Beta-cell function and mass in type 2 diabetes

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ABSTRACT

The aim of the work described here was to improve our understanding of beta-cell function (BCF) and beta-cell mass (BCM) and their relationship in vivo using the minipig as a model for some of the aspects of human type 2 diabetes (T2DM).

More specifically, the aim was to evaluate the following questions:
How is BCF, especially high frequency pulsatile insulin secretion, affected by a primary reduction in BCM or by primary obesity or a combination of the two in the minipig?

Can evaluation of BCF in vivo be used as a surrogate measure to predict BCM in minipigs over a range of BCM and body weight?

We first developed a minipig model of reduced BCM and mild diabetes using administration of a combination of streptozotocin (STZ) and nicotinamide (NIA) as a tool to study effects of a primary reduction of BCM on BCF. The model was characterized using a mixed-meal oral glucose tolerance test and intravenous stimulation with glucose and arginine as well as by histology of the pancreas after euthanasia. It was shown that stable, moderate diabetes can be induced and that the model is characterized by fasting and postprandial hyperglycemia, reduced insulin secretion and reduced BCM.

Several defects in insulin secretion are well documented in human T2DM; however, the role in the pathogenesis and the possible clinical relevance of high frequency (rapid) pulsatile insulin secretion is still debated. We therefore investigated this phenomenon in normal minipigs and found easily detectable pulses in peripheral vein plasma samples that were shown to be correlated with pulses found in portal vein plasma. Furthermore, the rapid kinetics of insulin in the minipig strongly facilitates pulse detection. These characteristics make the minipig particularly suitable for studying the occurrence of disturbed pulsatility in relation to T2DM.

Disturbances of rapid pulsatile insulin secretion have been reported to be a very early event in the development of T2DM and include disorderliness of pulses and reduced ability to entrain pulses with glucose. However, the role of reduced BCM and/or obesity in the development of these defects in humans is unknown. Therefore, the investigations were extended to include lean NIA/STZ minipigs where it was shown that a primary reduction of BCM leads to reduced insulin pulse mass but does not change periodicity of the pulses or the ability of glucose to entrain pulses. In contrast, obesity was found to be associated with reduced pulsatile insulin secretion and improved orderliness of glucose entrained pulses in the minipig. Furthermore obesity was associated with pancreatic lipid accumulation and increased beta-cell volume, although BCM relative to body weight was not changed. Finally, a combination of obesity and

reduced BCM resulted in severely disturbed insulin secretion and severe morphological changes. Thus, results from NIA/STZ minipigs suggest that not all of the defects of rapid pulsatile insulin secretion seen in human T2DM can be explained by a primary reduction of BCM mass or up to 2 weeks of mild hyperglycemia. Furthermore, based on the results from obese minipigs, obesity in itself induces small defects in rapid pulsatile insulin secretion and the combination of obesity and reduced BCM leads to further deterioration of BCF.

Another major characteristic of human diabetes is thought to be reduction of BCM and the ability to follow this parameter over time would greatly improve our understanding of disease progression and allow evaluation of pharmacological methods to increase BCM.

BCM cannot, at present, be measured in vivo in humans. We therefore set out to further validate data from smaller studies in lean non-human primates and minipigs showing a correlation between measures of BCF in vivo and BCM. In a large study in lean minipigs with a range of BCM, we found that a strong stimulation of insulin secretion with a combination of glucose and arginine resulted in the best correlation to BCM, as determined using stereology. A similar relationship was also shown in a group of both lean and obese animals, thereby supporting the application of similar methods to estimate BCM in humans over a range of body weights. Since changes in rapid pulsatile insulin secretion are detectable early in the development of diabetes and in obesity, we hypothesized that this parameter could also be highly correlated to BCM as it has been shown in smaller studies in lean minipigs. However, rapid pulsatile insulin secretion did not show a better correlation to BCM than combined stimulation with glucose and arginine, and thus analysis of pulses does not provide a better surrogate marker for BCM in the minipig.

To evaluate the weaker correlation of glucose stimulation compared to combined glucose and arginine stimulation in vivo with BCM, we further investigated BCF in lean, beta-cell reduced minipigs by studying BCF in vitro after isolation and perfusion of their pancreases to investigate the ability of the remaining beta-cells to compensate for the loss of BCM by increasing insulin secretion per BCM. The perfused pancreas was chosen in order to allow direct measurement of the insulin secretion without the effects of peripheral tissues. During the perfusion, it was shown that the remaining beta-cells were indeed able to compensate for the loss of BCM to a large extent in response to stimulation with glucose and glucagon-like peptide-1 but not in response to arginine. This shows that the type of stimulus applied is important for the ability to compensate for reduced BCM from the remaining population of beta-cells, and further supports the use of combined stimulation with glucose and arginine for estimation of BCM in vivo.

In conclusion, an animal model of reduced BCM and mild diabetes has been developed and characterized. The model has been used to evaluate effects of a primary reduction of BCM, showing a reduced rapid insulin pulse mass but normal periodicity and entrainability of the pulses, whereas obesity was associated with reduced rapid pulsatile insulin secretion. Thus, based on these data, the disturbed rapid pulsatile insulin secretion seen in T2DM humans may not directly be explained by the reduced BCM in diabetes, whereas obesity may be related to the reduced pulsatility. Furthermore, the model has been used to establish a correlation between extensive stimulation of insulin secretion in vivo and BCM obtained by stereology in both lean and obese animals. The ability to estimate BCM based on in vivo experiments in the minipig would allow longitudinal studies on changes in this parameter over time in the intact animal and support application of similar methods in humans. Such methods could be useful for the diagnosis and the measurement of the effectiveness of treatment of diabetes in humans in the future.

1. INTRODUCTION

Diabetes mellitus is a metabolic disorder characterized by chronic hyperglycemia with disturbances of carbohydrate, fat and protein

metabolism. The two most common forms of the disease are type 1 diabetes (T1DM) (which accounts for around 5-10% of cases) and type 2 diabetes (T2DM) (around 90% of cases) (ADA, 2007). T1DM is caused by autoimmune destruction of beta-cells, and insulin therapy is life-saving in this group of patients (ADA, 2007). The etiology of T2DM is multi-factorial, but defects in insulin secretion and action as well as a reduction of beta-cell mass (BCM) (ADA, 2007) are important factors in its development and the disease does not occur as long as there is an adequate population of beta-cells that produce sufficient amounts of insulin to maintain euglycemia.

Diabetes mellitus represents a major public health problem, which is expected to increase considerably over the coming decades (IDF, 2000). The global prevalence of the disease was reported to be approximately 150 million or 3.8% of the adult population around year 2000 and is expected to increase to around 300 million or 5.1% of the adult population by year 2025, and this is most likely an underestimate (Green et al., 2003). Furthermore, obesity is an important risk factor for T2DM (Mokdad et al. 2003) further adding to the increase in T2DM.

Sequelae of diabetes mellitus are severe, and include long term damage, dysfunction and failure of various organs, including retinopathy, nephropathy and neuropathy as well as increased mortality risk, especially due to cardiovascular disease (WHO, 1999, Casiglia et al., 2000). Due to the complications and increased mortality rate, diabetes is a debilitating condition for the patients, as well as a considerable economic cost for society. In the face of the rapidly growing prevalence of this major health problem, the study of T2DM and the search for efficient and safe strategies to prevent or treat the disease is of major importance, both from an ethical and an economical point of view.

BCM is thought to be reduced in T2DM (Maclean and Ogilvie, 1955, Westermark and Wilander, 1978, Saito et al., 1979, Klöppel et al., 1985, Clark et al., 1988, Sakuraba et al., 2002, Butler et al., 2003, Yoon et al., 2003, Deng, 2004), but since no techniques are currently available for evaluation of BCM in humans in vivo, the present knowledge about BCM in humans is mainly based on autopsy studies. Both beta-cell function (BCF) and insulin action can be evaluated in detail in humans in vivo, and since BCF and insulin sensitivity are closely related, both parameters are important in the development of T2DM (Bergman et al., 2002, Kahn, 2003, Tripathy et al., 2003). Thus, a progressive decline in both BCF and insulin action is characteristically seen when a person moves from normal glucose tolerance (NGT) through impaired fasting glucose and impaired glucose tolerance (IGT) to T2DM (Weyer et al., 1999, Tripathy et al., 2000, Chiasson and Rabasa-Lhoret, 2004, Weir and Bonner-Weir, 2004).

Given that reduced BCM and BCF are important factors in the pathogenesis of T2DM, increased knowledge about their relative contributions to this process could improve approaches to diagnosis, treatment and prevention of the disease. In this respect, animal models are particularly useful since specific metabolic changes can be induced and invasive techniques not applicable in humans can be used to investigate specific aspects of the disease. In principle, all animals that exhibit hyperglycemia and glucose intolerance due to insulin resistance and/or pancreatic beta-cell malfunction can be considered appropriate models for T2DM, but it should be kept in mind that not all aspects of the human disease are likely to be present in any one single animal model. Different syndromes that resemble human physiology and pathophysiology in various aspects which are useful for the study of human diabetes, occur spontaneously or can be induced in various species of animals.

2. DEVELOPMENT OF A MODEL OF MILD DIABETES IN THE GÖTTINGEN MINIPIG
2.1 BACKGROUND
Animal models are useful tools for investigation of the pathogenesis of human diabetes and for the development of new treatments for the disease. The use of animal models must be limited to specific and well defined characteristics that should be predictive of the disease in humans. Furthermore, the choice of animal model must in each case depend upon the aspect of the human disease in focus. The usefulness of such studies requires well-characterized animal models, where both the similarities and the differences to the disease in humans which are displayed in each model are known. A range of well characterized and widely used models based on rodents is available; for a recent review see (Anon., 2007). However, several of the widely used rodent models are based on monogenous defects and/or inbred strains of animals, thereby failing to reflect the more heterogenous nature of human T2DM. Furthermore, some of these models rely on defects in the leptin system, which does not seem to be a major cause of T2DM in humans. Due to the small size, several of the protocols relevant in humans may not be applicable in rodent models. Therefore, large animal models of diabetes are a valuable complement to rodent models in many ways, both for practical and physiological reasons.

2.2 CHOICE OF LARGE ANIMAL MODEL
Dogs, non-human primates and pigs are all of relevance as models in diabetes research. Non-human primates develop diabetes spontaneously, see (Hansen and Tigno, 2007), but the availability of these animals is very limited, so it can be difficult to obtain research naïve animals for studies, and invasive studies, such as the procurement of the pancreatic tissue, in such animals are rarely feasible. Furthermore, the behavioral needs of non-human primates are rather complex, so it is a challenge to house them under conditions that both allow experiments to be performed and allow natural behavior of the animals to ensure their welfare. Dogs also develop diabetes spontaneously, but the disease does not resemble T2DM (Catchpole et al., 2005) and no diabetic dog colonies are, to the author's knowledge, available for research. The pig offers several advantages in relation to diabetes research and is readily available, allowing age-matched and research naïve animals to be used in each study. The behavioral needs of the pig are also such that they can, to a large extent, be incorporated in the housing of the animals, even during experimental conditions, and the pig can easily be trained to allow performance of experiments in conscious, un-stressed animals, which is somewhat more challenging in non-human primates. The relatively small size of the Göttingen minipig makes this strain particularly suitable for experiments of longer duration. Of special interest in diabetes research are the similarities between the pig and humans in terms of nutrition requirements and the gastrointestinal tract, plasma lipids, pancreas development and morphology, cardiovascular system, skin and subcutaneous tissues, as well as metabolism and glucose tolerance, see (Larsen and Rolin, 2004 and 2007a) for more details.

For the purpose of the investigations in the present thesis, it can actually be seen as an advantage to use a model system where specific changes, such as reduced BCM or obesity, are induced at a known time with a well defined method, since this allows for evaluation of effects of that specific change on other aspects of physiology related to diabetes. In other settings, the use of a model of spontaneously developing diabetes could be a better choice, for instance when studying measures to prevent or delay development of the disease, but this would still require the choice of a model that mimics the aspects of T2DM to be investigated.

2.3 NORMAL GLUCOSE TOLERANCE AND INSULIN SECRETION IN THE GÖTTINGEN MINIPIG
After oral glucose (2 g/kg), the level of plasma glucose (up to 5-6 mM) in Göttingen minipigs (I, V) is quite low compared to that seen in humans after 1g/kg glucose (up to around 10 mM), but in both species, there is around a doubling of plasma glucose after the challenge. The insulin response to an oral glucose load rises to a maximum concentration of around 300-600 pM in the minipig (III,

V), which is about the same as is usually seen in humans (Perley and Kipnis, 1967, Ferrannini et al., 1985, Kelley et al., 1988, Tillil et al., 1988, Kruszynska et al., 1993). The insulin response to intravenous infusion of glucose to give plasma glucose concentrations around 20 and 30 mM is around the same or slightly lower in the minipig (plasma insulin around 300-750 pM) (I) than the response usually seen in humans (plasma insulin around 500-1500 pM) (Ward et al., 1984, Mikines et al., 1989, Van Haeften et al., 1990). Arginine can greatly potentiate insulin responses during hyperglycemia in both minipigs and humans, and although the absolute response after arginine at high glucose is lower in the minipig (plasma insulin around 1000 at 30 mM glucose) (I) than in humans (plasma insulin around 2000-3000 pM at 20-30 mM glucose) (Ward et al., 1984, Van Haeften et al., 1990), it does not level off during 10-30 minutes of intensive stimulation with glucose and arginine in either species (Van Haeften et al., 1990, I, MO Larsen, unpublished observations). However, pigs have been reported to clear an intravenous glucose load more quickly than humans (see Larsen and Rolin, 2004 for details).

Based on the lower fasting glucose and the lower glucose excursion after an oral glucose challenge in the pig compared to humans, the use of the human diagnostic criteria (WHO, 1999, ADA, 2007) for the diagnosis of diabetes in pigs would reflect a relatively more severe condition compared to humans. In our laboratory, we have therefore defined diabetes in pigs less strictly and classify abnormal glucose tolerance/ diabetes as significant fasting and glucose challenged (oral glucose load) hyperglycemia. For practical purposes, the classification of abnormal glucose tolerance is done by evaluating each group of animals during oral glucose challenge before and after reduction of BCM.

2.4 CHARACTERISTICS OF THE MODEL OF MILD DIABETES IN GÖTTINGEN MINIPIGS

Diabetes has not been shown to develop spontaneously in pigs, but whether it would do so with age after ad libitum feeding remains to be determined. For experimental purposes at present, therefore, diabetes must be induced in the pig.

Insulin requiring diabetes with almost no residual BCM can be readily induced with either streptozotocin (STZ) or alloxan (ALX) (see Larsen and Rolin, 2004 and 2007a for details), but to be able to study BCF after a primary reduction of BCM, a model of mild insulin deficient diabetes was developed using administration of a combination of nicotinamide (NIA) and STZ. The reason for using NIA was that our initial investigations in the minipig showed that the dose-response relationship for STZ in relation to hyperglycemia in the pig is very steep (I), and that NIA has been reported to provide partial protection of BCM in rodents (Masiello et al., 1998). To avoid any influence of estrus cycling, male animals were chosen for the model development. A model of mild diabetes has also been developed by the use of low dose ALX in the Göttingen minipig (Kjems et al., 2001).

Based on dose response experiments for both STZ and NIA (I), the combination of 67 mg/kg NIA and 125 mg/kg STZ was chosen as the standard dose for induction of mild insulin deficient, but not insulin requiring diabetes. This NIA/STZ model is characterized by mild fasting and postprandial hyperglycemia, reduced glucose- and arginine-stimulated insulin secretion and reduced BCM (I). Furthermore, the model is stable for at least 2 months (I), with unpublished data showing stability of both hyperglycemia and reduced insulin secretion and BCM for up to 4 months. Even though age, gender and nutritional status, which are all relevant for the response to STZ (see Larsen and Rolin, 2004 for details) were kept standardized in our experimental settings, the hyperglycemic response after NIA/STZ is variable between animals as is also normal glucose tolerance in this animal model. One main reason for this variability is the fact that the Göttingen minipig is an out bred strain, and because of the variability, it may be necessary to administer NIA/STZ to a greater number of animals in order to obtain a group of uniformly diabetic animals. Therefore, in our laboratory, we usually dose NIA/STZ in 20-50% more animals than are needed in the final experiment with diabetic animals, aiming at a success rate of 50-80% for induction of mild diabetes (with increases in both fasting and postprandial glycemia in individual animals). Furthermore, it is an advantage to characterize each animal both before and after administration of NIA/STZ to evaluate individual changes in glucose tolerance and insulin secretion. NIA in combination with STZ has been known to induce insulin producing tumors in the rat (Rakieten et al., 1971), but in all NIA/STZ minipigs evaluated to date in our laboratory (more than 150 in total), no such tumors have been observed.

The chemical reduction of BCM used in the model may not accurately mimic the reduction of BCM seen in human T2DM since the reduction of BCM in humans is thought to be a more gradual process with slower progression. However, the chemical reduction of BCM may better reflect the situation in humans than models using surgical reduction of BCM since partial pancreatectomy results in a residual population of healthy beta-cells and a reduction in the mass of other types of pancreatic endocrine as well as exocrine cells.

The mildly diabetic minipig has been used in our laboratories to evaluate several antihyperglycemic agents. Examples include the glucagon-like peptide-1 (GLP-1) analogue liraglutide (Ribel et al., 2002) and the dipeptidyl peptidase inhibitor valine pyrrolidide (Larsen et al., 2003b), that both showed effects in the model comparable with what has later on been demonstrated in humans with similar types of compounds (see von Geldern and Trevillyan, 2006 and Vilsboll, 2007 for details). Furthermore, metformin has been reported to reduce plasma glucose and increase glucose disposal in a more severe STZ diabetic pig model, although the potency of the compound seems to be lower than in humans (Koopmans et al., 2006).

2.5 CONCLUSIONS

The NIA/STZ minipig model displays two of the major characteristics of human T2DM, namely, reduced BCM (Maclean and Ogilvie, 1955, Westermark and Wilander, 1978, Saito et al., 1979, Klöppel et al., 1985, Clark et al., 1988, Sakuraba et al., 2002, Butler et al., 2003, Yoon et al., 2003, Deng, 2004) and reduced insulin secretion (Polonsky et al., 1988, Clark et al., 2001), and has been shown to be stable over several months. Another important feature of human T2DM is insulin resistance (Kolterman et al., 1981, DeFronzo et al., 1982) and although insulin resistance has been reported in severely diabetic pigs (Otis et al., 2003, Koopmans et al., 2006) and dogs (Reaven et al., 1977, Bevilacqua et al., 1985), this is not a characteristic in chemically induced models with mild diabetes (Reaven et al., 1977, Goodner et al., 1989, Larsen unpublished observations). The lack of insulin resistance is an obvious drawback of the NIA/STZ model in relation to some aspects of human T2DM. Therefore, we have evaluated the effects of obesity alone or in combination with reduced BCM. Results from these studies are discussed in detail in section 3.3. This type of model could further allow for study of the co-morbidities in relation to the combination of obesity and diabetes. Alternatively, for studies on insulin resistance, it seems that induction of more severe diabetes could provide an appropriate model.

With its current characteristics, the NIA/STZ model is relevant for studying acute and longer term effects of a primary reduction of BCM and mild hyperglycemia, and to study effects of pharmacological treatment aiming at correcting glycemia or improving BCF or BCM. The model has proven useful in relation to testing of anti hyperglycemic agents in our laboratory as a supplement to studies performed in rodents. For studies in relation to BCM, although it could be speculated that beta-cells would not be able to regenerate after STZ, this has been shown not to be the case, in rats, at least (Finegood et al., 1999).

3. BETA-CELL FUNCTION IN TYPE 2 DIABETES – WITH FOCUS ON HIGH FREQUENCY PULSATILE INSULIN SECRETION

3.1 BETA-CELL FUNCTION IN TYPE 2 DIABETES

It is well established that several defects in BCF are important contributing factors in human diabetes, and data from the United Kingdom Prospective Diabetes Study have suggested that reduced BCF is a progressive phenomenon that is present for a period before the onset of hyperglycemia in T2DM, (Holman, 1998). Insulin resistance is another major characteristic of human T2DM, and it could be speculated that long-term insulin resistance could lead to a secondary beta-cell failure. However, since the majority of obese (and thereby insulin resistant) subjects are not diabetic (Mokdad et al., 2003), a beta-cell defect seems to be involved in the development of the disease.

Recent data have shown several T2DM risk loci involved in pancreatic development and insulin secretion in a cohort of T2DM subjects, thereby potentially highlighting the importance of impaired adaptation of BCF to increased metabolic demands in the pathogenesis of T2DM (Sladek et al., 2007).

The gradual decrease in BCF seen in T2DM (Holman, 1998) is characterized by several defects including decreased insulin secretory capacity (Polonsky et al., 1988, Clark et al., 2001) and first phase insulin secretion (Brunzell et al., 1976), as well as an impairment of both ultradian (Polonsky et al., 1988, O'Meara et al., 1993b) and high frequency (rapid) pulsatile insulin secretion (Lang et al., 1981, Schmitz et al., 2001) and defective glucose sensing (Mao et al., 1999, Hollingdal et al., 2000). The mechanisms behind the reduced BCF in T2DM are still unclear, but gluco-, and lipo-toxicity are thought to have major roles. The possible causes for all defects in BCF related to T2DM are beyond the scope of this thesis, but more information can be found elsewhere (Bell and Polonsky, 2001, Ahren, 2005, Stumvoll et al., 2005, Robertson and Harmon, 2006). The main focus of the present chapter is on rapid pulsatile insulin secretion, since defects in this aspect of BCF are seen even in the pre-diabetic state. However, the clinical relevance of the pulses is still not certain (see below) and their role in the development of T2DM is unclear.

3.2 HIGH FREQUENCY PULSATILE INSULIN SECRETION

Rapid pulsatile insulin secretion was first reported in non-human primates (Goodner et al., 1977). Since then, several investigators have reported the same type of pulses in humans, but the pulse frequency reported has been variable. Several parameters such as sampling site, sampling frequency and sensitivity of the insulin assay used may affect the estimated pulse frequency. Thus, portal vein sampling has shown pulse intervals of around 4-5 minutes (Song et al., 2000, Porksen et al., 2002), and pulse interval determined in studies analyzing peripheral plasma sampled every minute with high sensitivity assays is also reported to be around 5 min (Porksen et al., 1997, Juhl et al., 2002, Meier et al., 2005). In contrast, studies using 2 minutes between samples from peripheral plasma and/or less sensitive insulin assays show a tendency for fewer pulses detected and pulse intervals around 10-12 min (Lang et al., 1979, Hansen et al., 1982, Peiris et al., 1992, O'Meara et al., 1993a). A study in dogs, specifically addressing the effect of sampling frequency, concluded that minutely sampling resulted in shorter pulse intervals and higher pulse frequency than 2 minutely sampling (Porksen et al., 1995), but whether the same can be shown in humans remains to be determined.

In normal physiology, around 70-75% of total insulin secretion in the fasting state is attributable to rapid pulsatile secretion in humans (Porksen et al., 1997, Porksen et al., 2002). The remaining insulin secretion derives from a continuous basal secretion or from less coordinated insulin bursts from individual beta-cells or islets. Rapid pulsatile insulin secretion can also be used to evaluate beta-cell sensitivity to glucose by entraining pulses in non-diabetic humans (Porksen et al., 2000). In brief, this technique involves short (usually 1 min) intravenous infusions of glucose every 10 minutes to induce pulsatile insulin secretion, thereby testing the ability of the beta-cells to translate a glucose stimulus into insulin secretion. In T2DM patients, glucose entrainment is reduced, showing an insensitivity of the beta-cells to glucose or an inability to translate the glucose signal into insulin secretion (Mao et al., 1999, Hollingdal et al., 2000).

3.2.1 High frequency pulsatile insulin secretion in type 2 diabetes and obesity

In T2DM patients, rapid insulin pulses are more irregular than in normal subjects (Lang et al., 1981, Schmitz et al., 2001). Abnormal pulsatile insulin secretion can be detected early in the development of both T1DM and T2DM since mildly IGT relatives of T2DM patients and islet cell antibody-positive relatives of T1DM patients show disturbed regularity of rapid insulin pulsatility (O'Rahilly et al., 1988, Bingley et al., 1992). Even in insulin resistant, but NGT, first degree relatives of T2DM patients, have rapid pulses been reported to be more irregular than in control subjects with no family history of T2DM, although their frequency and amplitude were still normal (Schmitz et al., 1997, Nyholm et al., 2000). As a further indication of the relationship between changes in rapid pulsatile insulin secretion and the development of abnormal glucose tolerance, obese subjects show an increased absolute, but reduced relative amplitude of rapid pulses, and an increased non-pulsatile (baseline) insulin secretion although no changes in periodicity have been reported compared to normal subjects (Hansen et al., 1982, Sonnenberg et al., 1994).

From the observations in humans, it is possible that a disturbed rapid pulsatile insulin secretion is a very early event in the development of diabetes. However, the exact mechanisms involved in the disturbed pulsatility as well as the consequences of such a disturbance can be difficult to reveal by studying the human disease, since it is not possible to accurately ascertain the precise stage of the development of the disease in man.

3.2.2 Clinical relevance of high frequency pulsatile insulin secretion

The clinical relevance of the rapid insulin pulses has been the subject of much debate, with several studies providing very conflicting data on this issue.

Pulsatile secretion of insulin has been reported to be more potent than continuous insulin delivery in suppressing hepatic glucose output from rat liver in vitro (Komjati et al., 1986) and in NGT (Paolisso et al., 1991) and T1DM humans (Bratusch Marrain et al., 1986), and to have a greater hypoglycemic effect/ increase glucose uptake in NGT (Matthews et al., 1983, Schmitz et al., 1986), T1DM (Paolisso et al., 1987) and T2DM humans (Paolisso et al., 1988a). Furthermore, pulse interval is correlated to peripheral insulin sensitivity (Peiris et al., 1992, Hunter et al., 1996), while pulsatile insulin delivery enhances insulin sensitivity (Ward et al., 1990) and has been reported to be important for modulation of pancreatic islet function (Paolisso et al., 1988b). In support of the effects found in shorter studies, long-term (2 weeks) pulsatile insulin replacement therapy in diabetic rats resulted in better glycemic control and small improvements in peripheral insulin sensitivity compared to continuous insulin replacement (Koopmans et al., 1996).

On the other hand, several studies have reported no difference in the effect of pulsatile or continuous insulin delivery on glucose uptake in dogs (Grubert et al., 2005) or humans (Verdin et al., 1984, Paolisso et al., 1986, Kerner et al., 1988), or on suppression of endogenous glucose production in humans (Paolisso et al., 1986). Furthermore, long term (4 weeks) pulsatile insulin replacement in diabetic non-human primates did not result in better glycemic control (Weigle et al., 1991).

Thus, the clinical relevance of rapid insulin pulses is still uncertain, but it has been suggested that it prevents insulin receptor down-regu-

lation (Matthews, 1991) and superior effects of pulsatile compared to continuous insulin delivery are often first seen after hours of infusion, further supporting this interpretation. However, from a physiological point of view, it is difficult to understand why such down-regulation should happen at normoinsulinemia, and if down-regulation occurs, it is clearly a problem that can be overcome in relation to subcutaneous delivery of insulin in the treatment of diabetes.

One main conclusion seems to be that it is more probable that rapid pulsatile insulin secretion is more important in relation to the liver compared to peripheral tissues, since insulin pulses in portal blood are much larger than those seen in the periphery, due to the hepatic extraction of insulin (Meier et al., 2005). Furthermore, the effects seem to be very dependent on basal glucagon-stimulated hepatic glucose output, since a study using somatostatin to suppress endogenous pancreatic secretion without replacement of glucagon failed to show any differences between effects of pulsatile and continuous insulin delivery on endogenous glucose production (Kerner et al., 1988). Furthermore, studies using high exogenous doses of insulin (that will suppress hepatic glucose out-put) also do not show any superior effect of pulsatile insulin (Verdin et al., 1984, Grubert et al., 2005). On the other hand, if glucagon is replaced above basal levels, pulsatile insulin infusion at physiological levels is not able to inhibit the stimulated hepatic glucose production (Paolisso et al., 1987).

In general, there is a lack of dose-response evaluation in many of the studies on effects of pulsatile versus continuous insulin delivery and the interaction with exogenous glucagon. In my opinion, rapid insulin pulses most probably exist as a biological phenomenon in humans, and to some extent they reflect BCF, although their clinical relevance is still uncertain. If they are clinically relevant, any change in insulin secretion or BCF that decreases this pulsatile insulin secretion could potentially result in reduced insulin action and thereby contribute to the development of hyperglycemia in T2DM.

3.3 INVESTIGATIONS INTO THE EFFECT OF REDUCED BETA-CELL MASS AND/OR OBESITY ON HIGH FREQUENCY PULSATILE INSULIN SECRETION USING THE MINIPIG MODEL

One main reason that it can be difficult to draw conclusions about the role of pulsatile insulin secretion in the development of diabetes based on studies in humans is that when, for instance, obese subjects are studied and show a reduced pulse amplitude (Hansen et al., 1982) or a correlation between insulin sensitivity and insulin pulse interval (Peiris et al., 1992), it is not possible to conclude whether these defects in pulsatility were the cause or simply a consequence of obesity. Similarly, when relatives of diabetic patients show disturbed regularity of rapid insulin pulsatility (O'Rahilly et al., 1988, Bingley et al., 1992), does that show that disease is progressing because of pulsatile defects, or that pulsatile defects are a very early symptom of the progress of the disease?

This problem could be circumvented by longitudinal studies in humans, or by using animal models where specific manipulations, not applicable in humans, can be done.

Therefore, one aim of the studies in the present thesis was to evaluate whether specific manipulations in the Göttingen minipig, such as reduction of BCM and/or induction of obesity, would affect rapid pulsatile insulin secretion.

3.3.1 High frequency pulsatile insulin secretion in the normal Göttingen minipig

Pulsatile insulin secretion was first characterized in the normal Göttingen minipig, in order to evaluate the model system. Pulses in portal and peripheral vein plasma were found to be highly correlated, thus indicating that the pulses seen in peripheral plasma are truly due to pulsatile secretion of insulin from the pancreas and not generated by oscillating hepatic or peripheral extraction or other peripheral effects (II). In humans, the liver preferentially extracts insulin delivered as pulses (Meier et al., 2005), so if anything, the effect

of the hepatic extraction of insulin would be expected to dampen the pulses seen in peripheral plasma, not increase them. Furthermore, in the minipig, the pulses seen in peripheral plasma with a frequency of around 6-10 minutes were easily detectable on the basis of plasma insulin profiles. Insulin had very rapid kinetics, resulting in minimal influence from re-circulating insulin, so that mathematical modeling was not required to detect pulses (II). This observation strongly supports the concept of rapid pulsatile insulin secretion in vivo showing that the pulses are a biological phenomenon and not a consequence of mathematical modeling in the minipig. Moreover, if glucose entrainment was used, the pulsatile pattern was even more marked, as has also been reported in humans (Porksen et al., 2000), demonstrating a high sensitivity of the beta-cells to glucose, even at lower glucose infusion rates than are usually applied in humans (4 vs. 6 mg/kg/min) (II) . In humans, the kinetics of insulin or C-peptide to be used for mathematical modeling are usually estimated based on injections of exogenous insulin or C-peptide in individual subjects (Polonsky et al., 1988, Porksen et al., 1997) or values from the literature are used. However, due to the rapid kinetics of insulin in the normal minipig, it is possible to estimate kinetics of insulin directly from plasma profiles of endogenous insulin. This characteristic of the pig could enable detection of changes in insulin kinetics due to metabolic changes even when endogenous insulin profiles are evaluated (II), whereas detection of such changes in humans based on endogenous profiles would not be possible. These characteristics make the Göttingen minipig a model of particular interest in the area of dynamics of insulin secretion due to the ease of insulin pulse detection even without the use of mathematical modeling.

Based on these studies in normal animals, the minipig was used as a model to study effects on rapid pulsatile insulin secretion after a primary reduction of BCM in the NIA/STZ model and/or induction of obesity.

3.3.2 Effects of a primary reduction of beta-cell mass on high frequency insulin pulses

Reduced BCM and defects in rapid pulsatile insulin secretion are both characteristic of T2DM in humans, but from the data available in humans, it is not possible to conclude whether the disturbed pulsatile insulin secretion is a consequence of reduced BCM or whether the two are just co-existing characteristics of the disease. It is difficult to imagine that disturbed pulsatile insulin secretion would be the cause of reduced BCM, and this aspect cannot be readily investigated even in an animal model.

We evaluated whether a primary reduction of BCM in the NIA/STZ minipig model and the ensuing moderate hyperglycemia would cause secondary defects in rapid pulsatile insulin secretion that mimic the defects seen in T2DM (IV), both under basal conditions and during glucose entrainment to also evaluate beta-cell sensitivity to glucose. In this model system, we found that a primary reduction of BCM induced hyperglycemia and impaired insulin secretion in response to a mixed meal as previously described. A clear rapid pulsatile pattern of insulin secretion was seen in NIA/STZ animals, with a reduced mean peak concentration and a reduced pulse mass, but with normal frequency and maintained relative pulsatile secretion. Beta-cells in NIA/STZ animals also showed normal responsiveness during glucose entrainment. Furthermore, there was a correlation between pulse mass and BCM, indicating a normal insulin secretion relative to BCM from the remaining beta-cells. Thus, a marked defect in BCF was present after reduction of BCM, but the changes in rapid pulsatile insulin did not reflect the irregular pulse pattern and lack of glucose entrainment reported in T2DM (Lang et al., 1981, Mao et al., 1999, Hollingdal et al., 2000, Schmitz et al., 2001), thereby indicating that these defects are probably not directly caused by the reduced BCM in diabetes. Furthermore, since 2 weeks of mild hyperglycemia did not result in disorderly pulsatility or reduced entrainability, this study does not support relatively short term glucotoxicity as a primary cause of disturbed pulsatility in

T2DM, but whether more pronounced or longer term hyperglycemia could affect pulses remains to be shown.

The results from our study in NIA/STZ minipigs are in accordance with previously published results in STZ diabetic non-human primates (Goodner et al., 1989) and ALX diabetic pigs (Kjems et al., 2001), whereas in partially pancreatectomized dogs, there is decreased pulse amplitude, reduced frequency and increased duration of individual pulses (Earnhardt et al., 2002). Thus, the surgical preparation of the dogs could possibly have affected insulin secretion by mechanisms other than just reduction of BCM. From these studies in animals, it seems that a reduction of BCM is not the major cause of all aspects of the disturbed rapid pulsatile insulin secretion seen in human T2DM.

3.3.3 Effects of obesity and obesity in combination with reduced beta-cell mass on high frequency insulin pulses

Since minor abnormalities in rapid pulsatile insulin secretion (increased absolute but reduced relative amplitude and increased baseline secretion) are seen in insulin resistant (i.e. obese) humans (Hansen et al., 1982, Sonnenberg et al., 1994), the effect of obesity in animal models could increase our knowledge about the sequence of events leading to the defect pulsatile insulin secretion. A study was therefore undertaken to evaluate the effect of relatively short-term (around 2 years) obesity and mild insulin resistance in minipigs, to investigate the hypothesis that disturbed rapid pulsatile insulin secretion is a consequence of obesity, and thus an early sign of beta-cell failure. Pulsatile insulin secretion was not evaluated in the animals before induction of obesity, since this would have required surgical implantation of central venous catheters twice in each animal over the duration of the experiment. However, since the animals included in the study were not selected to be obesity-prone, it would not be expected that this group of animals had abnormal rapid pulsatile insulin secretion or BCF in general before the study.

In the obese animals (VI), rapid oscillations in insulin appeared more irregular by visual inspection and there were more animals where a basal non pulsatile secretion could be seen from the plasma concentration curves. Furthermore, by deconvolution of data, we were able to demonstrate a reduction in the proportion of insulin secreted as pulses, whereas regularity of insulin secretion was slightly increased during glucose entrainment. These results indicate that even relatively short-term obesity can indeed induce small defects in rapid pulsatile insulin secretion that could be a sign of early failure of compensatory insulin secretion during insulin resistance. Furthermore, extensive fat deposition, both extracellularly and within the beta-cells was seen, which could, in the longer term, be detrimental to their function. Beta-cell volume was increased in response to obesity, as has also been reported in humans (Ogilvie, 1933, Klöppel et al., 1985, Butler et al., 2003, Yoon et al., 2003) as a further sign of compensation for obesity. The mechanism behind this increase in beta-cell volume has, in another study in obese minipigs, been shown to be via increased islet number indicating neogenesis of islets in response to obesity (Larsen et al., 2007b).

The changes in BCF and morphology seen in obese animals must be directly related to obesity and not to factors that were predisposing for obesity in these animals. Thus, based on this study in an animal model, at least the increased basal insulin secretion seen in humans (Hansen et al., 1982, Sonnenberg et al., 1994), would be expected to be a consequence rather than a cause of obesity. The apparent increase in orderliness of pulses during entrainment could simply reflect better glucose responsiveness in obese animals, indicating a compensatory mechanism to optimize insulin secretion. Alternatively, it could be a response to changes in plasma lipids which has been reported in humans (Schmitz et al., 2001), but plasma lipids were not measured in our study.

Insulin sensitivity was not assessed in individual animals before the induction of obesity, so it is not possible to determine the development in insulin sensitivity over the study period. Furthermore,

the only measure for insulin sensitivity applied in the study after induction of obesity (VI) was the HOMA index based on fasting glucose and insulin levels, showing a tendency towards insulin resistance in obese animals. This is not a very precise measure of insulin sensitivity, but for practical reasons, the application of the hyperinsulinemic euglycemic clamp was not possible in the study. We have, however applied the clamp technique in a small group of lean and obese pigs, showing a reduction in insulin sensitivity in relation to obesity (Larsen et al., 2003a), further supporting that obese animals do develop some degree of insulin resistance.

To further evaluate the effects of a combination of obesity and reduced BCM, we also included a group of NIA/STZ minipigs (VI) that were fed a high energy diet to induce obesity. In these animals, both reduced pulse mass and increased basal insulin secretion were seen, resulting in a smaller proportion of insulin being secreted as pulses. Plasma concentration curves of insulin from these animals were more irregular during basal conditions, and pulses were difficult to detect directly from these profiles by visual inspection of data. Non-pulsatile insulin secretion determined by deconvolution, both during basal and glucose entrained conditions, was increased and although pulses could still be entrained with glucose, pulse mass was markedly reduced. Furthermore, insulin responses to intravenous glucose and arginine were reduced although orderliness of insulin pulses based on regularity statistics was maintained in spite of obesity and reduced BCM. In a more severe model of diabetes, the Zucker Diabetic Fatty rat, impaired pulsatile insulin secretion and reduced entrainability of rapid insulin pulses by glucose has been reported (Sturis et al., 1994). In the rat model, the disturbances of pulsatile insulin secretion could not be totally abolished by preventing the rats from becoming hyperglycemic (Sturis et al., 1995), indicating that other mechanisms than glucosetoxicity are relevant for the development of abnormal rapid pulsatile insulin secretion in this model.

The impairment of pulsatile insulin secretion in obese and obese NIA/STZ minipigs resulted in impaired ability to fit insulin kinetic parameters to peripheral insulin oscillations (VI) whereas this was not seen in lean NIA/STZ minipigs (IV). Therefore, in future studies in obese animals, insulin kinetics should be obtained in individual animals based on intravenous injection of exogenous insulin.

The group size in our study (VI) was fairly small, but based on the many tests performed, there is a good indication that obesity in the minipig is associated with small defects in BCF and that the combination of reduced BCM and obesity leads to further deterioration. Thus, obesity in humans could potentially further impair BCF in subjects with relatively low BCM. It is surprising that the obese-STZ animals in our study did not develop overt diabetes since the combination of reduced BCM and obesity would be expected to result in more severe deterioration of metabolic control than reduced BCM in lean animals. This is further underlined by the observation in humans that both reduced BCF and insulin resistance are important risk factors for the progression from IGT to T2DM (Pratley and Weyer, 2002). Since the animals in our study were euthanized to obtain the pancreas for determination of BCM, we were not able to observe them for a longer period than what was included in the study (VI) and it remains to be determined whether the combination of reduced BCM and obesity over a longer period of time would translate to a more T2DM-like state in this animal model. In general, we have maintained lean animals with reduced BCM for up to 4 months in our laboratory and have not seen indications of reversion towards normoglycemia or development of more severe hyperglycemia in these animals.

3.4 CONCLUSIONS

Impaired BCF is a major characteristic of human T2DM. One of the early events seems to be defects in rapid pulsatile insulin secretion although the clinical relevance of these pulses is still uncertain. Due to the lack of longitudinal studies on this parameter in humans, ani-

mal models are useful to increase our understanding of the sequence of defects in BCF in the development of T2DM. In conclusion, based on studies in pigs, a primary reduction of BCM or glucotoxicity from mild hyperglycemia over 2 weeks could not explain all of the defects in rapid pulsatile insulin secretion seen in T2DM. Obesity in the minipig was associated with small changes in BCF and morphological changes, whereas a combination of reduced BCM and obesity led to severe defects in BCF and morphology, although it remains to be determined whether development of overt diabetes would be evident over a longer term study period. Thus, obesity in humans might cause small defects in BCF, even in subjects with adequate BCM, whereas obesity in combination with low BCM might further exacerbate the deterioration in BCF.

4. BETA-CELL MASS IN TYPE 2 DIABETES

4.1 INTRODUCTION

Reduction of BCM is a key feature of T1DM (Maclean and Ogilvie, 1955, Saito et al., 1979, Rahier et al., 1983, Klöppel et al., 1985, Kobayashi et al., 1997). In T2DM patients, most studies have reported a reduction, albeit modest (20-50%), of BCM (Maclean and Ogilvie, 1955, Westermark and Wilander, 1978, Saito et al., 1979, Klöppel et al., 1985, Clark et al., 1988, Sakuraba et al., 2002, Butler et al., 2003, Yoon et al., 2003, Deng, 2004), although a few studies have not been able to demonstrate a reduction of BCM (Stefan et al., 1982, Rahier et al., 1983, Kobayashi et al., 1997, Guiot et al., 2001). The largest of the studies showing reduced BCM in T2DM is based on biopsies from the pancreas (Butler et al., 2003) whereas only a few studies showing reduced BCM (Clark et al., 1988, Klöppel et al., 1985, Westermark and Wilander, 1978) or no change in BCM (Stefan et al., 1982, Rahier et al., 1983, Kobayashi et al., 1997) are based on data from the entire pancreas, and the group size in each study is fairly small. Furthermore, there is a considerable overlap between BCM in T2DM and NGT subjects (Maclean and Ogilvie, 1955, Westermark and Wilander, 1978, Klöppel et al., 1985, Sakuraba et al., 2002). Based on these factors, there is still some debate on the extent of reduction of BCM in T2DM.

The mechanism behind the loss of BCM in T2DM has been proposed to be increased apoptosis rate (rather than a loss of the ability to produce new beta-cells) (Butler et al., 2003) and, as is also seen for insulin resistance and reduction in BCF, both gluco-, and lipotoxicity are probably involved. A discussion of the mechanisms behind the changes in BCM is beyond the scope of this thesis, but more information can be found elsewhere (Del Prato, 2004, Donath and Halban, 2004).

4.2 PHYSIOLOGICAL IMPACT OF CHANGES IN BETA-CELL MASS

The endocrine pancreas has a significant capacity to adapt to conditions of increased insulin demand, such as in pregnancy or obesity, by increasing the functional BCM. In obese humans, several studies have reported increased beta-cell volume (Ogilvie, 1933, Klöppel et al., 1985, Butler et al., 2003, Yoon et al., 2003), whereas islet size is reported to be unchanged (Butler et al., 2003, Yoon et al., 2003).

A 20 to 30% reduction of BCM has been linked with very slight increases in glycemic levels (Weir et al., 1986), but hemi-pancreatectomy in humans has been shown to result in fasting and postprandial hyperglycemia and hypoinsulinemia (Kendall et al., 1990), although others have reported normal glucose levels in spite of reduced insulin secretion (Seaquist and Robertson, 1992). In pigs, impairment of glucose tolerance has been demonstrated to be related to the extent of pancreatectomy, with 40% reductions of pancreatic mass resulting in only mild changes, whereas an 80% reduction induced significant hyperglycemia (Lohr et al., 1989). Other studies have shown that reductions of BCM of around 60-75% results in reduced glucose tolerance in minipigs (Kjems et al., 2001, III), whereas in non-human primates a reduction of 50-60% causes severe hyperglycemia (McCulloch et al., 1991). However, a greater

than 80-90% reduction of BCM seems to be required before overt, insulin-dependent diabetes develops in humans (Saito et al., 1979, Gepts and Lecompte, 1981, Klöppel et al., 1985), pigs (III) and rats (Bonner-Weir et al., 1983). Furthermore, maintenance of even very low levels of BCF is of benefit for the ability to control HbA1c with exogenous insulin in T1DM patients, since the risk of hypoglycemia is much less in patients with residual BCF (Diabetes-Control, 1998).

Whether the ability to compensate for a reduction of BCM differs between species cannot be concluded from our present knowledge of BCF and BCM. However, most likely, the different results obtained are, to some extent, explained by the relatively small number of observations in each study, and the possibility that variability in BCF at very low values of BCM seems to occur.

In most (Teuscher et al., 1998, Ryan, 2002, Robertson, 2004), but not all studies in humans (Street et al., 2004) and in pigs (Mellert et al., 1999) and rats (Tobin et al., 1993), there is a strong relationship between islet mass used in transplantation studies and metabolic control as evaluated by glucose levels and insulin secretion, further underlining the importance of BCM in maintenance of normal glucose tolerance and insulin secretion.

Based on the above findings, therefore, in patients with T2DM, the moderate reduction of BCM reported is unlikely to be the only factor responsible for development of hyperglycemia. Most probably, reduced insulin action is also of key importance. BCF and insulin sensitivity are closely related, so that reduced insulin action will require a compensatory increase in BCF (Levy et al., 1991, Kahn et al., 1993, Kahn, 2003). However, since far from all insulin resistant (i.e. obese) subjects suffer from T2DM (Mokdad et al., 2003), beta-cell dysfunction and/or a reduction of BCM must be a prerequisite for development of T2DM. Furthermore, since the endocrine pancreas is continuously, although slowly, renewed, the reduced BCM seen in diabetes should probably be viewed as an imbalance between formation and destruction rates rather than a static situation (Finegood et al., 1995, Bonner-Weir, 2000, Butler et al., 2003).

4.3 EVALUATION OF BETA-CELL MASS IN VIVO

Knowledge about the dynamic changes in BCM could be of great importance for the early diagnosis as well as for the treatment of diabetes in humans. In relation to treatment of diabetes, the recent interest in the ability of incretin-related agents to maintain or even increase BCM (see Drucker and Nauck, 2006 for a recent review) has further emphasized the area of research concerning dynamics of BCM. Techniques that would allow non-invasive imaging of BCM in situ in the pancreas or in the liver after islet transplantation would be of high relevance. However, there are several practical problems to overcome before such a method can be used in humans. Most importantly, the small size of the pancreatic islets is less than the spatial resolution of current imaging technologies, so chemical agents must be used to mark beta-cells for imaging. For this to be effective, the agent used should be highly specific for the beta-cell and be rapidly cleared from the extracellular space (Sweet et al., 2004). Several methods for in vivo imaging are under investigation, see (Souza et al., 2006) for a recent review, but none are applicable to clinical use at present. Therefore, measurement of BCF as an estimate of BCM is, currently, the only method applicable in humans, and a validation of this approach in animal models is of great importance.

4.3.1 Correlations between beta-cell function and mass in lean animals

Correlations between in vivo functional tests and actual BCM have been reported based on relatively small group sizes in normal weight non-human primates (McCulloch et al., 1991) and pigs (Kjems et al., 2001). Furthermore, stimulation with a combination of glucose and arginine has been shown to be the most sensitive in vivo method to detect a reduction of BCM by partial pancreatectomy in dogs and by STZ in non-human primates (Ward et al., 1988, McCulloch et al., 1991).

Based on these observations, a study was performed to validate the relationship between BCF and BCM in a larger group of 30-40 normal weight minipigs, employing stimulation with arginine during hyperglycemia. To obtain a range of BCM, some animals were untreated whereas others had their BCM reduced with STZ alone or in combination with NIA. In this study (III), both fasting plasma glucose and the area under the curve for plasma glucose after an oral glucose load showed a curvilinear relationship to BCM, in agreement with what was previously shown in a smaller study in minipigs (Kjems et al., 2001) and which has also been shown in humans (Saito et al., 1979, Ritzel et al., 2007). Based on insulin responses, we found significant correlations between BCM and several parameters derived from an oral glucose tolerance test, but parameters based on extensive beta-cell stimulation with intravenous glucose and arginine gave considerably better linear correlations. This different correlation is most probably due to the smaller variability and higher levels of plasma glucose after intravenous glucose administration and the lack of the incretin effect compared to the oral route. Furthermore, since the best correlation in our study was obtained after combined stimulation with glucose and arginine, in agreement with previous results in dogs (Ward et al., 1988), non-human primates (McCulloch et al., 1991) and beta-cell transplanted humans (Ryan, 2002), it could be that at less extensive stimulation with glucose alone would allow the remaining beta-cells to compensate for moderately reduced BCM. On the other hand, studies in humans have indicated that the insulin response to intravenous glucose is lost when fasting plasma glucose is above 6-7 mM (Robertson, 2004), so this could also happen in animals with very low BCM, thereby causing a weaker correlation to BCM.

4.3.2 Compensation for reduced beta-cell mass in the perfused pancreas

To further investigate the ability to compensate for reduced BCM, we evaluated the isolated perfused pancreas from NIA/STZ and normal animals (V). The advantage of using this preparation is that it allows direct measurement of insulin secretion during perfusion without influence from other organs, such as extraction of insulin by the liver, variable blood flow and neural and endocrine factors. Furthermore, before the isolation of each pancreas, insulin response to an oral glucose challenge were evaluated in the same animals to allow direct comparison between in vivo and in vitro beta-cell responses. In this study (V), the remaining beta-cells were able to compensate for reduced BCM in vivo in response to the oral glucose load. A similar compensation was seen after stimulation in vitro with glucose, whereas the compensation was incomplete during GLP-1 stimulation, and was not seen during arginine stimulation. In the perfused pancreas from rats after 60% reduction of BCM by pancreatectomy, the relative insulin response to glucose was even lower than the relative response seen in sham operated animals (Bonner-Weir et al., 1983), which is in contrast to our results, whereas the lack of compensation during arginine stimulation in that study was comparable to our results in the minipig (V). In accordance with our results in the NIA/STZ minipig, the perfused pancreas from rats dosed with NIA/STZ, was able to compensate for reduced beta-cell content during glucose stimulation and showed dramatic response to arginine at low glucose levels (Masiello et al., 1998). Thus, the ability to compensate for reduced BCM seems to depend not only on the magnitude but also the type of stimulus and, to some extent, on the method used for reduction of BCM. The results from the perfused pancreas from both rats (Bonner-Weir et al., 1983) and pigs (V) further support the use of the combination of glucose and arginine for estimation of BCM in vivo.

4.3.3 Correlations between beta-cell function and mass in lean and obese animals

Since the majority of humans with T2DM are overweight or obese, a limitation of the studies in animal models on the relationship be-

tween BCF and BCM is that they only include normal weight animals. Similarly, the reports on correlations between transplanted islet mass and functional tests in humans do not include studies in obese subjects (Teuscher et al., 1998, Ryan, 2002, Robertson, 2004). Obesity is associated with insulin resistance, and this might lead to a compensatory increase in insulin secretion compared to lean individuals with a comparable BCM. Therefore, we performed a study to evaluate whether measures of BCF could be used to estimate BCM over a range of body weights in the Göttingen minipig (VII). To obtain a range of BCM, some lean and obese animals were treated with NIA and STZ. Furthermore, since small studies in lean pigs had shown a correlation between BCM and rapid pulsatile insulin secretion (Kjems et al., 2001, IV), we also determined this parameter to further extend these observations. In this study (VII), a significant correlation between several parameters of BCF and BCM was found. As had been shown in lean animals (III), extensive stimulation of insulin secretion resulted in the best correlations across the group of lean and obese animals. Furthermore, detailed analysis of pulsatile insulin secretion by deconvolution did not result in better correlations with BCM than the more simple methods such as intravenous stimulation with arginine during hyperglycemia, and does not, therefore, seem to represent a better surrogate measure for BCM in vivo. These results could indicate that similar methods might be relevant in humans, even over a range of body weights including obese individuals. However, due to the relationship between insulin sensitivity and BCF (Turner et al., 1979, Levy et al., 1991, Kahn et al., 1993), insulin sensitivity should be taken into account when performing such a comparison, and future studies should clarify the effect of body weight and insulin sensitivity on the relationship between BCF and BCM in more detail. Furthermore, the method could be evaluated more extensively in the minipig model, especially by including obese animals with more severe hyperglycemia.

4.4 CONCLUSIONS

Evaluation of BCM in vivo is not currently possible in humans and, therefore, the most feasible method at present is measurement of BCF as a surrogate marker for BCM. The relevance of this approach has been supported by several animal studies (McCulloch et al., 1991, Kjems et al., 2001, III, VII), and the use of such methods could be highly relevant for studies of dynamics of BCM in T2DM in order to increase our understanding of the pathology and treatment of the disease. From a clinical perspective, the method of using combined glucose and arginine function has recently been applied in human studies after islet transplantation with the purpose of estimating engrafted islet mass (Rickels, 2005).

Even with the development of in vivo imaging methods, some degree of variability is to be expected for the estimates of BCM and it could be speculated that the correlation between BCM determined by in vivo imaging and true BCM would not be much stronger than what can be obtained when using BCF as an estimate for BCM. Even when two very closely related parameters such as basal or first phase insulin and C-peptide levels (which should be very closely correlated since both insulin and C-peptide are secreted from the beta-cell in response to the same stimulus) in individual human subjects are correlated (Robertson et al., 1989), r-values are found to be in the same range as the 0.8 to 0.9 seen when correlating insulin responses to glucose and arginine in the minipig (VII). Based on this, the correlation between BCM and BCF can not be expected to be any better and the technique of using either BCF or in vivo imaging as an estimate for BCM will include inherent biological variation. Therefore, to further expand the use of such methods, it could be advantageous to study changes over time in individual animals, using estimates of BCM from BCF obtained in vivo. This is exactly the strength of an approach using in vivo based measures to estimate BCM, thus allowing evaluation of dynamics of BCM in individual minipigs over time as a method to follow disease development or effect of pharma-

cological intervention. Similarly, the application of the technique in humans could allow for evaluation of development of BCM either to follow effects of new pharmacological treatments in groups of patients or to follow disease development and adjust pharmacological treatment in individual patients.

5. CONCLUSIONS AND FUTURE RESEARCH
5.1 CONCLUSIONS
The main findings from the studies presented in this thesis can be summarized as follows:

A minipig model of moderate diabetes characterized by mild fasting and postprandial hyperglycemia, reduced insulin secretion and reduced BCM has been developed (I) and can be combined with obesity to allow study of these two co-morbidities in combination (VI). The model in lean animals has been shown to be stable for at least 2-4 months and is of relevance for acute and chronic studies of glucose metabolism, BCF and BCM. The model has been used for several mechanistic studies in relation to BCF and BCM as well as for testing of new pharmacological strategies for treatment of human T2DM. In our laboratory, we have maintained pigs with reduced BCM for up to 4 months and during this period we have not seen any indications of either reversion or further deterioration of the glucose intolerant/diabetic state.

Based on the mechanistic studies performed in the model, the following conclusions can be drawn:

The Göttingen minipig is highly relevant for the study of rapid pulsatile insulin secretion due to the fast kinetics of insulin and the ease of pulse detection (II). When the model was used to evaluate the consequences of a primary reduction of BCM on rapid insulin pulses, a reduction of pulse mass and mean peak concentration was found, but not all of the defects in rapid pulsatile insulin secretion found in humans (including disorderliness and reduced entrainability with glucose) could be demonstrated in the minipig (IV). Thus, the results from this animal model do not support reduced BCM as the single cause for disturbed rapid insulin pulses in human T2DM. Furthermore, since 2 weeks of mild hyperglycemia did not cause disorderly pulsatility or reduced entrainability, this study does not support short-term, mild glucotoxicity as a primary cause of disturbed rapid pulsatility in T2DM.

Rapid insulin pulses in obese minipigs appeared more irregular and more animals with baseline non-pulsatile insulin secretion were observed compared to lean animals (VI). In line with this, the proportion of insulin secreted as pulses was reduced, whereas regularity during glucose entrainability was increased. Thus obesity in itself could induce small defects in rapid pulsatile insulin secretion and resulted in pancreatic fat infiltration and an increase in beta-cell volume. Both BCF and pancreas morphology further worsened when reduced BCM and obesity were combined in the minipig (VI). Thus, obesity in humans could potentially cause deterioration in BCF, especially in subjects with relatively low BCM. It is surprising that the obese-STZ animals in our study did not develop overt diabetes since the combination of reduced BCM and obesity would be expected to result in more severe deterioration of metabolic control than reduced BCM in lean animals. It remains to be determined whether the combination of reduced BCM and obesity over a longer period of time would translate to a more T2DM-like state in this animal model.

The study in lean animals on the relationship between BCF and BCM (III) confirmed that extensive stimulation of insulin secretion with a combination of glucose and arginine resulted in the best correlation with BCM determined by stereology. Of special interest is the finding that this correlation was maintained when studying a group including both lean and obese animals (VII). This finding supports future evaluation of similar methods in humans even across a range of BMI. Based on the study in the perfused pancreas from minipigs, it was found that the remaining beta-cells are, to some extent, able to compensate for reduced BCM (V), but this ability depends to a large extent on the type of stimulus applied. This study further supports the use of a combination of glucose and arginine to get the best surrogate marker for BCM.

5.2 FUTURE RESEARCH
Investigations in large animal models remain an important supplement to the large amount of research done in rodents. The minipig model of diabetes and the methods described in this work are relevant for studies of dynamics of BCF and BCM in vivo and for evaluation of both acute and chronic effects of new anti hyperglycemic agents, thereby providing new insights into both the pathogenesis and treatment of T2DM.

Based on the correlation between BCF and BCM, longitudinal studies to follow these parameters in individual animals over time to define effects of obesity of longer duration either alone or in combination with reduced BCM, could be feasible, since it still remains to be shown whether these animals would develop more severe hyperglycemia and insulin resistance over time. If a more severe type of diabetes could be developed by these methods, the use of the model might be increased to also encompass the role of insulin resistance in T2DM. Since insulin resistance might influence BCF in such a model, the correlation between BCF and BCM could be expected to change in more severe states of insulin resistance than has been investigated in the present thesis. To evaluate this relationship, the development of methods to obtain sequential biopsies from the pancreas in individual animals during longitudinal studies would be relevant, although the information from such biopsies would only be relative beta-cell volume, not total BCM. Before using data based on such biopsies, it would have to be verified that beta-cell volume in the biopsy area actually reflects total pancreas beta-cell volume.

Furthermore, the models and techniques described here could be very useful for investigations of effects of pharmacological treatment to improve BCF and maintain or improve BCM. An example of such agents is the GLP-1 analogues, of which the first (exenatide) has recently been introduced as a clinical treatment in T2DM. This group of compounds has been reported to improve BCM in rodents, but results from studies in non-rodents are not yet available; see (Drucker and Nauck, 2006) for a recent review of this drug class. There are, however, studies indicating that the increase in BCM, or maybe more correctly the normalization of BCM, may be related to the metabolic state of the animal model studied, especially the level of glycemia, since some studies in NGT (Bock et al., 2003) or diabetic rodent models (Rolin et al., 2002, Sturis et al., 2003, Gedulin et al., 2005), have failed to demonstrate an absolute increase in BCM after treatment with GLP-1 analogues. Therefore, it would be of great interest to investigate the effects of such agents on BCM in a non-rodent model, and the NIA/STZ minipig could be very useful in this respect. In relation to such a study, however, it remains to be determined whether the model is able to regenerate BCM. It could be speculated that this would not be possible after STZ dosing, but in rats, at least, this is not the case (Finegood et al., 1999). On the other hand, the stability of the model for up to 4 months indicates that, if beta-cell regeneration is possible, it is a slow process, and the study period for such an investigation would be considerable. Furthermore, for this type of study, the lack of a positive control with a documented effect of BCM is a complicating factor.

Apart from additional preclinical studies as mentioned above, the validated correlation between BCF and BCM in animal models supports the application of similar approaches in humans. A method to follow BCM over time in humans would be an important tool in the early diagnosis of disease in high risk individuals, as well as in the evaluation of new strategies to maintain or increase BCM. Imaging technologies may be improved in the future to allow such investigations, but the correlation to actual BCM from such methods may not be higher than what is seen when using BCF as an estimate of BCM. Therefore, the use of in vivo measures of BCF as an esti-

mate of BCM remains a highly relevant strategy and further validation of methods to follow dynamics of BCM in humans would greatly improve the possibility to follow development and treatment of T2DM, and therefore would be of value for both patients and society.

Abbreviations

ALX:	Alloxan
BCF:	Beta-cell function
BCM:	Beta-cell mass
GLP-1:	Glucagon-like peptide-1
IGT:	Impaired glucose tolerance
NGT:	Normal glucose tolerance
NIA:	Nicotinamide
STZ:	Streptozotocin
T1DM:	Type 1 diabetes mellitus
T2DM:	Type 2 diabetes mellitus

7. References

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