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ABSTRACT

It has been shown that there are patients with type 1 diabetes (T1D), whose sera induce an increased activity of voltage-gated Ca^{2+}-channels in pancreatic β-cells, resulting in increased cytoplasmic free Ca^{2+} concentration ($[Ca^{2+}]_i$) and apoptosis. Purification of the protein in the active fraction of T1D sera revealed that the observed effects were mediated by apolipoprotein CIII (apoCIII), and this protein was shown to be increased in serum from T1D patients. To be able to evaluate the importance of apoCIII *in vivo* for the development of T1D there is a need for a suitable animal model. We used the animal model diabetes-prone BB rat (DPBB) that spontaneously, at the age of around 60 days, develops a human-like T1D. Isolated islet cells cultured overnight in the presence of 10% sera from 60 days old prediabetic BB rats had a higher increase in $[Ca^{2+}]_i$ upon depolarization with K^+, an impaired glucose-induced insulin secretion and a decreased viability, compared to cells exposed to age-matched control sera. The prediabetic sera with this effect are referred to as positive (pos). The effect on $[Ca^{2+}]_i$ was abolished when an antibody against apoCIII was present during culture. The relative amounts of apoCIII in pos, neg and control sera from 60 days old rats were evaluated and the content in pos sera was significantly higher.
To investigate the effects of apoCIII *in vivo*, DPBB rats were treated with either active or inactive antisense against apoCIII between the age of 12 to 40 days. The apoCIII antisense treatment significantly delayed the onset of diabetes.

Wild bank voles (*Myodes glareolus*) develop T1D and a picornavirus, named Ljungan virus (LV), has been isolated from these animals. If CD-1 mice, that normally do not carry LV, are infected with this virus *in utero* and exposed to stress after birth, the male offspring get type 2 diabetes. In BB rats LV was found in both prediabetic- and diabetic DPBB rats, as well as in diabetes-resistant rats. To evaluate if the presence of virus influences the onset of T1D, prediabetic rats were given antiviral treatments, which prolonged the prediabetic phase with approximately one week. The interplay between LV and diabetes is complicated and still not understood, and our data does not exclude a role of this virus in the development of diabetes.

LIST OF PUBLICATIONS

I. Lowering apolipoprotein CIII delays onset of type 1 diabetes
R. Holmberg, E. Refai, A. Höög, R.M. Crooke, M. Graham, G. Olivecrona P.O. Berggren, L. Juntti-Berggren *Manuscript*

II. The BioBreeding rat diabetes model is infected with Ljungan virus
B. Niklasson, T. Hultman, R. Kallies, M. Niedrig, **R. Nilsson**, P.O. Berggren, L. Juntti-Berggren, S. Efendic, Å. Lernmark, and W.Klitz *Diabetologia*. 2007 Jul;50(7):1559-60

III. Antiviral treatments reduce severity of diabetes in Ljungan virus-infected CD-1 mice and delay onset in diabetes-prone BB rats **R. Holmberg**, W. Klitz, M. Blixt, P.O. Berggren, L. Juntti-Berggren, B. Niklasson *Microbiol Immunol*. 2009 Oct;53(10):567-72.

Related publications:

Type 1 diabetic serum interferes with pancreatic beta-cell Ca(2+)-handling
N. Dekki, **R. Nilsson**, S. Norgren, S.M Rössner, I. Appelskog, C. Marcus, O.Simell, A.Pugliese, R.Alejandro, C. Ricordi, P.O. Berggren, L. Juntti-Berggren
Biosci Rep. 2007 Dec; 27(6): 321-6.

Activin B receptor ALK7 is a negative regulator of pancreatic beta-cell function
P. Bertolino, **R. Holmberg**, E. Reissmann, O. Andersson, P.O Berggren, CF. Ibáñez. *Proc Natl Acad Sci U S A*. 2008 May 20;105(20):7246-51.

Transthyretin binds to glucose regulated proteins and is subjected to endocytosis by the pancreatic beta-cell
N. Dekki, E. Refai, **R. Holmberg**, M. Köhler, H. Jörnvall, P.O. Berggren and L. Juntti-Berggren *Manuscript*

CONTENTS

LIST OF ABBREVIATIONS

ApoAI	Apolipoprotein AI
Apo AIV	Apolipoprotein AIV
ApoCIII	Apolipoprotein CIII
BSA	Bovine serum albumin
$[Ca^{2+}]_i$	Cytoplasmic free Ca^{2+}-concentration
CVD	Cardiovascular disease
DNA	Deoxyribonucleic acid
DPBB	Diabetes prone BioBreeding
DRBB	Diabetes resistant BioBreeding
EC	Endothelial cell
ELISA	Enzyme linked immune sorbent assay
EMC	Encephalomyocarditis
ERK1/2	Extracellular-signal-regulated kinases 1/2
GAD	Glutamic acid decarboxylase
HAECs	Human aortic endothelial cells
HDL	High-density lipoprotein
HEPES	N-(2-Hydroxyethyl)piperazine-N´- (2-ethanesulfonic acid)
HLA	Human leukocyte antigen
HPLC	High pressure liquid chromatography
IAA	Insulin auto-antibodies
ICA	Islet cell antibodies
IL-6	Interleukin 6
IL-8	Interleukin 8
IHC	Immunohistochemistry
i.p.	Intraperitoneal
IRE	Insulin-response element
LDL	Low-density lipoprotein
LPL	Lipoprotein lipase
LV	Ljungan virus
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide
MAPKs	Mitogen-activated protein kinases
NOD	Non-obese diabetic
PCR	Polymerase chain reaction
PKC	Protein kinase C
RIA	Radio immuno assay
RNA	Ribonucleic acid
RT-PCR	Reverse transcriptase-polymerase chain reaction
T1D	Type 1 diabetes
T2D	Type 2 diabetes
TFA	Trifluoroacetic acid
TNF-α	Tumor necrosis factor- α
SIR	Standardized incidence ratios
SPF	Specific pathogen free

VLDL	Very low-density lipoprotein
VCAM-1	Vascular cell adhesion molecule-1
VP1-4	Viral protein 1-4

1 INTRODUCTION

1.1 TYPE 1 DIABETES

Type 1 diabetes (T1D), previously called insulin-dependent or juvenile diabetes, is the result of an autoimmune destruction of the pancreatic β-cells. The disease can occur at any age, but is often diagnosed in children and young adults (ADAExpertCommittee 1997).

During the first half of the 1990s, the overall incidence of T1D varied from 0.1 in Venezuela to 38 in Sardinia and 41 per 100 000/ year in Finland. In Sweden, the incidence was 30 per 100 000/ year (DIAMOND 2006). There has been a rapid global increase of T1D during the last decades. In Europe the incidence of T1D in children under 15 years of age in 2005 is estimated as 15 000, in 2020, the predicted number of new cases is 24 400 (Patterson et al. 2009). Not only is the incidence increasing, but there is also a shift towards a younger age at diagnosis. This has occurred in a relatively short time period and can therefore not be due to genetic reasons, but more likely changes in the environment.

Development of autoimmune T1D includes genetic susceptibility, dysregulation of the immune system and environmental factors (Ermann et al. 2001). (Figure 1).

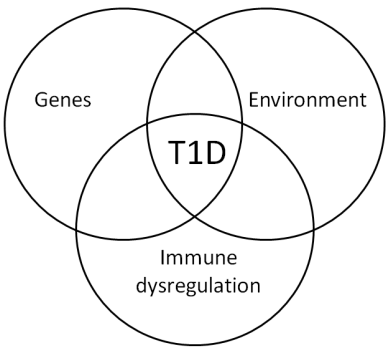

Figure 1. Etiology of T1D.

1.2 GENETIC DISPOSITION

The autoimmune process leading to T1D starts well before the disease is clinically present. To prevent the disease, it is necessary to identify subjects at risk. In the general population the risk to get the disease is about 0.5%. The family history of T1D is important, but it only identifies 13% of future cases (Dahlquist et al. 1985). To compare the relative risk in family members of T1D patients to the risk of those with no family history, standardized incidence ratios (SIRs) were calculated. SIR for T1D in offspring of diagnosed parents was 8.23, in singleton siblings 11.92, in twins 21.88 and in monozygotic twins 32.33 (Hemminki et al. 2009). This rather high familial risk is explained by a combination of shared genes and environment.

Many genes are referred to as susceptibility genes that by definition increase or modify disease risk. Susceptibility genes are neither necessary nor sufficient for disease development. Some gene carriers may never develop the disease, whereas some non-carriers do. The loci on chromosome 6 containing the HLA-DR and HLA-DQ regions is a major susceptibility interval accounting for about 45% of the total aggregated risk for T1D (Atkinson et al. 2001).

1.3 ENVIRONMENTAL FACTORS

Although the nature of the environmental "triggers" is unknown it is believed that it could be a specifically timed, short-lived event that converts a non-diseased to a pre-diseased individual. Environmental factors that have been suggested are:

- Different viruses as e.g. congenital rubella, enteroviruses and coxsackie B. There are several hypotheses how viruses induce or accelerate autoimmunity. One is the so called "molecular mimicry" where the virus shares structures with the autoantigen (Christen et al. 2010). Another is that viral infection can activate polyclonal lymphocytes or increase the immunogenicity due to target organ inflammation (Goldberg et al. 2009).

- Exposure to cow's milk in infancy. There are theories that a specific sequence of bovine albumin may cross-react with islet proteins (Akerblom 1996). The cow's milk is believed to trigger the gut immune system, which has been shown to be involved in the development of T1D in several studies (Kolb et al. 1999).

- Various toxins, such as N-nitroso compounds. Nitrate can be found in meat products and vegetables and is after transformation in the gut to N-nitroso compounds regarded as a risk factor for T1D. Antioxidants may protect the β-cells from being damaged by these compounds (Akerblom et al. 2002).

- There is also a possible, but debated, role of stressful life events in the development of T1D (Sepa et al. 2006).

1.4 IMMUNE DYSREGULATION

Inflammation, from the Latin word inflammatio for fire, is the biological response to infection, tissue injury or tissue malfunction (Medzhitov 2008). For the tissue not to be damaged an exact control of this process needs to be preserved. Although it remains unclear which immune system components or mechanisms that play the major role in β-cell destruction, most studies point towards the cellular immune system as playing the key role. Furthermore, multiple interrelated flaws in the immunoregulation may underlie the failure to form a tolerance to self-antigens that results in T1D. As the autoimmune disease process starts, autoantibodies to islet cell antigens develop. The most commonly used markers of anti-islet immunity are autoantibodies to insulin (IAA), glutamic acid decarboxylase (GAD), and ICA512/IA2A (Verge et al. 1998; Leslie et al. 1999; Knip et al. 2008). At the time of diagnosis about 90% of white children have at least one of these autoantibodies (Atkinson et al. 2001).

1.5 BB RAT

To be able to test preventive treatments for T1D it is necessary to use an accurate animal model that resembles the human form of the disease. For T1D there are mainly two models that are “human like”, namely the NOD (non-obese diabetic) mice and the DPBB (diabetes prone BioBreeding) rats. Their similarities and differences are listed in table 1. The BB rat was chosen because there is no gender difference and 100% of the DPBB rats develop the disease within a very narrow time window.

The BioBreeding rat was discovered at BioBreeding laboratories in Canada in 1974 (Nakhooda et al. 1977). Individuals with an autoimmune T1D were observed in a colony of outbred Wistar rats and continuous breeding resulted in a high incidence of T1D. In parallel to the breeding of the diabetic animals, a diabetes resistant strain was developed, this strain is referred to as DRBB.

Although the DPBB rat shares many characteristics with the human T1D, it must be stressed that no model will posses all features of the human disease.

	Human	DPBB rat	NOD mouse
Genetic predisposition	Yes	Yes	Yes
MHC association	Yes	Yes	Yes
Environmental factors	Yes	Yes	Yes
Insulitis	Yes	Yes	Yes
GAD antibodies	Yes	Yes	Yes
Lymphopenia	No	Yes	No
Other autoimmune diseases	Yes	Yes	Yes
Insulin-dependent	Yes	Yes	Yes
Long prediabetic period	Yes	Yes	Yes
Obesity	No	No	No
Sex differences	No	No	Yes
Secondary complications	Yes	Yes	Yes

Table 1. Characteristics of T1D in humans, DPBB rats and NOD mice. (Partial reproduction from Textbook of Diabetes, second edition, volume 1, p. 16.8)

1.6 APOLIPOPROTEIN CIII

Apolipoprotein CIII (apoCIII), is a 8.8 kDa polypeptide mainly synthesized in the liver and to a lesser extent in the intestine. The gene is located between the apoAI and the apoAIV genes on the long arm of chromosome 11(Karathanasis 1985; Jong et al. 1999). There are several pathways so far known to be involved in the regulation of apoCIII gene expression. One interesting, from the point of view of T1D, is the reduction by insulin via the IRE (insulin-response element) promoter (Chen et al. 1994). Peroxisome proliferator- activated receptors (PPARs) have been shown to reduce (Vu-Dac et al. 1998), whereas activation of NF-κB (Gruber et al. 1994; Ooi et al. 2008) and insulin resistance (Altomonte et al. 2004) induce apoCIII gene expression.
ApoCIII resides on the surface of apoB lipoproteins (e.g. chylomicrons, VLDL and LDL) and HDL and affects their metabolism. It does this mainly by inhibiting lipoprotein lipase (LPL), which metabolizes triglycerides and promote their clearance from the circulation (Kawakami et al. 2009). When the apoCIII gene was disrupted in mice, there was a 70% reduction in triglyceride levels (Gao et al. 2005), while an overexpression of human apoCIII in transgenic mice resulted in hypertriglyceridemia (de Silva et al. 1994). Under fasting conditions apoCIII is predominantly attached to HDL, whilst in the fed state it is mainly bound to apoB lipoproteins (Jong et al. 1999).

Several studies have shown that there is a relationship between apoCIII and cardiovascular diseases (CVD) (Lee et al. 2003; Kawakami et al. 2006, 113 Circulation; Kawakami et al. 2006, 114 Circulation; Kawakami et al. 2009). The nature of this relationship is not only due to modulations in lipoprotein metabolism. Inflammation has since long been regarded as an important part of the development of

arteriosclerosis. ApoCIII-rich lipoproteins, as well as apoCIII itself, increase the adhesion of monocytes to vascular endothelial cells (EC) (Kawakami et al. 2006, 113 Circulation). This apoCIII induced effect on monocytes was shown to be a result of activation of PKCα, NF-κB and β1-integrins in these cells (Kawakami et al. 2009). Furthermore, apoCIII increases the expression of vascular cell adhesion molecule-1 (VCAM-1) in ECs, thus facilitating adhesion of monocytes and thereby promoting atherogenesis (Kawakami et al. 2006, 114 Circulation). There are three isoforms of apoCIII, namely $apoCIII_0$, $apoCIII_1$ and $apoCIII_2$ referring to the number of sialic acid molecules bound to the protein. Of the total apoCIII present in the circulation each isoform contribute to about 10, 55 and 35%, respectively. Incubating human aortic endothelial cells (HAECs) with untreated apoCIII containing all isoforms, or desialyated apoCIII showed that only the sialylated apoCIII increased the concentrations of IL-6, IL-8, TNF-α and the expression of intracellular adhesion molecule (ICAM-1), all proinflammatory mediators (Hiukka et al. 2009).
An interesting observation has been made in the Lancaster Amish population where about 5% of the individuals are heterozygous carriers of a null mutation in the gene encoding apoCIII, resulting in a lifelong 50% reduction of the apoCIII levels, and a cardioprotective effect (Pollin et al. 2008).

ApoCIII has not only been related to CVD but also to diabetes. When sera from patients with T1D were investigated it was found that there are those that induce an increased activity of voltage-gated Ca^{2+}-channels in pancreatic β-cells, resulting in increased cytoplasmic free Ca^{2+} concentration ($[Ca^{2+}]_i$) and apoptosis (Juntti-Berggren et al. 1993). Purification of the protein in the active fraction of T1D sera revealed that the observed effects were mediated by apoCIII, and that the protein was increased in serum from T1D patients (Juntti-Berggren et al. 2004). It has been demonstrated that haplotypes in the apoCIII gene leading to augmented levels of apoCIII are associated with an increased susceptibility to T1D (Hokanson et al. 2006).

ApoCIII has also been shown to be up-regulated in serum from type 2 diabetic (T2D) patients with impaired β-cell function. When individuals with a family history of the disease were compared to individuals with no family history, the first group had increased levels of apoCIII. This would indicate that the altered level of apoCIII is due to a genetic disposition rather than the environment (Sundsten et al. 2008). It has been suggested that elevated circulating levels of apoCIII could contribute to β-cell death in T2D patients. The proposed mechanism of action for this is through activation of MAPKs p38 and ERK1/2 (Sol et al. 2009). Not only would the levels of apoCIII promote a deterioration of the β-cell function in T2D patients, but there are also epidemiological evidence for a correlation between the content of apoCIII in LDL and coronary events in these diabetic patients (Lee et al. 2003).

1.7 LJUNGAN VIRUS

In parts of Sweden, there is a cyclic change in rodent density with a peak every 3 or 4 years. In Grimsö (59°49´ N, 15°25´ E) small rodents have been collected yearly since 1973. During three following days once a year, 940 traps are set. The number of animals trapped per 100 trap nights is recorded. When the national census was used to calculate annual T1D incidence in the counties with cyclic rodent populations, a clear association between vole density and T1D incidence was seen (Niklasson et al. 1998). (Figure 2).

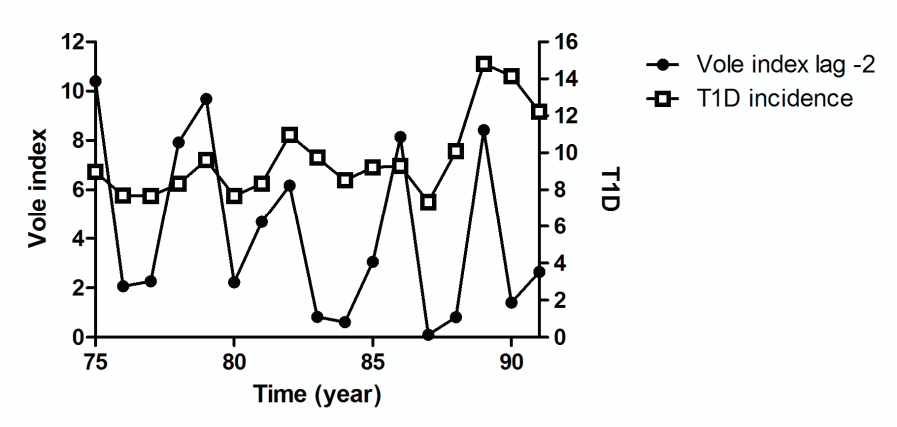

Figure 2. Vole density and T1D incidence.

Based on this association a search for an etiologic agent in small rodents was initiated. This resulted in the isolation of a novel picornavirus from the bank vole (*Myodes glareolus*) (Figure 3). The virus was named "Ljungan virus" (LV) after the valley in Västernorrland County, Sweden where it was originally isolated (Niklasson et al. 1999).

Figure 3. A bank vole.

B. Niklasson/ Apodemus

Picornaviruses have a protein capside containing three major polypeptides (VP1-3) and one minor polypeptide (VP4). They surround a singe-stranded RNA genome (7-8 kb) and appear as spherical particles of around 30 nm in diameter (Carrasco 1994).

The family of *Picornaviridae* is constantly growing. Other viruses that belong to this family are apthoviruses (e.g. foot-and-mouth), enteroviruses (e.g. polio, coxsackie B, meningitis), rhinoviruses (e.g. common cold), cardioviruses (infects rodents) and hepatovirus (e.g. hepatitis A) (Carrasco 1994). LV has similarities to all of them, but belongs to the genus of Parecho (Harvala et al. 2009).

The first observation of LV-related diseases was made in bank voles. When the animals where kept in captivity they developed T1D with polyuria, polydipsia, glucosuria, hyperglycemia, ketoacidosis, lysis of pancreatic islet β-cells and presence of GAD65-, IA-2 as well as insulin auto-antibodies (Niklasson et al. 2003, Ann N Y Acad Sci; Niklasson et al. 2003, Int J Exp Diabesity Res).

When the presence of LV antibodies was examined in children with newly diagnosed T1D and compared with healthy children, the T1D patients were more often antibody positive. The antibody titer was higher in the younger children. This might indicate that the children with T1D had been exposed to LV prior to onset (Niklasson et al. 2003, Int J Exp Diabesity Res).

2 AIMS

To investigate if sera from three different age groups (40-, 50- and 60-days old) of prediabetic DPBB rats interfere with intracellular Ca^{2+} handling and pancreatic β-cell function and viability in a similar way to what was previously found with human T1D sera. If the levels of apoCIII are increased, investigate if a reduction of the endogenous level of apoCIII will delay diabetes onset in DPBB rats. (Paper I)

In BB rats, as well as in humans, there have been studies showing evidence for that different viruses may play a role in the pathogenesis of T1D. Based on the observations of a possible connection between the presence of LV and diabetes I wanted to investigate if LV could be detected in our BB rat colony. (Paper II)

If LV could be detected in our prediabetic BB rats, investigate if antiviral treatment will influence the onset of the disease. To investigate further a possible link between LV and the development of diabetes, CD-1 mice, that develop type 2 diabetes if infected by LV *in utero*, will be subjected to antiviral treatment. (Paper III)

3 MATERIALS AND METHODS

3.1 ANIMALS

All experiments were carried out according to the approval from local Animal Experiment Ethics Committees. All animals had ad libitum access to standard food and water and were housed in a temperature- and humidity- controlled room with 12 hours light: dark cycles.

3.1.1 BB rats

BB rats were obtained from our breeding colony at Karolinska Institutet. The animals were housed under SPF (Specific Pathogen Free) conditions.

3.1.2 CD-1 mice

CD-1 (ICR) mice were obtained from Br Charles River laboratories, Germany. The mice were housed in individually HEPA ventilated cages.

3.2 MEDIUM

The medium used for isolation of pancreatic islets and cells, as well as for the experiments, was a HEPES buffer (pH 7.4) containing (in mM) 125 NaCl, 5.9 KCl, 1.2 $MgCl_2$, 1.28 $CaCl_2$ and 3 glucose. BSA was added at a concentration of 1 mg/ml. For cell culture S-MEM or RPMI 1640 culture medium, supplemented with 100 μg/ml streptomycin, 100 μg/ml penicillin, 2 mmol/l glutamine and 10% of the respective sera was used.

3.3 PREPARATION OF PRIMARY PANCREATIC ISLETS AND CELLS

Pancreatic islets from prediabetic and control BB rats were isolated by a collagenase technique (Lernmark 1974) and, when islet cells were used, disrupted into cells by Cell Dissociating Buffer. Islets were kept free floating in Petri dishes and cells were seeded onto either coverslips or into multiwell plates coated with poly-l-lysine.

3.4 QUANTIFICATION OF APOCIII IN SERA

To evaluate the levels of apoCIII in sera, we used Montage Albumin Deplete Kit (Millipore USA) that removes >65% of albumin from serum samples The collected samples from the columns were freeze-dried overnight and run on sep-Pak C18. The

eluted proteins were freeze-dried and thereafter dissolved in 100 ml 0.1%TFA and run on an ACE C18 10x0.21 cm column 20-60% and the area under the curve, where apoCIII elutes, was evaluated.
In sera from prediabetic rats, undergoing antisense treatment, we used AlbuSorb™ (Biotech Support Group) which removes >90% of albumin from the serum samples. Serum was added to a binding buffer with AlbuSorb powder. The samples were mixed and centrifuged and the supernatants were collected and freeze-dried overnight. The freeze-dried samples were dissolved in 100 ml 0.1%TFA and run on an ACE C18 10x0.21 cm column 20-60% and the area under the curve, where apoCIII elutes, was evaluated.
For both methods apoCIII was identified by MALDI mass spectrometry.

3.5 MEASUREMENTS OF $[Ca^{2+}]_i$

Cells attached to coverslips were loaded with 2 μM fura-2/ acetoxymethylester (Molecular Probes) and mounted on an inverted Zeiss Axiovert epifluorescence microsope connected to a Spex Fluorolog-2 system for dual-wavelength excitation fluorimetry. The emissions due to the two excitation wavelengths of 340 and 380 nm were used to calculate the fluorescence ratio 340/380, reflecting changes in $[Ca^{2+}]_i$ (Kindmark et al. 1992).

3.6 MEASUREMENTS OF INSULIN RELEASE

Dynamics of insulin release, when islets were stimulated with 11mM glucose, were studied by perifusing islets, mixed with Bio-Gel P4 polyacrylamide beads (Bio-Rad), in a 0.5 ml column at 37°C (Kanatsuna et al. 1981). The flow rate was 0.2 ml/min and 2 min fractions were collected and analyzed for insulin by radioimmunoassay using a rat insulin standard.

3.7 DETERMINATIONS OF CELL VIABILITY

Islet cells were seeded into a 96 well plate (5000 cells/well), coated with poly-l-lysine and exposed to 10% sera. After 24 hours, viability was determined by MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) assay (Mosmann 1983).

3.8 HISTOLOGICAL AND IMMUNOHISTOCHEMICAL TECHNIQUES

3.8.1 Insulin

Pancreatic tissue was removed and fixed in 4% formaldehyde solution overnight and

routinely embedded in paraffin. The pancreatic tissue was sectioned into 4 um thick sections and stained with Hematoxylin and Eosin and van Gieson's stain for collagen. The pancreatic β-cells were detected by immunohistochemistry. The tissue sections were incubated with an insulin antibody raised in Guinea Pig (diluted 1:200) overnight. The antibody binding in the sections was visualized using a standard Avidin-Biotin Complex method using diaminobenzidin as chromogen.

3.8.2 Ljungan virus

Formalin fixed paraffin embedded pancreas tissue was stained using LV VP1 specific polyclonal rabbit antisera and a mouse monoclonal antibody reactive to all known LV isolates but not to parecho- and cardio virus.

3.9 qPCR FOR DETECTING RELATIVE LEVELS OF APOCIII GENE EXPRESSION

RNA from snap frozen pancreatic islets, liver and spleen was isolated using RNA-Easy Kit with on-column DNase treatment (Qiagen). Subsequent cDNA conversion was carried out using a reverse transcriptase kit (Applied Biosystems). Real-time PCR studies (qPCR) for transcripts encoded by *apoCIII* were measured using intron spanning primers and SYBR Green with ROX kit (Invitrogen). qPCR detection and analysis were done using 7300 SDS (Applied Biosystems) and relative expression was measured using *β-actin* as a reference transcript. Values represent mean ± SEM, n=3-4. Primers for the full-length coding sequence of apoCIII were used for amplifying apoCIII coding transcripts from cDNA (25ng) of different tissues using Platinum-Taq (Invitrogen). The PCR product was run on a 2% agarose gel and the expected full-length transcript size for rat *apoCIII* was 306bp.

3.10 TRANSMISSION OF LJUNGAN VIRUS TO CD-1 MICE

Pregnant outbred CD-1 mice were infected i.p. with 1000 ID_{50} (determined by intra cerebral titration in suckling mice) units of LV145 SL two days after conception. Control animals were given an injection of saline. All female mice were kept in individual cages and followed for the development of gestational diabetes, pregnancy outcome and development of diabetes in the offspring. In all the pregnant mice mild stress was induced by adding glucose (100 g/L) to drinking water, performing weekly weights and a glucose tolerance test at the end of the pregnancy.

3.11 REAL TIME RT-PCR

Three independent real-time RT-PCR assays were used to identify and quantify LV infection. One PCR assay used primers and two different minor-groove-binder probes designed to amplify a 187-bp PCR product from the 5'-untranslated region of the LV genome. In addition we used primers designed to amplify a 103-bp PCR product from the 5'-untranslated region and a 164-bp product from the 2A region of the LV genome followed by a melting point analysis at the end of the amplification.

3.12 IN VIVO TREATMENTS

3.12.1 Treatment with antisense against apoCIII

DPBB rats were treated with apoCIII antisense (ISIS 353982) or an inactive control (ISIS 141923), n= 10 and 6, respectively. 25 mg/kg bodyweight were administrated i.p. twice per week between the age of 12 and 40 days.

3.12.2 Preparation of antiserum to LV

The brain from suckling NMRI mice infected intra-cerebrally at day 1 with the LV strains 145SL, 87-012 and 174F was used for immunization and infection. Antiserum to LV was made by infecting/immunizing adult mice with a 10% suckling mouse brain homogenate. The immune sera were collected from the adult mice 6-8 weeks after infection and pooled.

3.12.3 Antiviral agents

Pleconaril (VP-63843) an orally bio-available, broad spectrum anti-picorna-viral agent developed as a therapeutic tool in entero- and rhino viral infections. Ribavirin (1-beta-D-ribafuranosyl-1,2,4-triazole-3-carboxamide) is a synthetic nucleoside analogue with activity against a wide variety of both RNA and DNA viruses.

3.12.4 Anti LV treatment

At five weeks of age antiviral therapy to the male offsprings of CD-1 mice, using LV-specific murine antiserum or the oral antiviral compound Pleconaril (200 mg per kg body weight) or a combination of both, were given once a day for four consecutive days. The control groups of infected and uninfected male offsprings were untreated. From the age of 25 days all mice were subjected to stress by placing 4-5 male mice from different litters in the same cage. At 15 weeks of age the animals were subjected to a glucose tolerance test. Serum insulin levels and abdominal fat were measured.

A combination of Pleconaril and Ribavirin (200 mg of each per kg body weight) was used in the BB rat experiments. It was administrated orally with a tube connected to a syringe two times daily for nine consecutive days starting at day 38. A second group of BB rats were given a single dose i.p. of LV- specific murine antiserum, again at day 38.

3.13 ANALYSIS OF DIABETIC PARAMETERS IN THE CD-1 MICE

3.13.1 Analysis of insulin (CD-1)

Serum insulin was measured using a rat insulin ELISA kit

3.13.2 Analysis of abdominal fat (CD-1)

Abdominal fat in male mice was measured by dissecting out the bilateral scrotal fat cushion. The total weight of both fat cushions was used.

3.14 STATISTICAL ANALYSIS

3.14.1 Paper I

All statistical significance, except the evaluation of the antisense treatments, was evaluated by Student's t-test and p values < 0.05 were considered significant. Data are presented as means ± s.e.m.

The product limit method of Kaplan and Meier was used to estimate diabetes incidence after antisense treatment and the effect of the treatment was evaluated with a Log-rank (Mantel-Cox) Test, p values < 0.05 were considered significant.

3.14.2 Paper III

The glucose tolerance test in the CD-1 mice was evaluated by Fisher´s exact one sided test. Otherwise statistical significance in the CD-1 mice was evaluated by Student's t-test. P values < 0.05 were considered significant. Data are presented as means ± s.e.m.

Analysis of variance was used to test differences among treatments in the days-to-diabetes-onset variable in the BB rat experiment. The control groups for the antiserum and antiviral testing in the BB rat experiment were combined following an initial test. The three remaining treatments in the days-to-diabetes-onset variable were then analyzed separately. Specific pairwise differences among groups resulting from multiple comparisons testing following analysis of variance were evaluated with Bonferroni corrected tests. Data are presented as means ± s.e.m.

4 RESULTS AND DISCUSSION

4.1 PAPER I

The BB rat as an animal model for type 1 diabetes and the role of apolipoprotein CIII in the development of the disease

In this study the animal model DPBB rat that spontaneously, at the age of around 60 days, develops a human-like T1D was used. This model gives an excellent opportunity to study the impact of prediabetic interventions to prevent or delay the onset of the disease. Three prediabetic age groups 40-, 50- and 60 days, were investigated both *in vitro* and *in vivo*.

In pancreas taken from prediabetic rats at the age of 40 days there were fewer insulin-containing cells than in the age-matched controls. At the age of 50 days there was no difference, but at 60 days there was a pronounced decrease in the number of insulin positive cells. The insulin release data were in line with the morphological observations with a normalized glucose-induced secretion in the islets from 50 days old prediabetic rats. This phenomenon, with a temporary recovery, has been observed before (Bone et al. 1987; Sherry et al. 2006; Ablamunits et al. 2007; Pechhold et al. 2009). The reason for this transient improvement is not clear, but the explanation could be due to an increased cell replication, prior to onset of diabetes, that has been shown in several studies and suggested to be caused by the autoimmune attack (Bone et al. 1987; Sherry et al. 2006; Ablamunits et al. 2007; Pechhold et al. 2009).

Isolated islet cells cultured overnight in the presence of 10% sera from 60 days old prediabetic BB rats had a higher increase in $[Ca^{2+}]_i$ upon depolarization with K^+ , and a decreased viability, compared to cells exposed to age-matched control sera. Prediabetic sera, that induced this augmented increase in $[Ca^{2+}]_i$, are referred to as positive (pos). The relative amounts of apoCIII in pos, neg and control sera from 60-days old rats were evaluated and the content was significantly higher in the pos sera. To test if the increased level of apoCIII in pos sera was indeed the cause of the changed $[Ca^{2+}]_i$ response, cells were co-incubated with pos sera and an antibody against apoCIII, and this abolished the effect. These *in vitro* results from islet cells from BB rats confirmed our previous results, showing detrimental effects of apoCIII in both primary β-cells and β-cell lines (Juntti-Berggren et al. 2004).
The underlying cellular mechanisms whereby apoCIII impairs β-cell function are not known. Although most of the apoCIII is produced in the liver a local disturbance of expression of the protein within the pancreatic islets is a tempting working hypothesis. There is, to my knowledge, only one previous report of apoCIII in the islet (Waanders et al. 2009). This group did a quantitative proteomic analysis of pancreatic islets and among 6873 proteins they detected apoCIII. We have preliminary results showing the presence of apoCIII in rat islets (Figure 4). Our next aim is to see in which pancreatic islet cells apoCIII is expressed and if there is a

difference in diabetes prone compared to diabetes resistant animals.

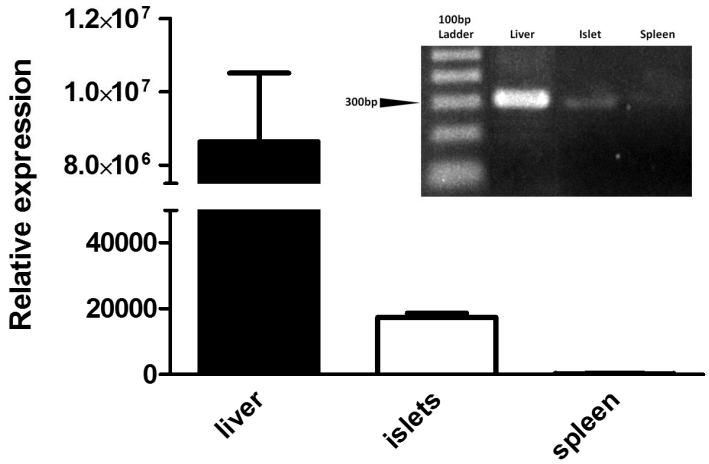

Figure 4. Relative expression of apoCIII gene in liver, islets and spleen from six weeks old Wistar rats, n = 3-4.

To investigate if apoCIII has effects *in vivo*, DPBB rats were treated with either active or inactive antisense against apoCIII by i.p. injections twice weekly between the age of 12 to 40 days. To verify that the antisense treatment had affected the endogenous levels of apoCIII, a blood sample was taken at the last day of treatment and the amount of the protein was found to be about 50% of the non-treated.
The major aim with this study was to clarify if a reduction of apoCIII in the prediabetic animal prolongs the time to onset of the disease. The obtained results are of importance showing an in average delay of 40 days, with a range between 71-158 days. The reasons for not achieving a total prevention could be due to the duration of the treatment or the doses given. In a pilot study the animals were injected following the same protocol, but with the aim to continue until diabetes onset. However, this resulted in an enlargement of the spleen in some animals and the treatment time was therefore shortened, which eliminated the side effects. A possibility to maintain the apoCIII levels down might be to give intermittent bolus doses or to do a partial knockout of the apoCIII gene in prediabetic animals. A lifelong 50% reduction of apoCIII, due to a mutation in the apoCIII gene, has been detected in a population of Amish people and this protected against CVD (Pollin et al. 2008). Therefore it would be of interest to see what the outcome would be with a similar genotype in a subject/animal predisposed to get T1D.

In summary, our results show that the DPBB rat model for T1D fulfills the criteria needed for testing our previous *in vitro* data on effects of apoCIII on β-cell function *in vivo*. The hypothesis, based on these *in vitro* results, that a lowering of apoCIII *in vivo* in the prediabetic animal would be protective, was proven to be true as there was a significant prolongation of the prediabetic phase. These data are encouraging with

regard to the development of a treatment regimen based on suppressing the levels of apoCIII that should be beneficial not only for diabetes, but also for individuals at risk for CVD.

4.2 PAPER II

Presence of Ljungan virus in the BB rat

In BB rats, as well as in humans, there have been studies showing evidence for that different viruses may play a role in the pathogenesis of T1D (Guberski et al. 1991; Hillebrands et al. 2003). In Sweden a co-variation of the incidence of diabetes in children and the 3-4 year population cycles of the bank vole has been reported (Niklasson et al. 1998). This co-variation initiated a search for a possible infectious agent, carried by the rodent and transferred to humans, and this resulted in the isolation of a picornavirus named Ljungan virus (LV), from the bank vole (Niklasson et al. 1999). What increased the interest for this virus was that T1D was diagnosed in captured wild bank voles (Niklasson et al. 2003, Ann N Y Acad Sci; Niklasson et al. 2003, Int J Exp Diabesity Res). Based on these findings prediabetic, diabetic diabetes-prone (DP), as well as diabetes-resistant (DR), BB rats from our colony were examined to elucidate if LV could be detected in them.

Virus isolation has proved to be difficult and infection may occur without a detectable antibody response (Niklasson et al. 1999). Even when a laboratory animal display severe disease symptoms and the presence of virus and viral RNA can be determined by IHC and PCR, serology tests can still be negative. A total of 26 BB rats, 16 of them from our colony and ten from a laboratory in the USA, were examined and all were LV positive by IHC. Positive staining was found in several organs, including heart, pancreas and brain, indicating a systemic infection. In the pancreas the staining was limited to the islets. IHC positive BB rats were confirmed by PCR. The virus was found in prediabetic, diabetic as well as DR rats. In addition, ten Wistar and five Sprague Dawley rats also showed positive staining by IHC, indicating that LV may be found in several laboratory rat strains, while at least CD-1 mice has shown to be non-carriers of the virus. The fact that LV can be found in samples not only from Sweden, but also from other countries, indicates that this virus is not only causing an incidental infection at a single location, nor is it limited to a specific strain or even species.

This study only shows the presence of LV in our BB rat strain, but no evidences for an involvement in the development T1D

4.3 PAPER III

Does treatment against Ljungan virus affect the development of diabetes in CD-1 mice and DPBB rats?

The background to this study is the following observations:

- Bank voles, which are carriers of LV, captured in the wild can have T1D (Niklasson et al. 2003, Int J Exp Diabesity Res) while those born in captivity develop T2D (Blixt et al. 2007).

- CD-1 mice, which do not normally carry LV, develop T2D at 10-15 weeks of age if infected by LV *in utero* and exposed to stress (Niklasson et al. 2006, Diabetologia).

- DPBB rats, that spontaneously develop T1D, have been shown to be carriers of LV (Niklasson et al. 2007).

The specific aims of this study were to investigate whether the T2D in the *in utero* infected CD-1 mice could be prevented and if the onset of T1D in the DPBB rats, that are natural carriers of LV, could be delayed by antiviral treatments.

Pregnant CD-1 mice were infected with LV i.p. two days after conception and thereafter exposed to mild stress (glucose in the drinking water, weekly weights and a glucose tolerance test). It was found that none of the mothers developed diabetes. After birth, the offspring were subjected to change in cage company since mixing the litters is known to be stressful for the animals. The male, but not the female, offspring developed diabetes at the age of 10-15 weeks with increased blood glucose, insulin levels and epididymal fat. A combination therapy, given at four consecutive days started at the age of five weeks, including antiserum and the antiviral drug Pleconaril, specific for the Picornavirus family, but not specific for LV, significantly reduced the blood glucose levels, the abdominal fat, and the serum insulin levels.
Our results are in line with previous observations that exposure to the virus in CD-1 mice is not enough, but that psychological stress has to be included for the induction of diabetes. Of interest to note is that it seems that females are more resistant to stress. Psychological stress has been discussed to increase the risk for diabetes (Sepa et al. 2006). During a stressful situation we are, by increasing the concentration of adrenaline and cortisol, preparing for fight or flight. Adrenaline causes an inhibition of insulin secretion and cortisol reduces the insulin sensitivity (Sepa et al. 2006). Our results clearly indicate that the viral infection plays a role for development of diabetes in this animal model, not normally susceptible for the disease, but that it needs to act in concert with other stressful events.

The second animal model used in this study was the DPBB rat. They were treated with a single dose of antiserum against LV at the age of 38 days or a combination of the antiviral drugs Pleconaril and Ribavirin, which has a broad-spectrum antiviral activity against both RNA and DNA viruses but is not specific for LV, twice daily for nine consecutive days starting at day 38. In the group treated with antiserum, the prolongation of the prediabetic phase was about four days and in the group treated with antiviral drugs, the onset was delayed with around one week.
Why the antiviral treatments did not delay the diabetes onset with more than approximately a week in our DPBB rats could be due to several reasons. We know that LV can not by itself induce T1D, as the DRBB rats are carriers, but do not get the disease. In the DPBB rat there is a genetic background promoting diabetes development any maybe the contribution of the virus is only playing a minor role in the progressive destruction of the β-cells. Another concern is that our treatment was not effective enough as the drugs used are not specific for LV and maybe did not totally eradicate the virus. Also the duration of the treatment might not have been optimal.
This is not the first time a virus has been connected to diabetes in BB rats. Kilham´s rat virus has been shown to induce diabetes in DRBB rats, but without affecting the onset of T1D in DPBB rats (Guberski et al. 1991). On the contrary, Cytomegalovirus infection accelerated T1D in DPBB rats, but did not induce diabetes in DRBB rats (Hillebrands et al. 2003).

To summarize our observations from this study, there is not a simple relationship between LV infection and diabetes. In the CD-1 mice we have indications that the viral infection plays a role, but is not enough *per se* to induce diabetes. However, in the DPBB rat model the picture is more difficult to interpret and at present it is unclear whether LV is involved as an environmental agent in the pathogenesis of T1D.

5 CONCLUDING REMARKS

We know from a considerable amount of studies that the development of diabetes is a complex process that includes several different parameters. Recent studies have drawn the attention to apolipoprotein CIII (apoCIII) and Ljungan virus (LV) as possible factors involved in the pathogenesis of the disease and they have been studied in this thesis.
From this study the following conclusions were reached:

Isolated islet cells cultured overnight in the presence of 10% sera from 60 days old prediabetic BB rats had a higher increase in $[Ca^{2+}]_i$ upon depolarization with K^+, an impaired glucose-induced insulin secretion and a decreased viability, compared to cells exposed to age-matched control sera. In resemblance with previously published results from human type 1 diabetes (T1D) sera, the levels of apoCIII were increased. These data confirmed that this animal model for T1D was suitable to study if decreasing the levels of apoCIII *in vivo* affects the onset of T1D. Treating prediabetic rats with an antisense against apoCIII reduced the endogenous levels of the protein and significantly delayed the onset of disease, thereby confirming our hypothesis that apoCIII is involved in the development of T1D. The main conclusion is that these data indicate that it would be beneficial to lower the levels of apoCIII in individuals at risk for developing T1D.

In BB rats LV was found in both prediabetic- and diabetic DPBB rats, as well as in DRBB rats. To evaluate if the presence of virus influenced the onset of T1D, prediabetic rats were given antiviral treatments, which prolonged the prediabetic phase with approximately one week.
In CD-1 mice antiviral treatment significantly reduced blood glucose and insulin levels as well as the amount of abdominal fat.
The interplay between LV and diabetes is complicated and still not understood, and our data does not exclude a role of this virus in the development of diabetes.

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