks i olika lösningar av glukos, fett och aminosyror. I dessa försök kunde vi inte visa några skillnader i insulinutsöndringen från de Langerhanska öarna mellan vår mus, som inte uttrycker HKL, jämfört med den från normala möss. Möjligen beror våra resultat på andra lipaser som kan ha en liknande funktion som HKL och som kompenserar för förlusten av HKL.

Artikel IV—Flera andra laboratorier i världen har gjort möss, som saknar HKL. Studier, som liknar våra, har också gjorts där. Märkligt nog har man fått väldigt olika resultat och egentligen har ingen tydlig roll för HKL i β-celler kunnat påvisas. Om man inaktiverar genen för HKL endast i β-celler, skulle man kunna få en bättre inblick i hur proteinet fungerar. I en mus, där ett protein är borttaget i alla kroppens celler, är det vanligt att ett annat protein kompenserar för det borttagna proteinet. För att bättre kunna undersöka HKLs funktion gjorde vi en vävnadsspecifik knock out, där HKL bara är avlägsnat

från β-cellerna. Den här musen beskrivs i artikel IV. I denna mus visade det sig att förlusten av HKL i β-cellerna hade stor betydelse för insulinutsöndringen. För att visa det injicerade vi glukos i musen och mätte därefter insulinnivåerna i blodet. Vi kunde också se förändringar i insulinsvaret i de Langerhanska öarna, när de tagits ut och stimulerats med olika lösningar av glukos, fett och aminosyror. Det verkar som om den första fasen av insulinutsöndringen påverkas mest. Vi tror därför att HKL faktiskt spelar en viktig roll i signalkedjan för insulinutsöndring i vår kropp.

Slutsats—Forskningen, som redovisas i denna avhandling, bidrar till förståelsen av hur lagringen av fetter påverkar insulinutsöndringen från β-cellerna. Den visar också hur denna process kan vara involverad i β-cellens kompensation vid insulinresistens och utvecklingen av typ 2 diabetes. Avhandlingen belyser även funktionen av HKL och fettsignalering i β-cellen, där HKL tycks vara en viktig faktor i reglering av insulinutsöndringen. Denna mekanism är hämmad i typ 2 diabetes. Det kan förhoppningsvis bli möjligt att dessa upptäckter kan initiera utvecklingen av nya läkemedel, som kan ge hjälp åt miljontals människor att leva på ett bättre sätt med sin typ 2 diabetes.

Acknowledgements

I would like to thank
My supervisors Hindrik Mulder and Cecilia Holm
Hindrik, thanks for your constant support and positive attitude. You have taught me many important things about science, and you have always been around to ask numerous questions at anytime. You have done a great job! Now it is time for me to try my own wings. Thank you Cecilia, for letting me join your group at times and for being my much needed HSL teacher.

The Hindrik Mulder group. You are wonderful! Without you it would not be half as interesting to go to work every day! Ulrika, my "new" room mate. You have come to be a true friend and a constant source of knowledge in the jungle of metabolic enzymes. Kalle, thank you for being a great male influence in a lab full of women, how come you blend in so well? Maria thanks for sharing stories about real life and for knowing the things I don't! Marloes, thank you for all your hard work on my manuscripts, for being a great friend and shared experiences about small children! Thomas, a former lab member, I really miss your wonderful jokes!

The Cecilia Holm group. Thank you Ann-Helen for teaching me everything I know about mice, and for being such a great friend. I really miss you at work! I believe we are all in mourning after your departure to Astra Zeneca.
Sara for expertise in all lab work and good times in the coffee room. Birgitta for never getting tired of my constant questions and for listening to my very bad sense of humor. Peter for being my favorite Danish man and for sharing my love for the moomins. Ola for being an excellent dive instructor (and scientist) and for introducing me to the love of my life. Cecilia Klint for early mornings and excellent discussions. Jörgen for always having something fun to say. Håkan for being a great room mate for some time and sharing my love for boy bands and really bad jokes. Christoffer for being just as crazy as me. Celine for being a nice continental influence in the lab, whose laughter is extremely contagious. Stephanie for interesting discussions about metabolism and *in vitro* experiments. Christian the new Danish guy for having a great sense of humor. Ulrika, for shared mice experiences and lessons about rye and GI.

The Eva Degerman group; Helena, Rebecca, Lena, Eva and Anki. Karin for being such a curious person and always spilling interesting gossip. Olga for returning! Linda, for a great time in Paris. Svante for being just himself. Especially thanks to Eva Degerman for being a great listener and a "real" doctor in times of need.

Maria Sörhede Winzell for discussing data and gossip with me.

Thanks Nils Wierup partner in snus and science, Doris Persson and Frank Sundler for excellent collaboration and valuable help with immunological staining and EM imaging.

Elisabeth, Åsa and Ingegerd. YOU are all wonderful and entertaining women! Thanks for all the great conversations in the coffee room!

Lars Thell, for excellent computer service.

All my non-lab friends, Helena, my komvux buddy. For letting me out of my boring lab/Lunda shell at times and being totally crazy. Katja for being such a great friend and setting me up with tickets to fun shows. Johanna my "cousin", for your everlasting friendship. Anette, my friend at The Swedish Embassy in DC. You believed in me when I did not even believe in myself. Thanks Manda for being a true friend and a great story of success. Thank you Anna for summer parties in Bollebygd.

Jenny K, Mia and Gesine, my fellow scientists and wonderful friends from the Brundin lab time. You always told me I could do this!! Thanks for fantastic friendship and inspiration!

My former study pals, Jenny and Petter. Thanks for all our fabulous parties and good times. May there be many more! Mattias thanks for lunches and friendship. Maria (Rödis) and Per, almost next door neighbors, thanks for spontaneous dinners and wine! The rest of the gang: Gustav, Emma and Per, Emelie, Malin and Kalle, Charlotte, Pär and Fredrik. You are all great.

The Ecology House group; Lina and Klas, Susanne, Marika, Christian, Janne and Mikael. I'm so happy that I did not choose the greener side, although I value your friendship enormously. Thanks for still inviting me to dissertation parties and beer nights!

All my co-authors on different articles.

Mamma, without you none of this would be possible. You have helped me in so many ways over the years. Åsa and Jenny, my beautiful sisters, thanks for always being there for me. Staffan and Joann for supporting me all the way from Las Vegas, USA.
Thanks to Hans and Tina Tornqvist for being interested in my progress and for being great friends to the whole family. The rest of my family, Christer, thank you for being a fantastic friend to me and a grandfather to all my children. Gun, Lotta, Christine and Fredde, thank you for great company and help with Johanna!

A special thanks to those who are not longer with us, Pappa and Eva. I am now the product of your constant speeches about the importance of an education; (even though I did not understand it until later).You helped making me the person I am today. I wish you could experience this with me.

Last but not least; Clara and Julia my beautiful daughters. You made me do this. Without you I would be nothing. Let this thesis inspire you in the future and know that you can do anything you want in life, even though it may seem impossible at times. Michael, no word could ever describe how happy I am to have met you. You have shown endless patience, support and love all the way. Johanna, my baby, now my thesis is finished and I will read a new bed time story every evening until you fall asleep.

Financial support for all studies were received by grants from The Krapperup foundation (to M. Fex), The Swedish research council (14196-03A:H.M.), The Albert Påhlsson, Åke Wiberg, Ingrid and Fredrik

Thuring foundation, The Swedish Diabetes Association, The Swedish Physiographic Society and the Faculty of Medicine, Lund University.

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