The bank vole (Myodes glareolus) – a novel animal model for the study of diabetes mellitus

MARTIN BLIXT
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Abstract

The bank vole (Microtus arvalis) develops glucose intolerance both when kept in captivity and in the wild state. Glucose intolerant bank voles kept in captivity exhibited polydipsia, polyuria, hyperglycemia, hyperinsulinemia, islet autoantibodies and a markedly changed islet structure resembling so-called hydropic degeneration. Islets showing hydropic degeneration have reduced β-cell mass. However, the relative islet size to total pancreas area was not changed.

Pancreatic islet isolated from glucose intolerant bank voles had an altered islet function showing signs of being exposed to an increased functional demand on their β-cells. Also, islets from male bank voles seem more affected than the islets from females. Islets isolated from glucose tolerant male bank voles cultured for 5 days at 28 mM glucose did not reveal any change in insulin gene expression or insulin biosynthesis rate. However, islets from female bank voles displayed a glucose concentration dependent response. This suggests that there is gender difference in that, islets of female more easily than islets of males adapt to elevated glucose concentration. Furthermore, islets isolated from glucose tolerant males had reduced insulin gene expression after exposure to proinflammatory cytokines for 48 hrs. This effect seemed to be NO-independent since only a minor elevation of nitrite accumulation in the medium was seen, and the use of iNOS inhibitor could not counteract the cytokine effect. The observed response seen in bank vole islets upon exposure to various glucose concentrations or proinflammatory cytokines is similar to those seen in studies of human islets. The bank vole may therefore represent a novel animal model for the study of diabetes. An unresolved issue is the role of the Ljungan virus which is found in the bank vole colony.

Bank voles developing glucose intolerance display features of both human type 1 and type 2 diabetes, where environmental factors seems to play an important role as determinant. Our findings suggest that bank voles bred in the laboratory may develop more of a type 2 diabetes. However, bank voles caught in nature instead may rather develop a type 1 form of the disease.

Keywords: Bank vole, pancreatic islets, diabetes mellitus, hydropic degeneration, proinflammatory cytokine

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List of Papers

This thesis is based on the following papers, which are referred to in the text by their Roman numerals.


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### Abbreviations

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<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>AMV</td>
<td>Avian myeloblastosis virus</td>
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<tr>
<td>ANOVA</td>
<td>Analysis of variance</td>
</tr>
<tr>
<td>BB</td>
<td>Bio-breeding</td>
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<tr>
<td>CD-1</td>
<td>Inbred mouse strain derived from ICR mouse</td>
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<tr>
<td>cDNA</td>
<td>Complementary deoxyribonucleic acid</td>
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<tr>
<td>Ct</td>
<td>Cycle threshold</td>
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<tr>
<td>CY2</td>
<td>Fluorescent dye fluorescein isothiocyanate</td>
</tr>
<tr>
<td>Db/db–model</td>
<td>Monogenic mouse model of obesity – leptin resistant</td>
</tr>
<tr>
<td>DNAse</td>
<td>Enzyme degrading deoxyribonucleic acid</td>
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<tr>
<td>dNTP</td>
<td>Deoxyribonucleotide triphosphate</td>
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<tr>
<td>ELISA</td>
<td>Enzyme–linked immunosorbent assay</td>
</tr>
<tr>
<td>Fa/fa–model</td>
<td>Monogenic rat model of obesity – leptin resistant</td>
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<tr>
<td>GAD65</td>
<td>Glutamic acid carboxylase isoform 65</td>
</tr>
<tr>
<td>GK</td>
<td>Goto Kakizaki inbred rat strain</td>
</tr>
<tr>
<td>HBSS</td>
<td>Hanks’ balanced salt solution</td>
</tr>
<tr>
<td>IA–2</td>
<td>Islet antigen–2</td>
</tr>
<tr>
<td>IFN–γ</td>
<td>Interferon gamma</td>
</tr>
<tr>
<td>IL–1β</td>
<td>Interleukin one beta</td>
</tr>
<tr>
<td>iNOS</td>
<td>Inducible nitric oxide synthase</td>
</tr>
<tr>
<td>IPGGT</td>
<td>Intraperitoneal glucose tolerance test</td>
</tr>
<tr>
<td>Jcl–ICR</td>
<td>Inbred mouse strain from Jackson laboratories</td>
</tr>
<tr>
<td>KK</td>
<td>Inbred mouse strain from Kasukabe in Saintama, Japan</td>
</tr>
<tr>
<td>KRBH</td>
<td>Krebs’ Ringer bicarbonate HEPES buffer</td>
</tr>
<tr>
<td>LADA</td>
<td>Latent autoimmune diabetes in adults</td>
</tr>
<tr>
<td>LV</td>
<td>Ljungang virus</td>
</tr>
<tr>
<td>NOD</td>
<td>Non obese diabetic inbred mouse strain</td>
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<tr>
<td>NYS</td>
<td>Nagoya–Shibata–Yasuda inbred mouse strain</td>
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<tr>
<td>Ob/ob–model</td>
<td>Monogenic mouse model of obesity – leptin deficient</td>
</tr>
<tr>
<td>PDX–1</td>
<td>Pancreatic–duodenal homebox – 1</td>
</tr>
<tr>
<td>RNAse</td>
<td>Enzyme degrading ribonucleic acid</td>
</tr>
<tr>
<td>RPMI</td>
<td>Roswell Park Memorial Institute</td>
</tr>
<tr>
<td>RT–PCR</td>
<td>Reverse transcriptase polymerase chain reaction</td>
</tr>
<tr>
<td>S.E.M.</td>
<td>Standard error of the mean</td>
</tr>
<tr>
<td>TBS</td>
<td>Tris buffered saline</td>
</tr>
<tr>
<td>TCA</td>
<td>Trichloroacetic acid</td>
</tr>
<tr>
<td>TNF–α</td>
<td>Tumor necrosis factor alfa</td>
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<tr>
<td>UV</td>
<td>Ultraviolet</td>
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Introduction

Diabetes mellitus is a group of metabolic disorders where the control of blood glucose homeostasis has been lost due to reduced ability to use carbohydrates as energy source. This is a consequence of a failing insulin hormonal signaling system. The exact etiology of diabetes mellitus is still unclear however, several theories have been presented arguing that genetic predisposition and environmental factors e.g. viral infections, dietary proteins, toxins as well as lifestyle may influence disease development [1-7]. The prevalence of diabetes mellitus is increasing rapidly. At present approximately 280 million adult individuals suffer from the disease. It is estimated that within 20 years this number will rise to over 400 million [8]. Diabetes mellitus can be divided into several variants of which type 1 and type 2 are the major diagnostic groups [9]. Type 1 diabetes mellitus is a chronic autoimmune disease that affects the β–cells in the pancreatic islet of Langerhans, which results in an insufficient endogenous insulin production [10]. Type 2 diabetes mellitus evolves from relative insulin deficiency that is a result of an increasing desensitization of insulin sensitivity in the peripheral tissue and an impaired β–cell function [11]. Ultimately both these conditions leads to increased blood glucose concentration and a failing β–cell mass. The diabetic patients’ incapacity to metabolize sufficient carbohydrates leads to increasing use of other fuels, preferably fat and protein. This shift in metabolism will lead to loss of normal physiological conditions and eventually result in presentation of the classical symptoms of diabetes; fatigue, polydipsia and polyuria depending on the graveness of the disease.

Historical aspects of diabetes mellitus

The first documented observation of diabetes is from the ancient Egypt preserved in the Ebers’ papyrus dated to 1552 BC [12]. In this papyrus there are descriptions of many diseases, symptoms and treatments including a phrase regarding a remedy that has been translated in two ways “"A medicine to drive away the passing of too much urine," and "To eliminate urine which is too plentiful.". These sentences indicate that diabetes may have been present at this point of time in history. Ebers was not the author but the man who became the owner of the papyrus in 1862 AD. The first known user of the word diabetes, which is Greek, “to pass through”, was Demetrios that lived in the late 2nd century BC in Apameia, today a part of Turkey [13]. It was
not until the late 18th century that William Cullen used the word mellitus that is “honey” in Latin [14]. One century later the German histologist Paul Langerhans published a description of distinct groups of cells seen throughout the pancreatic gland [15]. These groups of cells were later referred to as islets of Langerhans.

In 1921 Frederick Banting at the University of Toronto and his student assistant Charles Best successfully isolated insulin (initially called "iletin) from dog pancreases, using facilities and resources provided by John MacLeod. The findings of the experiments were published in early 1922. The same year in Toronto, Leonard Thompson, a 14 year old boy was the first human treated with insulin. The initial attempt made on January 11 was not satisfactory and James Collip who joined the team in December 1921 made further efforts to increase the purity of insulin. The second injection was made on January 23 and it was a success [16]. In 1923 Frederick Banting and John MacLeod were both awarded the Nobel Prize in medicine. They decided to share their prize sum with Charles Best and James Collip.

In 1958 the Nobel Prize in chemistry was awarded to the British molecular biologist Frederick Sanger for his work on the structure of proteins. Frederick Sanger was the first to describe the amino acid sequence of insulin [17]. In 1977 the American medical physician Rosalyn Sussman Yalow was awarded the Nobel Prize in medicine for the development of the radioimmunoassays of peptide hormones like insulin[18]. This assay has become a very important tool in biological and medical research. A major breakthrough in the research of islet biology was the development of islet isolation techniques. In 1964 the Swedish islet researcher Claes Hellerström at Uppsala University developed the micro dissection procedure that allowed the isolation of the endocrine islets from the exocrine pancreatic tissue [19]. This technique later became an enzyme based procedure and it is today frequently used worldwide in islet research [20, 21]. In 1967 there was a major breakthrough in insulin research when Donald Steiner discovered the insulin precursor - proinsulin and the insulin biosyntheses pathway [22].
Background

Animal models used in diabetes research

Perhaps the most famous experimental animal in the research history of diabetes mellitus was the diabetic dog Marjorie. The dog was pancreatectomised and thus developed diabetes. Pancreatectomised dogs seldom survived very long and usually died within a week or two as a consequence of elevated blood glucose. Marjorie was the first exception from this scenario as she stayed alive for an extended time period given injections with pancreatic extracts by Banting and Best [23]. Today we still use surgical removal to induce type 1 diabetes in animals. A less invasive action is to selectively destroy the endocrine pancreas or islet β–cells by toxins of which streptozocin [24] and alloxan [25] are two common examples. The use of streptozocin in one large single dose given to rodents will result in the destruction of the β–cells and insulin dependence, while multiple low doses may provoke diabetes and lead to a scenario more similar to the development of type 1 diabetes with activated immune cells as a key player [26].

Animal models that spontaneously develop type 1 diabetes have been bred in the laboratory. The two most commonly used in research are the non obese diabetic (NOD) mouse model and the BB rat (Bio Breeding Laboratories, Ottawa). Both these species develop autoimmune mediated type 1 diabetes with infiltrating T–cells, B–cells, macrophages and natural killer cells mounting to insulitis, a state of inflammation leading to the destruction of the islets [27, 28]. Also, a variety of islet auto–antibodies have been reported in both the NOD mouse and the BB rat [29, 30]. Islet autoantibodies are tools of the immune system that falsely identify islet constituents as harmful agents leading to disease development.

The NOD mouse model originates from the Jc1–ICR mouse strain. Diabetes presents at 12 – 30 weeks of age and there is gender difference with 90% of the females but only 10% of the males developing diabetes [31]. The BB rat is bred from the Wistar rat strain and the animals develop diabetes between 9 – 21 weeks of age [32].

Animal models of type 2 diabetes are a heterogeneous group characterized by insulin resistance and impaired insulin secretion. Obesity has a strong relationship with type 2 diabetes mellitus in humans. Animal models of monogenic obesity have therefore been used in research to gain insights into the human disease. An increasing functional demand on the β–cell in
some models, ob/ob mouse and fa/fa rats is compensated by a vast insulin production leading to hyperinsulinemia, while in the db/db mouse the animals develop hyperglycemia when their β-cells are unable to meet the required levels of insulin production [33]. The ob/ob, fa/fa and db/db mouse models are all a result of different changes in the leptin signaling system [34-36]. Leptin is a peptide hormone involved in the regulation of appetite.

The GK (Goto Kakizaki) rat bred from the Wistar rat, NSY (Nagoya–Shibata–Yasuda) mouse derived from the Jc1–ICR mouse and KK mouse are all examples of animal models of polygenic type 2 diabetes [37-39]. The GK rat is suitable to study the diabetes complications; retinopathy, neuropathy and nephropathy. The NSY mice spontaneously develop diabetes over time with impaired insulin secretion and mild insulin resistance in adult animals. The KK mouse is another model that with increasing age gradually develops insulin resistance with compensatory hyperinsulinemia and islet cell hyperplasia along with mild obesity. In contrast to the monogenic obesity type 2 diabetes models, obesity is not a major feature of these animals phenotype. In the type 2 diabetes models there is a pronounced gender difference favoring male animals, that to a high extent develop hyperglycemia while in females often less than a third is affected [39].

The Israeli sand rat (Psammomys obesus) is a good model when studying the effects of diet and exercise in the development of type 2 diabetes [40]. Sand rats kept in captivity and given laboratory chow, become obese, insulin resistant and hyperglycemic [41]. The glucose intolerant animals also develop hyperlipidemia and atherosclerosis when given a high cholesterol diet [42]. This model has an impaired insulin biosynthesis that results in increased circulating proinsulin also, a feature seen in human type 2 diabetes [43].

The bank vole

The number of rodents in the northern hemisphere has been shown to fluctuate from year to year. The fluctuations are synchronous and regular with density peaks every third to fourth year [44]. The cause of these population variations has not been explained although several theories including “suicide marches” and infectious agents have been presented over the years, none being widely accepted.

One strain of these rodents, the bank vole (Myodes glareolus previously known as Clethrionomys glareolus) is a small rodent with a body length of 7 – 13.5 cm, tail length of 3.5 – 6.5 cm and a body weight of approximately 12 – 35 g [45]. These animals do not hibernate i.e. are active throughout the year. During summertime they become nocturnal, however, for the rest of the year they are active both day and night. The main diet of the bank vole is vegetarian, including fruit, soft seeds, leaves, fungi, roots, grass, buds and
moss. They are also known to eat invertebrate food as snails, worms and insects occasionally. The bank vole has its breeding period from April to October and the gestation period is approximately 21 days long. The bank vole has an expected life span of up to 18 months [46].

In the north and the middle part of Sweden the bank vole population has synchronous cyclic density peaks. The population densities in the administrative provinces of Västerbotten and Örebro have been documented since 1973 [47, 48]. In 1998 it was shown that during the period 1973 – 1989 there was a correlation between the bank vole population density and the incidence of human type 1 diabetes mellitus [48].

In a subsequent study of bank voles kept in captivity it was shown that animals develop polydipsia and polyuria, symptoms that suggest that the animals have a disturbed glucose metabolism and there was also gender difference, where male bank voles to a larger extent were affected compared to the females [49]. Indeed, those animals that presented symptoms of glucose intolerance also displayed elevated levels of autoantibodies to glutamic acid carboxylase (GAD65), tyrosine phosphatase–like protein IA–2 and insulin [50]. These findings may suggest that the diabetic condition in these bank voles resembles the type 1 form of the disease [51-53]. Subsequently, it was shown that wild bank voles develop type 1 diabetes mellitus at density peaks [50].

The Ljungan virus

The bank voles have been found to host a novel picornavirus named the Ljungan virus (LV) related to both human parechovirus 1 and cardiovirus genus [54, 55]. The first LV was isolated from bank voles resident in the Ljungan valley along the Ljungan River in the Medelpad County, Sweden. Picornavirus particles have been found in the pancreas of the bank voles [54]. Also, it was recently reported that the BB rat model for type 1 diabetes is a carrier of the LV [56].

LV exposure often results in a chronic persistent or long lasting infection. Inoculation of CD–1 mice with the LV during gestation results in the development of glucose intolerance in the offspring [57]. Furthermore, environmental stress has proven to be an important factor playing a vital role for the development of diabetes in this mouse strain. Diabetic CD–1 mice have been tested for the presence of LV with a diagnostic method based on RT–PCR [58]. Although, these mice tested negative with this method there are several examples of animals later proven infectious. The false negative result in this case is probably a consequence of very low viral RNA copy number in persistently infected animals.

The breeding colony of bank voles at the Astrid Fagreus Laboratory, Karolinska Institute, Stockholm was originally established in Sweden from animals trapped in the provinces of Västerbotten, Småland and Skåne in the
1980:s [50]. The breeding colony is considered LV infected. Attempts to determine with accuracy if an individual diabetic bank vole is LV infected or not, have so far not been successful.

**Proinflammatory cytokines**

Cytokines are signal and effector peptides released mainly by the cells of the immune system to induce various responses in target cells. The cytokine signaling has shown to play an important regulatory role during the development of type 1 diabetes [59]. Besides modulatory effects of the inflammatory responses they are also believed to be important for the impairment of the β-cell function during the disease progression [60, 61]. Also, in type 2 diabetes the impaired β-cell function and the changes seen in peripheral tissues may be related to the effects of cytokine signaling [62-65].

Proinflammatory cytokines are a subgroup of cytokines that favors inflammation. The peptides are mainly released from activated macrophages and T-lymphocytes and contribute to immune cell activation and recruitment to the site of inflammation. The proinflammatory cytokines IL–1β, TNF–α and IFN–γ have been studied extensively in islet research and have been proposed to exert key roles for the detrimental effects seen on the β-cell in type 1 diabetes [60, 66]. IL–1β alone or in combination with TNF–α and/or IFN–γ has been suggested to mediate some of these effects through activation of the inducible nitric oxide synthase (iNOS) [67]. Activated iNOS produce nitrogen oxide free radicals (NO) that may perturb islet function and even be toxic to the β-cell and ultimately lead to cell death [68-70]. Nitric oxide synthase (NOS) exists in several isoforms all catalyzing NO biosynthesis via a reaction involving conversion of L-arginine to L-citruline [71]. The main production of NO in the pancreatic islet takes place in activated intra-islet macrophages but other sources of NO production have also been reported like the β-cell itself and endothelial cells [72-75]. NO is a very central effector molecule thus inhibition of NOS can prevent some of the effects seen when islets are exposed to proinflammatory cytokines [69, 76-79].

There is a high diversity between species in the response to these cytokines. In mouse islet cells the iNOS activation can be mediated by IL–1β alone or in combination with TNF–α and IFN–γ [80, 81]. Mouse islets exposed to IFN–γ alone show an impairment of insulin secretion though some conflicting studies exist [80, 82]. In rat islets IL–1β alone or TNF–α alone can activate iNOS though when used in combination there is a massive production of NO [69, 70]. However, in human islets a combination of IL–1β, IFN–γ or IL–1β, TNF–α and IFN–γ is needed to activate iNOS [83]. The synergistic effect of IFN–γ in cytokine mediated β-cell damage may depend on enhancement of the IL–1β induced gene expression in the target cells [84-
In human islets, IFN–γ alone induced minor effects like suppressed insulin secretion [83, 87, 88].

One explanation for the difference seen between species in regulation of iNOS expression in pancreatic islets has been suggested to be a result of variation in the requirement of transcription factors [89].

**Effects of high glucose**

Prolonged periods of hyperglycemia have proven harmful. The secondary complications often seen in kidneys, eyes, nerves and blood vessels in diabetic patients have been suggested to be a result of extended time periods with hyperglycemia [90]. However, not only these organs are affected. High glucose concentration also has negative effects on the insulin producing cells. An increased glucose load *in vivo* on islet β–cells has been proposed to participate in disease progression in type 1 and type 2 diabetes [91, 92]. In type 2 diabetes the formation of noxious free oxygen radicals damages the β-cell and affects the islet function [93]. However, in type 1 diabetes the destruction of the β–cell mass is primarily mediated by immune cells [94, 95].

An increased functional demand on isolated islets *in vitro* has shown that hyperglycemic–like conditions can alter islet function and be detrimental for the β–cell, but depending on the species of origin this effect varies [93-97].

Mouse islets cultured at 28 mM glucose for 3 days are not affected by an increased functional demand. However, after 1 week in culture the islets display decreased insulin content and enhanced glucose stimulated insulin release. These islets also display increased accumulation of insulin in their culture medium. Islets cultured at 5.6 mM glucose also had an increased (pro)insulin biosynthesis rate in short–term incubation at 1.7 mM glucose. With small variations between strains murine β–cells are affected after long term high glucose culture *in vitro*. However, it appears that they can adapt to this harsh environment and avoid glucose induced cell death [94, 98]. Rat islets cultured at high glucose for 2 days have reduced insulin content [99]. In some studies a reduced glucose stimulated insulin release could be seen [100]. However, in other studies no obvious effect was observed [97].

Islets isolated from the Israeli sand rat and cultured at high glucose have markedly decreased insulin content after 3 days. Further culture of these islets resulted in an increase in the proportion of proinsulin in the islets [101]. Moreover, in a study where islet cells from normal Psammomys obesus were explanted *in vitro* and cultured as monolayer it was found that an elevated glucose concentration (33.3 mM) for 10 days caused a pronounced reduction in glucose induced insulin secretion and insulin content. There was also increased proinsulin–related peptide in the culture medium [96]. Islets from animals kept on a high energy diet display glucose-induced apoptosis and reduced β–cell proliferation [102].
Human islets exposed to a prolonged culture period at high glucose have reduced insulin content, increased basal insulin secretion and reduced response upon glucose stimulation. These islets also have a reduced (pro)insulin biosynthesis rate and total protein biosynthesis rate. However, the cell viability was not changed although the glucose oxidation rate was reduced [93].

**Morphological changes of pancreatic islets in diabetes**

The clinical manifestation of diabetes mellitus is probably preceded by a prolonged period of β–cell mass destruction [103, 104]. The morphologic hallmark of type 1 diabetes has been considered the presence of inflammation lesions in the pancreatic islets [105, 106]. In these type 1 diabetic patients the partial or total islet destruction is supposed to be mediated by immune cells and proinflammatory cytokines [60, 107-109], while in type 2 diabetic patients the question of β–cell mass deterioration is more controversial. Previous studies have reported of unchanged β–cell mass in type 2 diabetic patients [110, 111], whereas more recent studies have shown that the β–cell mass is affected even in these subjects [112, 113]. The reduction of the β–cell mass in type 2 diabetic patients has been suggested to be a result of lipotoxicity as well as glucotoxicity [114, 115].

The Israeli sand rat develops morphological changes in the pancreatic islets in parallel with the development of hyperglycemia, when given high energy diet [116]. Islets from severely diabetic sand rats have vacuolization in their β–cells and deposits of lipids [116-118]. The severely altered islet structure seen in these animals is a result of prolonged disease progression and is apparent only at the end–stage of the disease [116]. Islets in glucose intolerant sand rats early during disease progression have degranulated β–cells, which can be seen as weak staining for insulin [119][122].

Microtus arvalis is another wild rodent that develops glucose intolerance. In accordance with the sand rat, early during disease progression, the pancreatic islet cells display degranulation and the animals develop hyperglycemia and hyperinsulinemia. In severely diabetic animals the pancreatic islet β–cells display vacuoles described as glycogen deposits and the animals become hypoinsulinemic [120]. Another animal model showing deposits of glycogen in the islets when cultured at high glucose conditions in vitro is the Wistar rat [121]. In these islets the glycogen appears to be localized to the β–cell while the other islet cells seem unaffected [122].

Hydropic degeneration is a degenerative change of the cell structure that resembles cloudy swelling. In rabbit islets hydropic degeneration has been demonstrated in the β–cell and proven to be glycogen deposits [123]. Morphologic changes resembling hydropic degeneration can be induced in both
rabbit α–cells and in rat β–cells using various chemical substances [124-126].
Aim

Paper I
In this paper we aimed to characterize the function of pancreatic islets isolated from glucose intolerant/diabetic and glucose tolerant/normal bank voles, in order to investigate if the bank vole could become a future model for the study of human diabetes.

Paper II
Herein we studied the function of pancreatic islets isolated from glucose tolerant/normal male bank voles after prolonged exposure to proinflammatory cytokines \textit{in vitro}.

Paper III
The aim of this study was to investigate functional alterations of islets isolated from glucose tolerant bank voles after prolonged exposure to various glucose concentrations \textit{in vitro}.

Paper IV
The focus of this study was to characterize the islet morphology of glucose intolerant/diabetic animals and further investigate if a relationship could be seen between structural changes in the islets and the observed animal phenotype.
Materials and methods

Animal and sample preparations (I – IV)

Bank voles (Myodes glareolus) were housed at Astrid Fagreus Laboratory, Karolinska Institute, Stockholm. The breeding colony was originally established from animals trapped in Sweden in the provinces of Västerbotten, Småland and Skåne [50]. Animals had free access to water and standard laboratory chew (LABFOR R3, Lactamin, Kimstad, Sweden) with an energy content of 3.01 kcal/g and were occasionally given pieces of vegetables. The experimental procedures were approved by the animals’ ethical committee in Stockholm (N248/03, N276/06) and in accordance with international guidelines (NIH publications no.85–23, revised 1985).

Animals were tested with an intraperitoneal glucose tolerance test (IPGTT). Blood glucose determinations were performed with an automated glucose meter (Paper I, Precision PCX; Abbott Inc., Stockholm, Sweden; Paper II, III, IV, Accu-Chek Aviva; Roche Diagnostics, Bromma, Sweden) on whole blood. Animals were injected with 2 g glucose/kg body weight and samples were taken from the retro–orbital sinus immediately prior to injection (0 min), at 60 and 120 min after glucose injection. A bank vole with 120 min blood glucose ≥11.1 mM was classified as glucose intolerant/diabetic. The animals were killed by cervical dislocation and exsanguinations via the carotid arteries. The animals were anaesthetized with Isoflurane (Abbott Inc.) prior to testing and killing. After killing the animals the pancreas was removed and kept on ice in Hanks’ balanced salt solution (HBSS; SBL Vaccine, Stockholm, Sweden) supplemented with 50 U/ml benzylpenicillin and 0.05 mg/ml streptomycin (Roche) or placed in 4 % formalin for 48 hrs. In the latter case the fixed pancreas was washed and stored in 70 % ethanol until embedded in paraffin. The tissue was sectioned (5 μm) and attached to POLYLYSINE™ glass slides (Menzel–Gläser; Braunschweig, Germany). Serum was prepared from the blood samples, frozen and stored at -70°C. The serum insulin level was later measured using high range rat insulin ELISA kit or rat insulin ELISA kit (Mercodia AB, Uppsala, Sweden) according to the instructions of the manufacturer.
Islet isolation and culture condition (I – III)

The pancreas was inflated with HBSS, cut in pieces and digested at 37°C with collagenase A (Roche) dissolved in HBSS to a final concentration of 3.125 mg/ml. Islets of Langerhans were handpicked using a braking pipette under a stereomicroscope and transferred to sterile non-attachment culture dishes containing culture medium RPMI 1640 with 11.1 mM glucose supplemented with 10 % fetal calf serum (FCS), 2 mM glutamine (Sigma–Aldrich Sweden AB, Stockholm, Sweden) and antibiotics (see above).

(I)
Islets from glucose tolerant/normal and glucose intolerant/diabetic female and male bank voles 4 to 44 weeks of age were either studied immediately after isolation or cultured at 37 °C, in humidified air + 5 % CO₂ (AGA, Stockholm, Sweden) for 7 days. Culture medium was exchanged every second day.

(II)
Islets from glucose tolerant/normal male bank voles 15–20 weeks of age were precultured in 5 days and then transferred to new culture dishes containing culture medium as above, with or without addition of IL–1β (25 U/ml; human; PeproTech EC Ltd., London, UK) alone or in combination with TNF–α (1000 U/ml; human; PeproTech) + IFN–γ (1000 U/ml; murine; PeproTech) and cultured for additional 48 hrs before tested. Bank vole specific cytokines are not commercially available. Also we added 2 mM amino-guanidine to randomly selected dishes with or without all three cytokines during the 48 hrs culture period.

(III)
Glucose tolerant/normal female and male bank voles 4–42 week of age were used to study the functional changes upon prolonged exposure to high glucose concentration. Islets from two bank voles of the same gender and similar body weight were pooled and distributed into three new culture dishes with 50 islets of equal size in each and further cultured for 5 days in culture media supplemented with glucose to a final concentration of 5.6, 11.1 or 28 mM before examined. The culture media was exchanged on day 1 and 3. In separate experiments islets were cultured in medium containing 11.1 mM D–glucose + 16.9 mM L–glucose to explore possible osmotic effects of a high glucose concentration.

Islet (pro)insulin biosynthesis rate (I – III)

The (pro)insulin biosynthesis analysis was performed using 10 islets in duplicate that were incubated for 2 hrs at 37°C in humidified air + 5 % CO₂ in

20
1.7 mM or 16.7 mM glucose, Krebs-Ringer bicarbonate buffer [127] with 10 mM HEPES (Merck Eurolab, Stockholm, Sweden), hereafter designated as KRBH, supplemented with 3H-labeled leucine to a concentration of 0.4 μM (120 Ci/mmol; American radiolabeled Chemicals, St. Louis, MO, USA) and 2 mg/ml bovine serum albumin (BSA, ICN Biochemicals, Irvine, USA). After the incubation the islets were washed with non–radioactive leucine (10 mM; Sigma–Aldrich) dissolved in HBSS and the islets were dispersed in 200 μl H₂O through sonication. A volume of 10 μl of the homogenate was mixed with 50 mM glycine (Merck Eurolab), 6 mM NaOH, 0.1 % Triton X–100 (Sigma–Aldrich) and 2.5 mg/ml BSA pH 8.8 and 5 mg Sepharose A (Amersham Pharmacia AB, Uppsala, Sweden). (Pro)insulin was precipitated using a polyclonal anti–serum raised in guinea pig against bovine insulin (Chemicon Inc., Hampshire, UK) and normal guinea pig serum was used to assess unspecific bindings. Another 10 μl homogenate was dissolved in 25 mM glycine, 3 mM NaOH and 1.25 mg/ml 0.305 M TCA (Merck) to calculate total protein synthesis rates. The pellet was dissolved in 0.15 M NaOH. Radioactivity was measured in a liquid scintillation counter after adding 4 ml of Ultima Gold scintillation fluid (PerkinElmer Life Sciences, Boston, USA).

Islet glucose oxidation rate (I – III)

The islet glucose oxidation rate was studied using duplicate or triplicate vials containing 10 islets in KRBH, supplemented with radioactive labeled D-[U-14C]glucose (16 Ci/mmoll; Amersham Pharmacia) and non-radioactive glucose to a final concentration of 1.7 mM or 16.7 mM. Islets were incubated at 37°C (O₂/CO₂; 95/5 %; AGA) under slow-shaking for 90 min. After the incubation antimycin A (Sigma–Aldrich) was added to a final concentration of 0.017 mM and 250 μl 1 M hyamine 10–X (PerkinElmer) in the outer vials reincubation at 37°C in 2 hrs. Radioactivity was measured in a liquid scintillation counter after adding 5 ml of Ultima Gold scintillation fluid (PerkinElmer) [128].

Medium insulin and proinsulin accumulation, islet insulin release and insulin content (I– III)

Media from the last 48 h of culture was collected for determination of medium insulin and proinsulin accumulation. Islets in triplicate of 10 were incubated at 1.7 mM glucose in KRBH supplemented with 2 mg/ml BSA at 37°C (O₂/CO₂; 95/5 %; AGA) for 1 h. Subsequently the buffer was replaced with KRBH supplemented with 16.7 mM glucose and incubated in an additional h. Islet insulin content was measured on islets pooled from the triplicates above after disruption in H₂O and extraction overnight at 4°C in 68–70
% ethanol (Solveco Chemicals AB, Stockholm, Sweden) supplemented with 0.13 mM HCl (Merck). The insulin concentrations in the culture media, the incubation buffers and the insulin extractions were measured with either a rat insulin high range ELISA kit or a rat insulin ELISA kit (Mercodia). The proinsulin concentration in the culture media was measured with a rat Proinsulin ELISA kit (Mercodia) according to the instructions of the manufacturer.

Medium nitrite accumulation (II)

Samples from the culture medium were mixed with 0.05 % N–(1–naphtyl)–ethylenediamine dihydrochloride (Sigma–Aldrich) and 0.5 % sulfanilamide dissolved in phosphoric acid (Merck). Samples were incubated at 60°C for 2 min followed by incubation at room temperature in 20 min. Nitrite levels were determined through spectrophotometric absorbance measurement at 546 nm. Culture media collected from wells without islets was used as control of the original nitrite level in the media.

RNA isolation, cDNA synthesis and Real Time PCR (II, III)

Total RNA was extracted from 50 islets using RNeasy Micro kit (Qiagen, Hilden, Germany) complemented with RNase–free DNAse (Qiagen) to ensure DNA free samples and eluted in RNAse free water. The RNA amount and purity was tested using the Nanodrop ND–1000 system (NanoDrop Technologies, Delaware, USA). Synthesis of cDNA was performed with Reverse Transcription System (Promega, Madison, USA) using 1:2 volume of total RNA and 0.025 μg/μl Oligo(dT)15 primer per cDNA synthesis reaction mixed with reaction buffer (5 mM MgCl2, 10 mM Tris–HCl (pH 9.0), 50 mM KCl, 0.1% Triton® X–100, 1 mM each dNTP, 0.5 U recombinant RNasin® ribonuclease inhibitor, 15 U AMV (Avian Myeloblastosis Virus) reverse transcriptase. The reactions were incubated at 42°C for 60 min followed by 99°C in 5 min and subsequently stored at -20°C.

The LightCycler Instrument (Roche) combined with sequence independent detection with SYBR Green I was used to amplify and analyze generated cDNA. The sequence of the primers used were:

- **insulin** (mouse)  
  Forward 5´-CCATCAGCAAGCAGGTTAT-3´  
  Reverse 5´-GGGTGTGTAGAAGAAGCCA-3´

- **PDX–1** (mouse)  
  Forward 5´-GGTGCCAGAGTTCAGCGCTA-3´  
  Reverse 5´-TTGTTTTTCCTCGGGTTCCGC-3´

- **β–actin** (mouse)  
  Forward 5´-CCACCGATCCACACAGAGTACTTG-3´  
  Reverse 5´-GCTCTGGCTCTCTAGCACC-3´

(TIB MOLBIOL Syntheselabor GmbH, Berlin, Germany) PCR amplification of 0.1 μg of cDNA sample was performed with 0.2 μM of each insulin
or PDX–1 primer and 0.5 μM of each β–actin primer in SYBR Green JumpStart ready mixture (FastStart Taq DNA polymerase, dNTP mix and SYBR Green I dye; Roche) and 3.5 mM MgCl₂ (Roche). For each reaction, the polymerase was activated by preincubation in 95°C 30 sec. Amplification was as follows: 5 sec at 94°C, 10 sec at 47°C and 15 sec at 72°C in 40 cycles. Cycle threshold (Ct) values were obtained for individual samples with the second derivative maximum method [129, 130]. The relative expression was calculated from the formula 2⁻(ΔCt), were ΔCt is the difference between the insulin Ct value or PDX–1 Ct value and the β–actin Ct value. RNase-free water was used as a negative control to assure contamination free reagents.

DNA quantification (III)

DNA content was measured with fluorometric assay using picoGreen (Molecular Probes, Eugene, CA, USA) labeling system according to the instructions of the manufacturer. The DNA content was measured in water homogenates in conjunction to determinations of the islet insulin content, total protein and (pro)insulin biosynthesis rates.

Islet cell viability (II)

Islet viability was determined using Hoechst 33342 and propidium iodide (Sigma–Aldrich) staining [131]. In each test 10 islets were incubated with 5 μg/ml bisBenzimide (Sigma–Aldrich) and 20 μg/ml propidium iodide for 20 min. The islets were washed in PBS and the emitted fluorescence was observed at 461 nm and 615 nm respectively after excitation with UV light. The nucleus morphology and the number of blue and red fluorescent cells were counted in a microscope (Leica Microsystems GmbH, Wetzlar, Germany) by a blinded observer.

Paraffin removal and antigen retrieval (IV)

All incubations were performed in room temperature and the water was deionized prior to use unless otherwise stated. The slides were washed twice for 10 min in xylene to ensure paraffin removal. The sections were rehydrated using an ethanol dilution series finishing in water. The antigen retrieval was performed using the pressure cooker 2100 retriever system (Prestige Medical, California, USA) and the BORG–decloaker™ RTU buffer (Biocare Medical, California, USA) according to the description of the manufacturer. After boiling the retriever buffer was removed and the sections were washed twice with heated Hot Rinse buffer (Biocare Medical). Subsequently, the buffer was exchanged and the slides washed twice for 5 min with water and twice for 5 min in Tris–buffered saline pH 7.6 with TWEEN
TBS was hereafter used as wash buffer and solvent for antibody preparations.

Haematoxylin staining (I, IV)
The tissue was counterstained using Mayer’s haematoxylin solution (Histolab, Göteborg, Sweden). The excess color was removed by washing in non-deionized water in 10 min. The sections were dehydrated using an ethanol dilution series finishing in ethanol. The slides were mounted with cover glasses (Menzel–Gläser) using the Microm CTM6 system (Microm International GmbH, Walldorf, Germany). The sections were examined in bright field microscope (Leica Microsystems GmbH) using 40x and 400x magnifications.

Fluorescence staining (IV)
Unspecific binding of the secondary antibody was blocked during 1 h incubation in a humidified chamber using normal donkey serum (1:20; Jackson ImmunoResearch Laboratories, West Grove, Pennsylvania, USA). The primary antibodies were monoclonal antiserum raised in chicken against human insulin (1:1000; Immunsystem, Uppsala, Sweden) and human glucagon (1:100; a kind gift from Professor Anders Larsson, Uppsala University Hospital, Sweden) as well as monoclonal antiserum raised in sheep against somatostatin (1:75; Guildhay, Guildford, UK) and human pancreatic polypeptide (1:1; SeroTech, Oxford, UK). Excess blocking buffer was removed prior to application of the primary antibody thereafter the slides were reincubated in the humidified chamber at 4°C over night. The secondary antibodies were raised in donkey against chicken or sheep and conjugated with cyanine dye–Cy2 (1:200; IgG, Jackson ImmunoResearch Laboratories). Unbound primary antibody was removed by washing thrice for 5 min. The secondary antibody was applied to the sections and the slides were reincubated in the humidified chamber for 1 h and subsequently washed thrice for 5 min. The slides were mounted with cover glasses (Menzel–Gläser) using 87% glycerol (Calbiochem, California, USA). The islets were photographed using a fluorescence microscope with filter cube L5 (emission filter 527/30 nm) at 200x magnification (Leica Microsystems GmbH). The specificity of the antibodies was tested using different dilutions of the antibodies as well as by omitting the primary antibodies.

Morphological evaluation (IV)
Glucose tolerant/normal and glucose intolerant/diabetic male and female bank voles 9–28 weeks of age were used to study the morphology of the
endocrine pancreas. The total islet area as percent of the total pancreatic area was measured in 2 sections at least 300 μm apart stained with haematoxylin.

The insulin, glucagon and somatostatin positive areas and total islet area were studied on slides stained using immunohistochemistry and fluorescent dyes. For each animal we measured the islet positive area of insulin, glucagon, somatostatin and pancreatic polypeptide in 10 islets from 2 sections and expressed the results as percent of the total islet area.

Statistical analysis (I–IV)
Mean values were calculated from duplicate or triplicate measurements and then considered as one separate observation (n). In paper I, II and IV each observation (n) represents a single donor. In paper III each observation (n) represents pooled islets from two donors matched by gender and body weight. Values are expressed as means ± S.E.M., and groups of data were compared using Student’s unpaired or paired t-test, one or two way ANOVA using Bonferroni correction, Fisher’s exact test or Mann-Whitney rank sum test. We also tested if an independent variable could be used to predict a dependent variable using linear regression and Galton log-normal regression analysis [132]. Observed differences were considered statistically significant when P ≤ 0.05. Statistical analysis were performed using SigmaStat© (SPSS Inc., Chicago, IL, USA).
Results and discussion

Glucose intolerance in bank voles (I)

Bank voles caught in nature and subsequently bred in captivity develop a disturbed glucose homeostasis. The bank vole breeding colony at the Astrid Fagreus Laboratory has been shown to have a glucose intolerance/diabetes incidence of approximately 20% when maintained on a standard laboratory diet. The animals developed glucose intolerance early in life and the majority of all diagnosed bank voles were between 5 to 20 weeks of age. In this colony it appears as if more males than females developed glucose intolerance/diabetes early during this period.

We used an intraperitoneal glucose tolerance test (IPGTT) as a diagnostic method to determine the glucose tolerance status of the animals in the colony. Based on the IPGTT test results two groups of glucose intolerant/diabetic animals could be distinguished, those with blood glucose close to the cutoff value of 11.1 mM glucose (~70%) and those with severely elevated blood glucose (>16.7 mM). The glucose intolerant/diabetic animals were also suffering from hyperinsulinemia, and although without obvious signs of obesity, they displayed a trend towards slightly higher body weight than glucose tolerant bank voles. We also observed that male bank voles classified as glucose intolerant/diabetic alone were hyperglycemic.

The bank voles suffering from glucose intolerance/diabetes have previously been reported to display features of human type 1 diabetes. The massive destruction of the islet β-cells and the presence of islet autoantibodies in these animals support the notion of a new possible animal model of type 1 diabetes [133]. However, the symptoms described above are more similar to type 2 diabetes–like condition. Perhaps the condition observed in the glucose intolerant/diabetic bank voles are a mixture of the two variants. In humans there are a somewhat similar diabetic condition known as latent autoimmune diabetes in the adults (LADA) in which patients with type 2 diabetes phenotype exhibit islet autoantibodies [134]. The diabetic bank vole shares features with this condition.

Pancreatic islets isolated from glucose intolerant/diabetic male bank voles displayed an increased basal insulin secretion acute after isolation compared to islets from glucose tolerant/normal male bank voles. After one week in culture the islets also increased their response to glucose and the basal insulin secretion was now even more elevated compared to the acute performed
experiments. After culture the glucose stimulated insulin release was elevated in islets isolated from glucose intolerant/diabetic males compared to the basal insulin secretion of such islets. Furthermore, the insulin content of islets from glucose intolerant/diabetic males was elevated after culture. Islets isolated from glucose intolerant/diabetic female bank voles did not have these features. However, islets isolated from glucose tolerant/normal female bank voles had increased insulin content acute after isolation compared to islets from normal males.

These findings may indicate a gender difference in that the β-cells of the glucose intolerant/diabetic males were more affected by an increased functional demand in vivo than the females. Indeed, an increased functional load on the β-cells may well exist during development of both type 1 diabetes [94] and type 2 diabetes in rodents [135]. Furthermore the altered function observed acutely in islets isolated from glucose intolerant/diabetic male bank voles could be reversed by culture and did not represent a permanent dysfunction. It has previously been shown that islets isolated from prediabetic NOD mice had an impaired glucose stimulated insulin secretion, but the β-cell function could then subsequently be restored after culture [95]. This does not exclude that bank voles developing diabetes have β-cells that degenerate and islets that gradually disappear from the pancreas.

**NO independent reduction in insulin biosynthesis (II)**

The proinflammatory cytokines, IL–1β, TNF–α and IFN–γ together reduced the relative contribution of labeled (pro)insulin to the total protein biosynthesis rate in bank vole islets after 48 hrs exposure. This was mainly a result of a decreased (pro)insulin biosynthesis rate. Further, these islets also had markedly reduced insulin gene expression and a tendency towards reduced PDX–1 gene transcription. The proinflammatory cytokines also led to reduced islet insulin content and increased basal insulin secretion in the bank vole after 48 hrs exposure. IL–1β alone had the same effect on insulin content after only 24 hrs exposure, however, the basal insulin secretion was not affected at this time point compared to non–exposed control islets. The proinflammatory cytokines had little effect on the glucose stimulated insulin release and no effect on the insulin accumulation in the medium. These findings suggests that the reduction in islet insulin content was not attributed to an enhanced exocytosis and not related to an altered glucose metabolism, but rather was due to a decline in the formation of insulin. This pattern is partly similar and partly different from what has been seen in similar experiments using islet preparations from other species. In rat pancreatic islets, IL–1β induced strong suppressive effects on all functions described above and it is likely that impaired glucose metabolism is a key factor [70, 88, 97]. Concerning mouse pancreatic islets, exposed to IL–1β, the insulin content and
(pro)insulin biosynthesis rates were depressed, medium insulin accumulation and glucose oxidation rates essentially unaffected, but glucose stimulated insulin release reduced by about 50 % [136]. Furthermore, human islets cultured with IL–1β alone for a prolonged period can be shown to have a stimulated β–cell function [137]. When a combination of cytokines was added a marked increase in medium insulin accumulation was present as well as a decrease in islet insulin content, a decline in relative glucose induced insulin release (16.7/1.7), but no change in glucose oxidation rate [83].

NO is often assessed by nitrite medium accumulation in cell culture experiments. Bank vole islets exhibited a modest increase in medium nitrite after 48 hours cytokine exposure. When aminoguanidine, a preferential iNOS inhibitor was added to these islets [138] the medium nitrite level was reduced, but the reduction in islet insulin content was not affected suggesting that the decline in islet insulin content was not NO–dependent. It is likely that an iNOS and NO–independent pathway is responsible for the actions we observed in proinflammatory cytokine exposed bank vole islets. iNOS and NO–independent pathways have been suggested previously to be based on experiments with human islets [83], rat–derived β–cell preparations [139, 140] and pancreatic islets from mice with an inactivated iNOS gene [141]. In experiments performed with the latter type of islets it has been shown that mRNA expression for the transcription factor PDX–1 was also reduced by cytokines [142]. Indeed, this was what we saw in bank vole islets after exposure to IL–1β + TNF–α + IFN–γ though the effect was more pronounced on the insulin gene expression where a 50 % reduction could be seen.

Prolonged exposure to high glucose (III)

Isolated bank vole islets displayed a markedly elevated basal insulin secretion and reduced insulin content compared to control islets (11.1 mM glucose) after prolonged exposure to high glucose conditions in vitro (28 mM glucose). However, only a minor effect was seen on glucose stimulated insulin release compared to controls. The reduced insulin content in islets cultured at high glucose suggests that the islets were not able to adjust to the increased functional demand. Bank vole islets maintained at low glucose condition (5.6 mM glucose) showed a reduced basal insulin secretion and glucose stimulated insulin release while the islet insulin content was essentially unaffected compared to control islets (11.1 mM glucose). The stimulation index, the fraction between the insulin release upon a glucose challenge and the basal insulin secretion, reveal that the islets maintained at high glucose conditions were less responsive compared to controls suggesting that their functional ability was exceeded. In contrast to the bank vole islets maintained at low glucose conditions displayed increased stimulation index compared to controls. The latter finding suggests that the control islets are
not cultured at an optimal glucose concentration. The increased response seen at 5.6 mM glucose suggests that this glucose concentration, using the RPMI 1640 culture medium, may be the most favorable culture conditions for bank vole islets. It has previously been shown that human islets cultured in RPMI 1640 have an optimal glucose concentration lower than the standard 11.1 mM glucose [143]. In similarity to our experiments in bank vole human islets also display a similar response in stimulation index and in insulin content with increasing glucose concentration in the culture medium [143]. The selection of culture media appears to be of importance, at least when studying isolated rat islets \textit{in vitro}. Normal rat islets cultured at high glucose has in some studies shown to express a decrease in glucose stimulated insulin secretion so-called desensitization [100]. However, in other studies high glucose culture has been shown to have no obvious harmful effect on the islets [97].

Islets isolated from female bank voles and maintained at low glucose condition had reduced (pro)insulin biosynthesis and reduced insulin gene expression compared to control islets. In islets isolated from male donors no difference was detected in the (pro)insulin biosynthesis rate or the insulin gene expression in response to the different glucose concentrations used in the culture media. These results may suggest that female islets are more suited, than male islets, to adapt their (pro)insulin biosynthesis rates and insulin gene expression to the increasing functional demand.

The insulin accumulation in medium was not changed after islet culture in high glucose compared to the control group. However, the proinsulin fraction in this medium, expressed as percent of total medium insulin accumulation, was increased. The insulin and proinsulin concentration in medium with a low glucose concentration collected after islet culture displayed a decline compared to control islets. The increased proinsulin amount in the culture medium with high glucose concentration may suggest that the islet insulin production capacity was exceeded. The Israeli sand rat shares some of these features. Diabetic sand rats kept under non–fasting conditions have markedly increased proinsulin levels in their serum [144]. Also, pancreatic extracts from these animals revealed that they had depleted insulin stores. These findings indicate that the functional demand on the β–cell \textit{in vivo} has surpassed the capacity to produce insulin. In a study where islet cells from normal Psammomys obesus were explanted \textit{in vitro} and cultured as monolayers it was found that an elevated glucose concentration (33.3 mM) for 10 days caused a pronounced reduction in glucose induced insulin secretion and depletion of islet insulin stores as well as increased proportion of proinsulin related peptides [96].
Islet morphology and function (IV)

Bank vole pancreatic islets develop a markedly changed islet structure containing balloon-like cells and severe reduction of the number of endocrine cells. The changed structure of these islets resembles so-called hydropic degeneration [123]. Hydropic degeneration was observed to be present only in islets from bank voles classified as glucose intolerant/diabetic following IPGTT.

The severely changed islet structure seen in islets with hydropic degeneration did not affect the relative islet area of the total pancreas area. However, the loss of endocrine cells resulted in a reduced insulin positive area compared to total islet area in these islets. In pancreas from female donors, islets with hydropic degeneration also had reduced glucagon and somatostatin positive areas. This difference was not apparent in islets from male donors.

Microtus arvalis is another vole that as a response to feeding on a low fiber diet develops similar islet structural changes as presently could be found in the severely affected bank voles [120]. The vacuoles observed in these animals were described as enlarged β-cells with glycogen deposits. Another animal model showing deposits of glycogen in the islets when cultured at high glucose conditions in vitro is the Wistar rat [121]. This may suggest that the vacuoles seen in islets from severely affected bank voles could reflect glycogen deposition.

Similar to the severely affected bank voles the glucose intolerant sand rat develops markedly changed islet structure [118]. In the sand rat this altered islet structure is a result of prolonged disease progression [116]. This altered islet structure has been presented as a result of extensive cell death that leads to the formation of vacuoles in the β-cells [102]. Based on this change seen in severely diabetic sand rats it is reasonable to assume that the altered islet structure seen in severely affected bank voles may be due to increased cell death. However, previous studies have shown that islets exposed to prolonged period of elevated glucose levels in vitro does not reveal any change in islet viability nor could increased cell death be observed in islets exposed to proinflammatory cytokines despite a suppressed function.

The observed extensive morphological islet changes seen in diabetic bank voles has made us suspect that a subpopulation of islets has been studied throughout this project namely those that we were able to isolate upon the collagenase isolation procedure. It may well be that the islets with the most deteriorated structure, could not be retrieved upon islet isolation. We found that the islet yield during isolation from a glucose tolerant/normal animal is approximately 90 islets per pancreas, while in severely diabetic animals it usually decreases to 60 islets or less.

The serum insulin level was elevated in female bank voles with islets showing hydropic degeneration. However, this increase was not as clear in male bank voles. But when both female and male serum insulin were plotted
the bank vole serum insulin could be predicted from the non–fasting blood glucose, using the Galton log–normal regression [132] (P<0.018). It is possible that with increasing functional demand the bank vole β–cell an initial period of hyperinsulinemia will occur but eventually it will lead to loss of insulin production and hypoinsulinemia.

Although the islet structure was severely changed and the number of endocrine cells reduced morphologic screening of pancreatic sections from bank voles of various age have not revealed any clear signs of insulitis, which argues against a classical human type 1 diabetes pathogenesis. In human type 2 diabetic patients, the loss of β–cell mass does not affect the fractional islet area which is consistent with our finding in the bank vole [112, 113]. Bank vole has been considered as a diabetes model showing features of both type 1 and type 2 diabetes. However, our present findings suggest that the bank voles bred in the laboratory for a prolonged period is more likely a diabetic animal model showing mainly features of type 2 diabetes. In this study of glucose intolerant/diabetic animals we have observed elevated serum insulin levels, increased body weight, reduced cell mass, markedly changed islet structure and the absence of insulitis, all signs of type 2 diabetes mellitus. These observations are made on a 30 years old breeding colony and we cannot exclude that bank voles caught in nature instead develop a type 1 form of the disease. This argues for a decisive role for interactions between environmental factors in the diabetes development in bank voles.

Ljungan virus (I–IV)

The role of the LV in the bank vole pathology is still unclear. Whether or not the LV infection has contributed to the development of glucose intolerance/diabetes in the bank vole still remains to be revealed. Therefore, we cannot exclude that the LV may be the agent responsible for the development of diabetes in bank voles. Furthermore, such infection may have influenced our findings when cytokines were added to the isolated pancreatic islets or when the islets were exposed to an increased functional demand.
Conclusions

**Paper I**
We report that bank voles to a significant extent develop glucose intolerance/diabetes in the laboratory, a condition which was accompanied by hyperinsulinemia. When islet functions were studied *in vitro*, signs of an increased functional demand on the β-cells were seen, and that there might be a gender difference in that the β-cells of males are more affected than the islets of the females.

**Paper II**
The pancreatic islets isolated from glucose tolerant bank voles are affected by prolonged exposure to proinflammatory cytokines. This effect seems to be essentially NO-independent, and a key cellular event appears to be a decreased level of insulin mRNA leading to a decline in insulin translation that result in a depletion of insulin stores in the β-cells and a lowered insulin response to a glucose challenge.

**Paper III**
We found that islets isolated from female and male bank voles display signs of dysfunction after high glucose culture *in vitro*, although this did not cause direct toxicity and/or cell death. A gender difference was observed suggesting that the islets of the females may more readily adapt to the elevated glucose concentration than islets of male bank voles.

**Paper IV**
The bank vole has been considered as a diabetes model showing features of both type 1 and type 2 diabetes. In this study we report that islets in severely affected animals have markedly changed structure, reduced β-cell mass and hyperinsulinemia, all of which are characteristics of a type 2 diabetes. These findings suggest that bank voles bred in the laboratory may develop similar features to type 2 diabetes. However, it cannot be excluded that bank voles caught in nature instead rather develop a type 1 form of the disease. This argues for a decisive role for interactions between environmental factors in the diabetes development in bank voles.
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