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SERUM PROTEINS IN TYPE 1 DIABETES

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**Karolinska
Institutet**

Stockholm 2007

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Printed by Repro Print

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ISBN 91-7357-057-5

*Vilken tur att det inte är med livet som med en bok,
att man kan läsa det sista kapitlet först*

ABSTRACT

We have previously shown that there are patients with type 1 diabetes (T1D) whose sera interfere with cytoplasmic free Ca^{2+} -concentration ($[\text{Ca}^{2+}]_i$), resulting in β -cell apoptosis. In this study the frequency of sera, that induces this effect, was found to be around 35% in T1D patients, and 25% in first degree relatives (FDR) of T1D patients. The effect occurred in subjects of different gender, age and ethnicity, and was not related to the presence of autoantibodies. Hence, in a defined subgroup of patients with T1D and FDR a defect Ca^{2+} -handling may aggravate the autoimmune response and thereby the development of β -cell destruction, and it might be possible to interfere early on with Ca^{2+} -induced β -cell apoptosis.

Purification and fractionation of T1D sera, that affected $[\text{Ca}^{2+}]_i$, revealed that the protein that gave a higher increase in $[\text{Ca}^{2+}]_i$ upon depolarization, was apolipoprotein CIII (apoCIII). ApoCIII increases the activity of voltage-gated Ca^{2+} -channels. A proof for apoCIII being the responsible serum component was that the addition of an antibody against apoCIII abolished, not only the effects of apoCIII on $[\text{Ca}^{2+}]_i$ and cell death, but most importantly the effects of T1D sera itself on β -cell function and survival.

During the procedure to purify the serum factor, we observed a band at 14 kDa on SDS/PAGE that was stronger in T1D than control sera. The protein in the band was identified by sequence analysis to be the monomeric form of transthyretin (TTR). TTR is a transport protein and exists mainly as a tetramer in sera from healthy individuals. In T1D sera the concentration of TTR was decreased, whereas the concentration of the monomer was increased. Physiological concentrations of the tetrameric form of TTR was shown to affect the β -cell stimulus-secretion coupling, promoting glucose-induced increases in $[\text{Ca}^{2+}]_i$ and insulin release, which resulted from a direct effect on glucose-induced electrical activity and voltage-gated Ca^{2+} -channels. The tetrameric form of TTR also protected against apoCIII induced β -cell death. The monomer was without effect on glucose-stimulated insulin release and β -cell death. Therefore, conversion of TTR tetramer to monomer may be involved in the development of β -cell failure in T1D.

TTR was shown to bind to glucose regulated proteins (Grp), 78, 94 and 170 in both the membrane and cytosolic fractions of islet cell homogenates, and was internalized into the β -cell via a clathrin-dependent pathway, indicating the involvement of receptor-mediated endocytosis. The Grp complex of Grp78, 94 and 170 may serve as a plasma membrane protein responsible for the binding and uptake of TTR into the β -cell. These data may suggest that the effects of TTR on β -cell function and survival are due to intracellular effects of the protein.

In conclusion, there is a group of patients with T1D and FDR that have sera that affects $[\text{Ca}^{2+}]_i$ and β -cell survival. The serum factor responsible for these effects was identified to be apoCIII, which is increased in T1D sera. The concentration of TTR tetramer is decreased, whereas that of the TTR monomer is increased in T1D sera. The tetrameric form of TTR was shown to have a positive role in the β -cell stimulus-secretion coupling. These data support the possibility to develop a diagnostic test to identify individuals at risk to be subjected to Ca^{2+} -induced β -cell damage and thereby aggravation of the autoimmune response in T1D.

ISBN 91-7357-057-5

LIST OF PUBLICATIONS

This thesis is based on the following papers, which in the text will be referred to by their Roman numerals:

- I. **Nancy Dekki**[†], Rebecka Nilsson, Svante Norgren, Sophia M Rössner, Ioulia Appelskog, Claude Marcus, Olli Simell, Alberto Pugliese, Rudolfo Alejandro, Camillo Ricordi, Per-Olof Berggren och Lisa Juntti-Berggren (2006). Defect pancreatic β -cell Ca^{2+} -handling after exposure to serum from type 1 diabetic patients and first-degree relatives. *Submitted*
- II. Lisa Juntti-Berggren^{*}, Essam Refai^{*}, Ioulia Appelskog, Mats Andersson, Gabriela Imreh, **Nancy Dekki**[†], Sabine Uhles, Lina Yu, William J Griffiths, Sergei Zaitsev, Ingo Leibiger, Shao-Nian Yang, Gunilla Olivecrona, Hans Jörnvall and Per-Olof Berggren (2004). Apolipoprotein CIII promotes Ca^{2+} -dependent β -cell death in type 1 diabetes. *Proc. Natl. Acad. Sci. USA*; 101, 10090-10094
- III. Essam Refai^{*}, **Nancy Dekki**^{*†}, Shao-Nian Yang, Gabriela Imreh, Over Cabrera, Lina Yu, Guang Yang, Svante Norgren, Sophia M Rössner, Luca Inverardi, Camillo Ricordi, Gunilla Olivecrona, Mats Andersson, Hans Jörnvall, Per-Olof Berggren och Lisa Juntti-Berggren (2005). Transthyretin constitutes a functional component in β -cell stimulus secretion coupling. *Proc. Natl. Acad. Sci. USA*; 102,17020-17025
- IV. **Nancy Wenna**[†], Essam Refai, Jawed Shafqat, Moin Ishrat, Hans Jörnvall, Per-Olof Berggren och Lisa Juntti-Berggren. Transthyretin binds to glucose regulated proteins and is endocytosed into the pancreatic β -cell. *Manuscript*

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LIST OF ABBREVIATIONS

Ab	Antibodies
ApoCIII	Apolipoprotein CIII
BSA	Bovine serum albumin
$[Ca^{2+}]_i$	Cytoplasmic free Ca^{2+} -concentration
Cch	Carbamylcholine
CCV	Clathrin coated vesicle
DIG	Digoxigenin
ECL	Enhanced chemiluminescence
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme linked immuno sorbent assay
FCS	Fetal calf serum
FDR	First degree relatives
GAD	Glutamic acid decarboxylase
Grp	Glucose regulated protein
HB	Homogenization buffer
HEPES	N-(2-Hydroxyethyl)piperazine-N'-(2-ethanesulfonic acid)
HLA	Human leukocyte antigen
HPLC	High pressure liquid chromatography
HSP	Heat shock protein
IAA	Insulin autoantibody
ICA	Islet cell autoantibodies
LB	Lysis buffer
LAMP-1	Lysosomal associated membrane protein 1
MAB	Maleic acid buffer
MHC	Major histocompatibility complex
NBT/BCIP	Nitro-Blue Tetrazolium Chloride 5-Bromo-4-Chloro-3'-Indolyphosphate p-Toluidine Salt
PBS	Phosphate buffered saline
PM	Plasma membrane
PVDF	Poly vinylidene difluoride
RIA	Radio immuno assay
SDS	Sodium dodecyl sulfate
T1D	Type 1 diabetes
TBS	Tris buffered saline
TFA	Trifluoroacetic acid
Tris	Tris(hydroxymethyl)aminomethane
TTR	Transthyretin

1 INTRODUCTION

Diabetes mellitus has been recognized as a disease since antiquity. A papyrus from the 3rd Dynasty of Egypt, by the physician Hesy-Ra, shows the earliest description of diabetes. The word diabetes was first used by the Greek physician Aretaeus from Cappadocia in the 2nd century AD. The exact words of Aretaeus (translated by Francis Adams, London, 1856):

“Diabetes is a wonderful affection, not very frequent among men, being a melting down of the flesh and limbs into urine. Its cause is of a cold and humid nature, as in dropsy. The course is the common one, namely, the kidney and bladder; for the patients never stop making water, but the flow is incessant, as from the opening of aqueducts. The nature of the disease, then, is chronic, and it takes a long period to form; but the patient is short lived, if the constitution of the disease be completely established; for the melting is rapid.”

In type 1 diabetes (T1D) there is a deficiency of insulin. T1D can, according to recommendations from the American Diabetes Association, be divided into two forms:

- Type 1a – where there is an autoimmune destruction of the pancreatic β -cells
- Type 1b – a non-immune mediated diabetes with severe insulin deficiency without known etiology.

From now on T1D will refer to the type 1a form.

Research over the past 30 years has established that T1D is an autoimmune disease. The evidences for autoimmunity are:

- Infiltration of lymphatic cells into the islets of Langerhans as a sign of inflammation
- Autoantibodies
- Other autoimmune disorders are associated with T1D
- Inherited susceptibility

However, the direct mechanisms involved in the initiation and progression of the disease, are still not identified. A difficulty is that there is often a long prodromal phase between the start of insulinitis and the onset of disease. Moreover, T1D is not a homogeneous disease and the events involved can probably differ between different patients. From an epidemiological point of view, there is an interesting international variation in incidence. A Finnish child has, compared to a child in Japan, and a child in the Zunyi region of China, a 40 and a 100 times, respectively, higher risk to develop T1D. The etiology is complex and imperfectly understood, but there are three cornerstones (Figure 1):

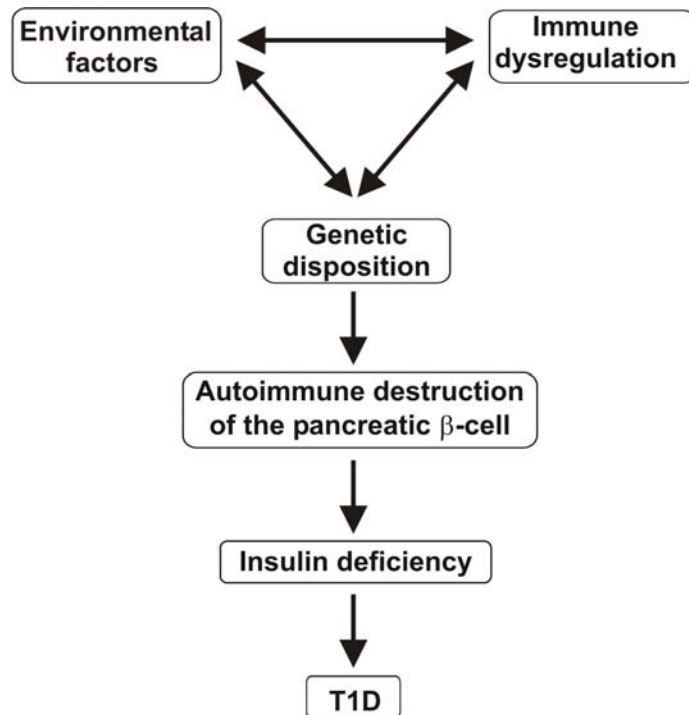


Figure 1. Etiological events involved in the development of T1D.

1.1 INHERITED SUSCEPTIBILITY

T1D is associated with several genes at different loci. The strongest linkage is with the human leukocyte antigen (HLA) genes lying within the major histocompatibility complex (MHC) region on the short arm of chromosome 6 (Atkinson and Eisenbarth, 2001). More than 90% of patients who develop T1D have either DR3, DQ2 or DR4, DQ8 haplotypes, whereas fewer than 40% of normal controls have these haplotypes (Devendra et al., 2004)

1.2 AUTOANTIBODIES

The first detectable sign of β -cell autoimmunity is the appearance of autoantibodies. There are four autoantibodies that have been shown to predict T1D (Knip et al., 2002). These include islet cell antibodies (ICA), insulin autoantibodies (IAA), autoantibodies to the 65 kDa isoform of glutamic acid decarboxylase (GAD), and the tyrosin phosphatase-related IA-2 molecule (Baekkeskov et al., 1990; Bonifacio et al., 1995; Bottazzo et al., 1974; Palmer et al., 1983). Presence of two or more autoantibodies gives a higher probability of developing the disease. In family studies, including relatives of patients with T1D, detection of multiple autoantibodies is associated with a higher risk of developing T1D (Kukko et al., 2005; Verge et al., 1996).

1.3 ENVIRONMENTAL FACTORS

There are several facts that support the role of exogenous agents in the development of T1D. Of all cases of T1D, 10% occur in people of European origin, with Finland having the highest rate of incidence (Gillespie, 2006). Preliminary results from the Finnish study TRIGR (Trial to Reduce IDDM in the Genetically at Risk) suggest that it is possible to manipulate the appearance of β -cell autoimmunity by eliminating cow's milk from the diet of infants (Akerblom et al., 2005). In another study from Finland they found that early enterovirus infections and early exposure to cow's milk are risk factors for T1D (Vaarala et al., 2002). Several other studies have also found evidence for a relationship between enteroviral infections and T1D (Coulson et al., 2002; Honeyman et al., 2000). Other observations that support the hypothesis of an environmental trigger are that there is a seasonal variability, temporal variation from year to year and that β -cell autoimmunity is not necessarily induced at the same time in all genetically susceptible siblings within a family (Knip et al., 2005).

1.4 SPECIFIC BACKGROUND

In our studies we have previously shown that there are patients with newly diagnosed T1D whose sera induce an increased activity of voltage-gated Ca^{2+} -channels in insulin-secreting β -cells, resulting in increased cytoplasmic free Ca^{2+} -concentration ($[\text{Ca}^{2+}]_i$), and apoptosis (Juntti-Berggren et al., 1993). These *in vitro* findings are interesting and may be part of the autoimmune reaction associated with the destruction of the pancreatic β -cells *in vivo* in a subpopulation of diabetic patients. The aim of this thesis was to further investigate these serum effects.

2 AIMS

1. Investigate the frequency of sera from patients with T1D of different gender, age, ethnic background and duration of the disease, and first-degree relatives (FDR) of T1D patients, with and without autoantibodies, that induce the observed effects on $[Ca^{2+}]_i$ and β -cell apoptosis.
2. Identify and characterize possible serum factor(s) responsible for the changes in $[Ca^{2+}]_i$ and β -cell apoptosis.
3. Study molecular mechanisms whereby the identified serum factor(s) interfere with the function of the β -cell.

3 MATERIAL AND METHODS

3.1 PREPARATION OF PRIMARY BETA-CELLS

Adult *ob/ob*-mice from a local colony were used (Hellman, 1965). Pancreatic islets were isolated by a collagenase technique and cell suspensions were prepared as previously described (Lernmark, 1974; Nilsson et al., 1987) or with a non-mechanical disruption method, followed by incubation with Versene 1:5000. Thereafter, the islets were disrupted into cells in an enzyme free Hanks based solution. Cells were seeded onto glass coverslips or kept in suspension and cultured in RPMI 1640 culture medium, supplemented with 10% fetal calf serum (FCS), 2 mM glutamine 100 U/ml penicillin and 100 µg/ml streptomycin.

3.2 HUMAN PANCREATIC ISLETS

Human islets were isolated and quantified as described before (Ricordi et al., 1988). The islets were cultured in CMRL 1066 culture medium supplemented with 10 % FCS, 2 mM Glutamax, 100 U/ml penicillin, 100 µg/ml streptomycin and 5.5 mM glucose

3.3 CELL LINE

The RINm5F cell line is a clonal insulin producing cell line established from a transplantable rat islet cell tumour (Oie et al., 1983).

3.4 MEDIUM

The basal medium used for isolation of the pancreatic islets and for further experiments was a HEPES buffer (pH 7.4) containing (in mM): 125 NaCl, 5.9 KCl, 1.2 MgCl₂, 1.28 (mouse) or 2.56 (human) CaCl₂, 25 HEPES and 3 glucose.

3.5 SERUM MATERIAL

The first group of T1D patients was identified in Stockholm, Sweden, and included 22 Caucasian children (age 1-15 years) and 60 adults (age 17-39 years) with newly diagnosed T1D. The second group included 56 patients from the U.S. with a heterogeneous ethnic background, divided into three subgroups depending on the duration of the disease; 0-12 months, 1-10 years and >10 years. The age of onset of T1D in this group was between 3-36 years. In addition, serum from 15 autoantibody (ab) positive and 102 ab negative first-degree relatives (FDR) of patients with T1D, were studied. Control sera were collected from healthy age matched children and from adult healthy blood donors, of which none had diabetes-related ab. A third group of sera from Finland consisted of 31 samples from Caucasian Finnish children with newly diagnosed T1D and matched healthy controls. All serum samples were heat-inactivated by incubation at 56°C for 30 min, identically sterile-processed and stored frozen at -20°C until used.

3.6 PURIFICATION OF SERA

Serum samples from diabetic patients that induced an enhanced response in $[Ca^{2+}]_i$ were centrifuged and the supernatant was passed through a 0.45 mm sterile filter. Samples were loaded on Sep-Pak C₁₈ preconditioned with 0.1% trifluoroacetic acid (TFA). After application, the sample proteins were eluted with 60% acetonitrile (ACN) in 0.1% TFA and were then lyophilized. Batches of the lyophilized sample were

dissolved in 0.1%TFA and injected into a reverse phase (RP)-HPLC with a Vydac C₁₈ column. The separation was made by using a linear gradient of 20-60% ACN in 0.1% TFA. The collected fractions were lyophilized.

3.7 IDENTIFICATION OF SERUM PROTEINS

Fractions of diabetic sera, that induced an enhanced increase in [Ca²⁺]_i, was identified and lyophilized. Primary sequence of the serum protein was obtained for 20 N- and 5 C-terminal degradations. Protein molecular weights were determined by electrospray mass spectrometry. To determine the position of glycosylation, the proteins were digested with trypsin and separated with HPLC using a Vydac C18. The separated fractions were applied to mass analysis.

Lyophilized material from diabetic and control sera were submitted to two SDS/PAGEs where one gel was stained with Coomassie brilliant blue and one was blotted on a poly vinylidene difluoride membrane (PVDF). The protein bands of interest were cut out and digested in the gel with trypsin. After digestion, the material was run on a micro HPLC. Fractions were collected and mass fingerprinted by MALDI mass spectrometry. The results were confirmed by amino acid sequencer analysis for 15 cycles.

3.8 MEASUREMENTS OF [Ca²⁺]_i

Changes in [Ca²⁺]_i were recorded in cells pre-incubated with 10% T1D-, FDR- or control serum (Paper I), apoCIII (Paper II), TTR or TTR monomer (Paper III and IV) in RPMI 1640 culture medium with or without FCS. Cells were attached to coverslips and incubated in HEPES buffer containing a fluorescent intracellular Ca²⁺ indicator fura-2/acetoxymethyl ester. The coverslips were mounted as a bottom of an open chamber and cells were perfused with medium. Fluorescence signals were recorded with a Spex Industries Fluorolog-2 system for dual-wavelength excitation fluorimetry, connected to an inverted epifluorescence microscope. 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 [Ca²⁺]_i.

3.9 PATCH CLAMP

3.9.1 Whole cell recordings

Whole-cell Ca²⁺ currents were recorded in mouse pancreatic β-cells, incubated overnight with apoCIII (Paper II), TTR (Paper III) or the vehicle, by using the perforated-patch variant of the whole-cell patch-clamp recording technique. Electrodes were pulled from borosilicate glass capillaries on a horizontal programmable puller and filled with a solution containing (in mM): 76 Cs₂SO₄, 1 MgCl₂, 10 KCl, 10 NaCl, and 5 HEPES (pH 7.35), as well as amphotericin B (0.24 mg/ml) to permeabilize the cell membrane and allow low-resistance electrical access without breaking the patch. The cells were bathed in a solution containing (in mM): 138 NaCl, 10 tetraethylammonium chloride, 10 CaCl₂, 5.6 KCl, 1.2 MgCl₂, 5 HEPES and 3 D-glucose (pH 7.4). Whole-cell currents induced by voltage pulses (from a holding potential of -70 mV to several clamping potentials from -60 to 50 mV in 10 mV increments, 100 ms, 0.5 Hz) were filtered at 1 kHz and recorded. All recordings were made with an Axopatch 200 amplifier at room temperature (RT) (≈ 22°C). Acquisition and analysis of data were done using the software program pCLAMP6.

3.9.2 Membrane potential recordings

Perforated-patch whole cell recordings were also employed to register membrane potential in the β -cells incubated overnight with TTR (Paper III). Typical electrode resistance was 2–4 M Ω . Electrodes were filled with a solution containing (in mM) :76 K₂SO₄, 1 MgCl₂, 10 KCl, 10 NaCl, and 10 HEPES (pH 7.35), as well as amphotericin B (0.24 mg/ml). The cells were bathed in a buffer containing (in mM): 138 NaCl, 5.6 KCl, 2.6 CaCl₂, 1.2 MgCl₂, 5 HEPES, and 3 glucose (pH 7.4) for 1 hour before membrane potential registration. Membrane potential was recorded during perfusion with 3 and 16.7 mM glucose in current-clamp mode with an Axopatch 200 amplifier. All recordings were made at 34°C.

3.10 MEASUREMENT OF INSULIN RELEASE

3.10.1 Mouse

Dynamics of insulin release were studied by perfusing islet cell aggregates mixed with Bio-Gel P4 polyacrylamide beads 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 radio immuno assay (RIA) using a rat insulin standard.

3.10.2 Human

Insulin release was measured in islets mixed with Bio-Gel P4 polyacrylamide beads in a 0.27 ml column at 37°C. Fractions were collected every minute at a flow rate of 0.1 ml/min. Insulin content was analyzed with ELISA. PicoGreen double-stranded DNA (dsDNA) quantification reagent kit was used to quantify dsDNA in the human islets from each perfusion column. The amount of insulin released is presented as μ U/ng of dsDNA.

3.11 FLOW CYTOMETRIC ANALYSIS OF CELL DEATH

Cells from *ob/ob* mice or RINm5F cells were cultured for 48 h in the presence of 10% T1D or control serum with or without the presence of anti-apoCIII (Paper II), apoCIII (Paper II+III), total TTR or TTR monomer (Paper III), or the vehicle. The whole cell population was collected and stained with GFP-conjugated annexin V (Paper II) or PI (Paper II+III) and analyzed on a FACScan using CELLQuest acquisition software. FACS gating, based on forward and side scatter, was used to exclude cellular debris and autofluorescence. Typically 10 000 cells were selected for analysis.

3.12 PREPARATION OF WHOLE CELL LYSATES

Cells were washed with cold PBS and lysed on ice in lysis buffer (LB) containing; 250 mM NaCl, 50 mM tris(hydroxymethyl)aminomethane (Tris), 1 mM EDTA, 0,1% SDS, 1% NP-40, 0,5% sodium deoxycholate and protease inhibitor cocktail.

3.13 PREPARATION OF MEMBRANE AND CYTOSOL FRACTIONS OF ISLET HOMOGENATE

Islets were homogenized on ice in homogenization buffer (HB) containing (in mM): 20 HEPES (pH 7.5), 1 MgCl₂, 2 EDTA and protease inhibitor cocktail. The homogenate was centrifuged and the supernatant was collected and centrifuged in an ultracentrifuge. The supernatant which contained the soluble proteins (cytosol) and the pellet (membranes) were collected and resuspended in HB. All working steps were performed on ice.

3.14 NUPAGE ELECTROPHORESIS AND WESTERN BLOT ANALYSIS

Protein concentration was measured with the Bradford protein determination assay. Equal amounts of protein (10-20 µg) from cytosol or membrane fractions, CCV fractions or eluates from protein interaction with Dynabeads RP C18 were mixed with NuPage sample buffer and separated over a gradient NuPage-gel (4-12%). After electrophoresis, the protein bands were either stained with coomassie brilliant blue or transferred to a PVDF membrane. The membrane was blocked with 5% non-fatty powdered milk in a buffer containing: 10 mM Tris (pH 7.5), 100 mM NaCl and 0.05% Tween20 (TBS-T) for 1h or with blocking reagent in a buffer containing in M: 0.1 maleic acid (pH 7.5) and 0.15 NaCl (MAB). Thereafter, the membrane was incubated with the appropriate primary antibodies, washed with TBS-T or MAB with the addition of 0.3% Tween (MAB-T) and incubated with secondary horseradish peroxidase-conjugated antibodies or polyclonal sheep anti-digoxigenin Fab fragments conjugated with alkaline phosphatase. The immunoreactive bands were visualized using ECL PlusTM immunoblotting detection system or with alkaline phosphatase NBT/BCIP.

3.15 TTR ADSORPTION TO DYNABEADS RP C18

Magnetic beads (Dynabeads RP C18, 50 mg/ml) were washed with 0.1% TFA. TTR or transferrin (40 µg) were adsorbed to the beads and incubated at RT for 2 min. The beads were washed with 0.1% TFA and thereafter incubated with cytosol or membrane fractions, for 1 h. Elution was made with 60% ACN for 2 min. All incubations were done at room temperature (RT). Eluates obtained were run on NuPage gel electrophoresis and on a RP-HPLC with an Ace C18 column (10 x 0.2 cm). The separation was made using a linear gradient of 20-60 % ACN in 0.1% TFA acid for 30 min at 0.2 ml/min.

3.16 LABELING OF TTR WITH DIGOXIGENIN AND ALEXA488

FLUOROPHOR

TTR was labeled with Digoxigenin-succinimidyl ester (DIG-NHS) or Alexa488-tetrafluorophenyl-ester (Alexa488-TFP). Non-bound DIG-NHS and Alexa488-TFP were separated on a Sephadex G-25 column, respectively a Bio-Rad Biogel P-30 fine resin. Unbound Alexa488-TFP was collected and used for control experiments.

3.17 LIGAND BLOTTING

Aliquots of equal amounts of protein (20-40 µg) from cytosol or membrane fractions were run on NuPage (4-12%) electrophoresis. After electrophoresis, proteins were transferred to a nitrocellulose membrane and then blocked for 4h with 5% non-fatty powdered milk in TBS-T or blocking reagent in MAB containing TTR or TTR-DIG. The washing procedure and further incubations steps were done as described above.

3.18 ENDOCYTOSIS STUDIES

In the following preparations, before adding TTR-DIG, the cells were kept at 4°C to normalize to basal metabolism.

3.18.1 Clathrin coated vesicle preparation

Isolated β -cells from 5-6 *ob/ob*-mice were incubated with TTR-DIG for 15 min. Preparation of CCV-fractions was done as described before (Hammond and Verroust, 1994).

3.18.2 Internalization studies

β -cells were incubated with TTR-DIG for different time points; 15, 30, 60, 120 or 180 min. The cells were washed with phosphate buffered saline (PBS) and lysed in LB. Controls were made with isolated cells, incubated for 60 min with TTR-DIG and then incubated with pronase in RPMI 1640 culture medium, containing 20 mM HEPES (pH 7.6) to eliminate surface bound proteins. The cells were digested for 50 min at 4°C. The reaction was stopped by adding an equal volume of 10 mg/ml bovine serum albumin (BSA). The cells were washed with PBS and then lysed in LB. Protein concentration was measured with the Bradford protein determination assay and 10 μ g of protein was electrophoresed on NuPage (4-12%) gel, followed by transfer and detection of DIG.

3.19 DETECTION OF TTR-DIG AND TTR-ALEXA488 IN SINGLE BETA-CELLS

β -cells attached to coverslips were incubated with TTR-DIG for different time points in RPMI 1640 culture medium. The cells were washed in PBS and fixed in 4% formaldehyde. Permeabilization was done with 0.3% Tween20 and thereafter the cells were incubated with polyclonal sheep anti-DIG Fab fragments conjugated with alkaline phosphatase. Control cells were incubated with levamisol to inhibit endogenous alkaline phosphatase. Alkaline phosphatase precipitates in the β -cells were visualized in a light microscope.

Cells attached to coverslips were incubated with TTR-Alexa488 or only Alexa488 for 16 h in RPMI 1640 culture medium. The cells were washed in HEPES buffer and the coverslips were mounted on an open chamber with a high rubber ring to allow the cells to be alive in buffer. The cells were visualized in a confocal microscope.

3.20 STATISTICAL ANALYSIS

Statistical significance was evaluated by Student's paired and un-paired t-test. *P*-values <0.05 were considered significant. Data are presented as means \pm SEM.

4 RESULTS AND DISCUSSION

4.1 PAPER I

4.1.1 Studies on sera from different populations

We have previously reported that there are sera from patients with T1D that interfere with Ca^{2+} -handling (Juntti-Berggren et al., 1993). However, this study was performed on a limited number of patients and gave no information of the frequency of subjects that have components in their sera that induces changes in $[\text{Ca}^{2+}]_i$, and thereby disturbs the function of the β -cell (Juntti-Berggren et al., 1993). In the first paper the aim was therefore to investigate the frequency of sera from patients with newly diagnosed T1D that affects $[\text{Ca}^{2+}]_i$, as well as if this serum effect remains when the patient has had diabetes for a longer period of time. Also, we wanted to examine if the serum effects are present in both children and adults and in subjects of different ethnic background. Furthermore, we wanted to clarify if there are sera from FDR of T1D patients, with and without ab, that affect $[\text{Ca}^{2+}]_i$. To investigate this serum samples were collected from Swedish Caucasians, both children and adults, Finnish Caucasian children and adult Americans with a mixed ethnic background. In the American group we also had samples from FDR. The increase in $[\text{Ca}^{2+}]_i$ upon depolarization with K^+ , was compared between cells exposed to control sera and to cells exposed to sera from patients with newly diagnosed T1D or FDR to T1D patients. Those T1D or FDR sera that gave a higher increase in $[\text{Ca}^{2+}]_i$ than control sera are referred to as positive and those that were similar to control sera, negative. An example of such a comparison is shown in figure 1D Paper I.

Swedish patient population

The Swedish Caucasian patient population consisted of 22 children and 60 adults with newly diagnosed T1D. In the group of children there were 12/22 (54%) that had serum that induced higher increases in $[\text{Ca}^{2+}]_i$ upon K^+ depolarization. Unfortunately, ab were not measured in these patients at the time of diagnosis. Within the group of adults with T1D, 24/60 (40%) had a higher increase in $[\text{Ca}^{2+}]_i$ compared to control subjects. In this group, ab were measured in almost all patients. There was no correlation between those sera that induced enhanced increases in $[\text{Ca}^{2+}]_i$ and the presence of ab (Table 1, Paper I).

American subject population

Among the American subjects, with a mixed ethnic background, there were totally 14/56 (25%) patients with T1D whose sera affected the $[\text{Ca}^{2+}]_i$. The patients were then divided into different subgroups depending on the duration of the disease, and there were positive sera in each group; 0-12 months: 2/10, 1-10 years: 7/29, >10 years: 5/17 had positive sera. In the group of FDR of T1D patients, with and without ab, 3/15 and 32/102 (20 % and 31%), respectively, had sera that gave a higher increase in $[\text{Ca}^{2+}]_i$ compared to sera from healthy controls. As in the Swedish Caucasian group no correlation was found between sera that induced increased changes in $[\text{Ca}^{2+}]_i$ and the occurrence of ab (Table 1, Paper I).

Finnish patient population

In the Finnish serum material, consisting of serum from Caucasian children with newly diagnosed T1D, there were 8/31 (26%) sera that promoted enhanced changes in $[\text{Ca}^{2+}]_i$.

Data of the presence of ab was only available for three positive children where two of them had ab.

The influence of T1D sera on Ca^{2+} -handling and cell death has also been studied in neuronal cells. These studies show that serum from patients with T1D, with or without neuropathy, affect $[\text{Ca}^{2+}]_i$ and cell death in insulin secreting cell lines, primary rat islets and neuronal cell lines (Caraher et al., 1999; Conroy et al., 2002; Pittenger et al., 1993; Pittenger et al., 1995; Pittenger et al., 1997; Zanone et al., 2003). In contrast to our experiments, where the effect was not acute, an increase in $[\text{Ca}^{2+}]_i$ was shown in neuroblastoma cells acutely exposed to T1D sera (Pittenger et al., 1997). Since these sera were collected from T1D patients with neuropathy, the presence of additional or different factors are possible. Also, it may reflect a difference in response between neuroblastoma and insulin-secreting cells. Moreover, T1D sera have been shown to be cytotoxic in neuronal cells, causing cell death and inhibiting cell growth after two days of culture (Pittenger et al., 1993; Pittenger et al., 1995).

We have shown that the increases in $[\text{Ca}^{2+}]_i$ and apoptosis could be prevented by the L-type Ca^{2+} -channel blocker verapamil (Juntti-Berggren et al., 1993). Other studies show similar results where the L-type Ca^{2+} -channel blocker nifedipine has been used and partially prevented the cytotoxic effect of T1D sera in rat islets and neuronal cell lines, suggesting that an increase in Ca^{2+} -influx to the cells is involved in the toxic effect (Caraher et al., 1999; Conroy et al., 2002). Of interest to note is that we have investigated the effect of diabetic sera on primary cerebellar granule cells. T1D sera promoted the growth of a unique cell with an irregular triangular soma, classified as a neuronal cell due to the presence of low voltage-gated Ca^{2+} -currents and long neuronal dendrites (Chandra et al., 2001). Hence T1D sera, from a subgroup of patients, not only interfere with the pancreatic β -cell, but also with other types of cells, which could be of importance for the development of diabetic complications.

This study shows that there are patients with T1D, of different gender and ethnicity, which have factor(s) in their sera that affect intracellular Ca^{2+} handling with negative consequences for cell function and survival. The fact that serum from a group of FDR also affects $[\text{Ca}^{2+}]_i$, may be used in the development of a diagnostic test to identify individuals at risk to be subjected to Ca^{2+} -induced β -cell destruction.

4.2 PAPER II

4.2.1 Identification of the serum factor

To reveal the identity of the factor in T1D sera that increases $[Ca^{2+}]_i$, diabetic sera were fractionated with RP-HPLC and the yielded fractions were subsequently tested for changes in $[Ca^{2+}]_i$ in β -cells. One fraction, eluting between 52-60% ACN, was identified to induce a higher increase in $[Ca^{2+}]_i$, upon depolarization with high concentrations of K^+ , to open the voltage-gated L-type Ca^{2+} -channels. This fraction was further purified with repeated RP-HPLC runs. All obtained fractions were tested and one positive fraction was identified (Figure 2A-D, Paper II). The sequence information of the protein in this fraction was identified by both N- and C-terminal degradations and the sequences obtained were identical to those of human apolipoproteinCIII (apoCIII).

ApoCIII is a 79 residue, 8.8-kDa polypeptide (Brewer et al., 1974), mainly synthesized in the liver and to a smaller extent in the intestine (Wu and Windmueller, 1979; Zannis et al., 1985). ApoCIII has three known isoforms that differ in terms of glycosylation, CIII₀ (no sialic acid), CIII₁ (one sialic acid residue), and CIII₂ (two sialic acid residues), contributing to approximately 10%, 55%, and 35%, respectively, of total plasma apoCIII (Holdsworth et al., 1982; Kashyap et al., 1981). Mutagenesis of the glycosylation site and expression in stable cell lines suggest that intracellular glycosylation of apoCIII is not required for its intracellular transport and secretion, and does not affect the affinity of apoCIII for plasma very low density (VLDL) and high density (HDL) lipoproteins (Roghani and Zannis, 1988). ApoCIII plays an important role in metabolism of triglyceride-rich lipoproteins (Fredenrich, 1998) by inhibiting the activity of lipoprotein lipase (Ginsberg et al., 1986; Krauss et al., 1973; Wang et al., 1985). Overexpression of human apoCIII in mice results in severe hyperglyceridemia (Ito et al., 1990), whereas disruption of the apoCIII gene in mice leads to a 70% reduction in triglyceride levels (Maeda et al., 1994). The apoCIII gene is part of the apoAI/CIII/AIV/AV gene cluster on chromosome 11q23-q24 (Protter et al., 1984). Insulin is involved in the regulation of the apoCIII gene by down-regulating it at the transcriptional level (Chen et al., 1994).

The concentration of apoCIII has been found to be higher in diabetic patients than in healthy subjects (al Muhtaseb et al., 1992; Blackett et al., 1988; Bren et al., 1993; Briones et al., 1984; Joven et al., 1989; Krauss et al., 1973; Manzato et al., 1993; Reverter et al., 1993). The purified apoCIII from our T1D sera was analyzed by massspectrometry for subcomponent identification. The major components had masses that corresponded to the mono- and diglycosylated isoforms of apoCIII (Figure 2E, Paper II). The levels of apoCIII were evaluated as area under the curve from the second RP-HPLC (Figure 3A, Paper II). In T1D sera the levels of the glycosylated isoforms of apoCIII were four-fold higher than in control sera.

To test if apoCIII really was the serum factor we were looking for, β -cells were incubated with the protein, using concentrations found in diabetic patients. The effects on changes in $[Ca^{2+}]_i$, upon depolarization, were similar to those obtained by the product isolated from T1D sera. ApoCIII also induced an increased cell death. A proof for that apoCIII is the responsible serum component was that the addition of an antibody against apoCIII abolished, not only the effects on $[Ca^{2+}]_i$ and cell death by apoCIII, but most important by T1D sera itself.

To elucidate the molecular mechanisms underlying the effects on $[Ca^{2+}]_i$, the activity of voltage-gated Ca^{2+} -channels were analyzed in β -cells exposed to apoCIII. Cells treated with apoCIII displayed larger Ca^{2+} -channel currents than control cells during depolarization in the range -10 to 10 mV, from a holding potential of -70 mV. These data are in line with our previous study (Juntti-Berggren et al., 1993) where we also had

effects, not only in insulin-secreting cells, but also in GH₃ cells, within the range of physiological depolarizations.

Still it is not known if apoCIII exerts its effects bound to the plasma membrane (PM) or if it is internalized into the β -cell. In a preliminary study immunocytochemistry was applied to study the uptake of apoCIII. Mouse β -cells were preincubated for 15, 30 and 60 min with apoCIII, followed by incubation with a primary polyclonal rabbit anti-human apoCIII and a secondary antibody coupled to Alexa488. The cells were investigated with confocal microscopy and apoCIII could be detected in the β -cells already after 15 min (Figure 2A). When omitting the primary antibody or the preincubation with apoCIII, no un-specific staining could be visualized (Figure 2D-E). These data suggests that apoCIII is taken up into the β -cell.

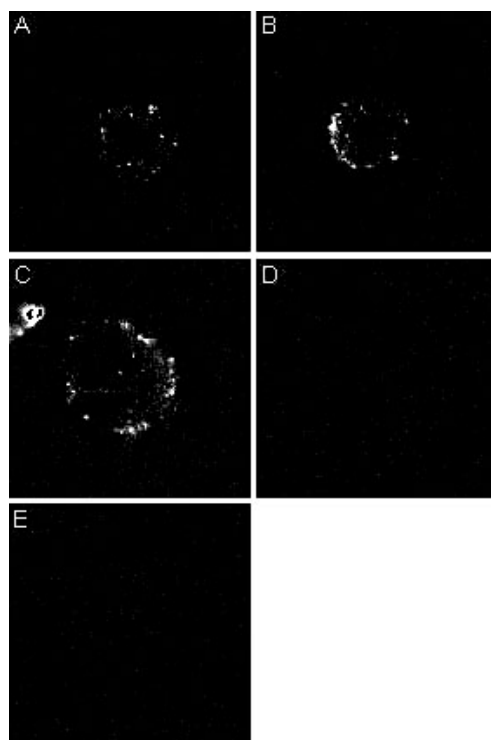


Figure 2. Internalization of apoCIII into the β -cell. Preincubation with apoCIII for (A) 15 min (B) 30 min and (C) 60 min. (D) Preincubation with only the secondary antibody and (E) preincubation where apoCIII was excluded.

In conclusion, the results in paper II suggest that the Ca²⁺-dependent cytotoxic effect of T1D serum in the pancreatic β -cell is mediated by apoCIII. Recently, further support for this notion was obtained when it was reported that an apoCIII gene haplotype block, leading to increased apoCIII levels, is associated with susceptibility to T1D in humans (Hokanson et al., 2006).

4.3 PAPER III

4.3.1 Transthyretin and the beta-cell

During the procedure to purify apoCIII from sera, we observed that a protein band at 14 kDa appeared much stronger in sera from T1D patients than controls, when serum from children and adults with T1D and healthy controls were run on SDS/PAGE. Sequence analysis revealed that the protein in the band was the monomeric form of transthyretin (TTR).

TTR is produced in the liver and in the epithelial cells surrounding the choroid plexus of the brain (Dickson et al., 1985; Soprano et al., 1985). In addition, TTR has been found to be produced in the retina and the pancreatic islets (Itoh et al., 1992; Jacobsson et al., 1990). TTR is a transport protein for thyroxin and, in association with retinol-binding protein, for retinol (van Jaarsveld et al., 1973). TTR is a serum protein consisting of four identical subunits of 14 kDa each (Blake et al., 1978). It has a complex equilibrium between different quaternary structures in serum (Pettersson et al., 1987). In serum TTR mainly exists as a tetramer (Figure 3A), with only a small amount of monomer present *in vivo* in healthy subjects (Blake et al., 1978; Sekijima et al., 2001) (Figure 3B).

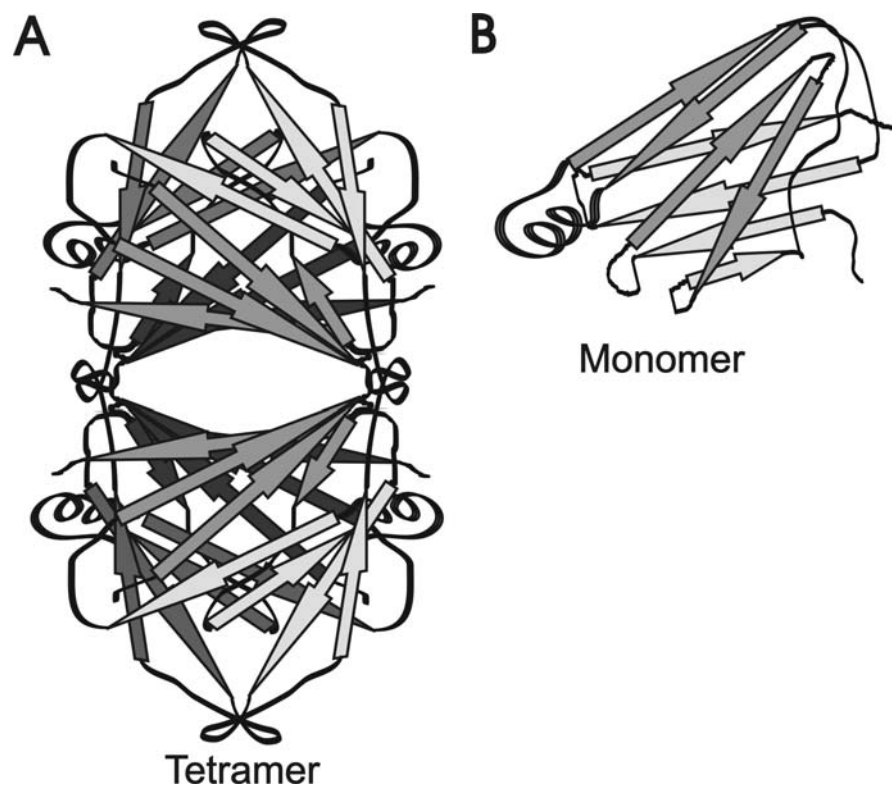


Figure 3. The TTR tetramer (A) and monomer (B).

There is a group of diseases called TTR amyloidoses in which misfolded TTR aggregates in different tissues (Kelly, 1998; Sousa and Saraiva, 2001). More than 80 point mutations in the TTR gene have been described. Several of these mutations are related to familial amyloidotic polyneuropathy (FAP). In another amyloidotic disease, senile systemic amyloidosis (SSA), aggregates of wild-type TTR is mainly seen in the heart (Westermarck et al., 1990).

In T1D patients the concentration of TTR in serum, mainly reflecting the tetramer, is decreased (Jain et al., 1992; Kemp and Frindik, 1991; Kobbah et al., 1988). However, nothing regarding possible effects of TTR on the β -cell function has been reported, therefore we incubated isolated β -cells overnight with physiological concentrations of TTR. The cells exposed to TTR had a higher increase in $[Ca^{2+}]_i$ subsequent to stimulation with glucose and depolarizing concentrations of K^+ , than control cells incubated with the vehicle. This effect was abolished when the cells were coincubated with TTR and an antibody against TTR.

The activity of voltage gated Ca^{2+} -channels was analyzed and Ca^{2+} -currents, registered from TTR-treated cells, depolarized to -20 mV, were significantly enhanced as compared to those from control cells. Mouse β -cells are equipped with multiple types of voltage-gated Ca^{2+} channels that become activated at different potentials (Schulla et al., 2003). Our finding that there was an effect at -20 mV with a tendency of increased currents also at -10 mV, most likely reflects that TTR up-regulates both the L_D - and R-type Ca^{2+} -currents (Namkung et al., 2001; Yang and Berggren, 2005; Yang and Berggren, 2006; Yang et al., 1999). These data support that TTR acts on the voltage gated Ca^{2+} -channels, because the effects occurred in the range of physiological depolarizations (Rorsman and Trube, 1986; Yang and Berggren, 2005; Yang and Berggren, 2006).

In trying to elucidate if TTR mobilizes Ca^{2+} from intracellular stores, β -cells, preincubated with TTR tetramer overnight, were stimulated with carbamylcholine (Cch) and depolarizing concentrations of K^+ in the presence and absence of extracellular Ca^{2+} . Preliminary data show that there was no difference in the increase in $[Ca^{2+}]_i$ in a Ca^{2+} free medium, after stimulation with Cch (Figure 4). However, when Ca^{2+} was present in the extracellular medium a more pronounced increase in $[Ca^{2+}]_i$, upon stimulation with Cch and K^+ , was observed in TTR treated cells (Figure 4). This implies that the effects of TTR on changes in $[Ca^{2+}]_i$, mainly reflects Ca^{2+} influx over the PM, also then influencing Ca^{2+} mobilization from intracellular pools.

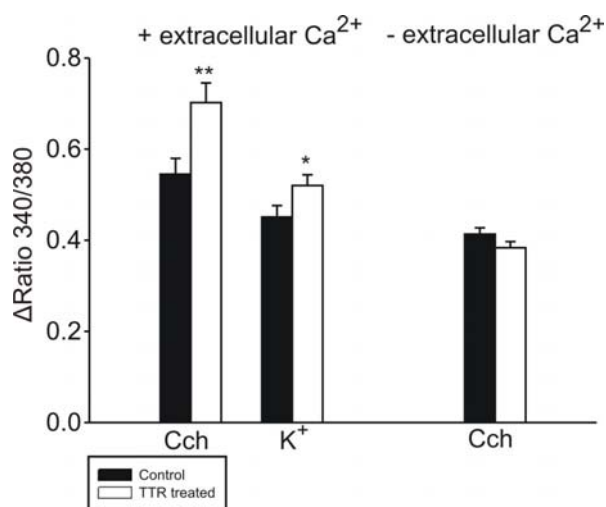


Figure 4. Effects on $[Ca^{2+}]_i$ in mouse β -cells preincubated with TTR tetramer.

To investigate if the increases in $[Ca^{2+}]_i$ were paralleled by changes in insulin release, mouse β -cells were incubated with TTR and thereafter stimulated with glucose and KCl in a perfusion system. TTR enhanced both basal and glucose-stimulated insulin release, while no difference on K^+ -induced insulin secretion was seen. The reason maybe that TTR have an additional interaction with the metabolic steps involved in glucose-stimulated insulin release. Therefore, it was of interest to analyze changes in β -cell membrane potential, a parameter determined by glucose metabolism. There was no difference in membrane potential at 3 mM glucose, but at 16.7 mM glucose TTR treated cells had a higher frequency of action potentials than control cells. These results indicate that TTR indeed enhances glucose metabolism and thereby increases glucose induced electrical activity.

Since our new observation was that the band on SDS/PAGE, representing TTR monomer, was of a higher intensity in T1D patients, than in normal subjects, the monomer was purified from commercially available TTR and tested on β -cells. The concentrations, 1 and 2 μ g/ml, were chosen based on the only study, to our knowledge, where the levels of monomer have been analyzed. The concentration in sera from control subjects was reported to be 0.6 ± 0.2 μ g/ml (Sekijima et al., 2001).

In β -cells, exposed to the two concentrations of monomer, stimulation with glucose and depolarizing concentrations of K^+ gave a more pronounced increase in $[Ca^{2+}]_i$, compared to control cells. Glucose and K^+ stimulated insulin release was not affected, but basal insulin secretion was higher in cells incubated with 2 μ g/ml TTR monomer. A very interesting finding was that, whereas TTR tetramer partly protected against apoCIII-induced β -cell death, the monomer was without effect.

These data demonstrate that TTR tetramer constitutes a functional component in the β -cell stimulus-secretion coupling. The observation that there is an increase in the monomeric form of TTR in T1D sera may indicate that the conversion of tetramer to monomer is associated with the development of β -cell failure and destruction in T1D.

4.4 PAPER IV

4.4.1 Binding and endocytosis of TTR

Several studies, in different cell types, have presented evidences for that TTR is endocytosed via a receptor mediated pathway (Divino and Schussler, 1990a; Divino and Schussler, 1990b; Kuchler-Bopp et al., 2000; Vieira et al., 1995; Vieira and Schneider, 1993). Membrane associated proteins of 90, 100 and 115 kDa have been proposed as putative TTR receptors, but they have not yet been identified (Kuchler-Bopp et al., 2000; Sousa and Saraiva, 2001; Vieira et al., 1995; Vieira and Schneider, 1993).

Dynabeads were utilized to study TTR binding to cytosol and/or membrane proteins. The TTR coupled beads were incubated with the cytosol and membrane fractions from β -cells and possible protein interactions were analyzed with gel-electrophoresis and masspectrometry after elution. Protein bands obtained were of an approximate molecular weight of 70, 98 and 110 kDa for the membrane fraction, and of 98 kDa for the cytosol fraction. The identity of these bands was revealed by mass mapping using MALDI masspectrometry, and corresponded to the masses of glucose-regulated proteins (Grp) 78, 94 and 170. The results were confirmed by N-terminal amino acid sequencer analysis. Further analysis of the proteins bound to the Dynabeads-TTR complex, were performed with RP-HPLC. This resulted in identification of Grp78, and Grp94 in the membrane fraction, but the cytosol fraction contained too little amount of protein material. However, ligand blotting demonstrated that TTR binding proteins, both in the membrane and cytosol fractions were of a molecular weight of 70, 98 and 110 kDa. The fractions were electrophoresed again and protein bands of interest were cut out for mass mapping by MALDI masspectrometry and corresponded to Grp94 and Grp170 in the membrane fraction, and Grp170 in the cytosol fraction. These data suggest an interaction between TTR and Grp78, Grp94 and Grp170 in the membrane fraction, and Grp94 and Grp170 in the cytosol fraction.

Grps are molecular chaperones with protective properties. They are stress proteins, located in the ER, and are induced by conditions such as oxidative stress, defective Ca^{2+} -homeostasis and glucose deprivation (Lee, 1992). Grp78, Grp94 and Grp170 are Ca^{2+} -binding proteins (Koch et al., 1986; Lievremont et al., 1997; Macer and Koch, 1988) and help to regulate Ca^{2+} -homeostasis in the cell, thereby protecting it from unphysiological increases in $[\text{Ca}^{2+}]_i$ that can result in cell death (Liu et al., 1998; Miyake et al., 2000). The Grps are involved in protein folding, protein translocation and regulation of protein secretion from the ER. Grp 78 and Grp94 have been shown to have trans-membrane domains that spans the ER and can therefore be seen in both membrane and luminal fractions of the cells (Kang and Welch, 1991; Reddy et al., 2003). A number of studies have revealed the presence of Grp78 and Grp94 in the PM in different cell types (Arap et al., 2004; Calvert et al., 2003; de Crom et al., 1999; Delpino and Castelli, 2002; Reddy et al., 1999; Robert et al., 1999; Triantafilou et al., 2001; Wiest et al., 1997). In liver cells, Grp94 was observed within the PM in coated pits, which suggests its involvement in receptor-mediated endocytosis, a process described for several receptors (Brown and Goldstein, 1986). Grp170 can associate directly or indirectly with Grp78 and Grp94 suggesting that these three Grps may function as a multimeric complex (Meunier et al., 2002). Internalization of TTR was studied in β -cells pre-incubated with TTR-DIG. TTR was taken up into the β -cell in a time dependent manner, with a peak at 60 min followed by a plateau, which is in line with studies in other cell types (Kuchler-Bopp et al., 2000).

To verify that TTR was really internalized into the β -cell, proteins bound to the surface of the cell were removed by proteolytic digestion with pronase. Western-blot analysis showed that TTR was still present after the enzyme digestion. The internalization was

also studied at single cell level using immunocytochemistry. Fixed β -cells were pre-incubated with TTR-DIG and TTR was present in 13% of the β -cells after 15 min and in 22% after 60 min. In living β -cells, incubated overnight with TTR-Alexa488, there were punctuate structures throughout the cell. No DIG or Alexa488 could be detected in control cells.

Endocytosis is a cellular process by which eukaryotic cells internalize material from the extracellular medium. A specific and saturable uptake of TTR in hepatomas and astrocytes, consistent with the existence of a TTR-receptor, has been reported (Divino and Schussler, 1990a; Divino and Schussler, 1990b). The initial step in receptor-mediated endocytosis occurs through different mechanisms, where the best characterized is via clathrin-coated pits. Most receptor-ligand complexes accumulate at clathrin-coated pits of the PM. Internalization of TTR has been described to occur through a clathrin-dependent pathway in chicken oocytes, which suggests a receptor-mediated endocytosis of TTR (Vieira et al., 1995; Vieira and Schneider, 1993). TTR has been shown to be present in coated-pits in choriocapillaries (Smith et al., 1989). However, there is a study in an ependymoma cell line, where they claim that endocytosis of TTR is clathrin-independent (Kuchler-Bopp et al., 2000). In order to clarify if the endocytosis of TTR in β -cells is clathrin-dependent, clathrin-coated vesicles (CCV) were isolated from β -cells pre-incubated with TTR-DIG. Immunodetection showed that CCV and TTR were present in the same fraction. These data indicate that TTR is endocytosed into β -cells via a clathrin-dependent receptor-mediated pathway.

TTR from human plasma has been shown to bind to the human lysosomal associated membrane protein 1 (LAMP-1) (Chang et al., 2004). To investigate if the endocytosed TTR is taken up into lysosomes, colocalization of TTR-Alexa488 and Lysotracker Red, which is a red-fluorescent dye that stains acidic compartments, was studied in living β -cells. Preliminary data does not show any colocalization.

5 CONCLUSIONS

From this study the following conclusions were reached:

- In the populations we investigated, around 35% of patients with T1D, and 25% of FDR of T1D patients, had sera that affected $[Ca^{2+}]_i$. This effect occurred in subjects of different gender, age and ethnicity, and was not related to the presence of autoantibodies.
- The observed cytotoxic effects of T1D serum in the pancreatic β -cell were shown to be mediated by apoCIII, and the level of this protein was increased in diabetic sera. The apoCIII induced increases in $[Ca^{2+}]_i$ reflect an activation of the voltage-gated Ca^{2+} -channels.
- TTR is a serum protein that in physiological concentrations has a positive role in the pancreatic β -cell stimulus-secretion coupling. This results from a direct effect on glucose-induced electrical activity and voltage-gated Ca^{2+} -channels. TTR also protected against apoCIII induced β -cell death.
- The concentration of TTR was decreased in T1D sera, whereas that of the monomeric form of TTR was increased in sera from both children and adult patients with T1D. The monomer was without effect on glucose-induced insulin release and apoptosis. Conversion of TTR tetramer to monomer may thus be involved in the development of β -cell destruction in T1D.
- TTR was shown to bind to Grps in the pancreatic β -cell. The protein is internalized via a clathrin-dependent receptor mediated endocytosis. The Grp complex of Grp78, 94 and 170 may serve as a plasma membrane protein responsible for the uptake of TTR into the β -cell.

6 ACKNOWLEDGEMENTS

This work has been performed at the Rolf Luft Research Center for Diabetes and Endocrinology, Department of Molecular Medicine and Surgery, Karolinska Institutet, Stockholm, Sweden. I wish to express my gratitude to all people that supported me during my theses work. In particular I want to thank:

Lisa Juntti-Berggren, my main supervisor for your never ending enthusiasm and your positive and cheerful attitude. I am grateful for that you always took time for me when I needed your help, weekdays as well as weekends. Thank you for all the time we spent discussing our interesting projects and other topics. It has been a privilege to work with you.

Per-Olof Berggren my co-supervisor for giving me the opportunity to perform my thesis work at this department and for showing great interest in my work and giving me constructive criticism.

Katarina Breitholz, Christina Bremer, Britt-Marie Witasp for all invaluable administrative assistance and help.

Annika Lindgren and **Hannelore Rotter** for always being there when I needed help in the lab and all our funny chats in the culture room. You are amazing!

Lennart Helleday for always taking time to help me with the computer and the SPEX system.

Essam Refai for our nice collaboration and hectic (egyptian...) discussions. Thank you for being there through hard times in the lab, always cheering me up, and not to forget, for all the candy!

Rebecka Nilsson for being the best colleague! Thanks for all advice and funny chats!

Shao-Nian Yang for your outstanding expertise and help during my studies and for being my supervisor throughout my master degree project. Thank you for thoroughly teaching me several methods.

My co-authors not already mentioned: **Hans Jörnvall, Gunilla Olivecrona, Ingo Leibiger, Over Cabrera** (Over, thank you for the greatest mojitos!), **Martin Köhler, Gabriela Imreh, Jawed Shafqat, Luca Inverardi, Camillo Riccardi, Svante Norgren, Ioulia Appelskog, Claude Marcus, Sophia Rössner, Alberto Pugliese, Rudolfo Alejandro, Olli Simell, Sabine Uhles, William Griffiths, Mats Andersson, Sergei Zaitsev, Lina Yu, Guang Yang and Moin Ishrat** for productive collaborations.

Jenny Johansson, Slavena Mandic, Stefania Cotta-Doné for being such great friends. Thank you for all our nice dinners and party evenings. I hope we will continue our friendship through life.

Daniel Nyqvist, Lena Lilja and Juliette Janson for all the pleasant time we spent together. Especially thank you for the “hard/rough” jokes and pub evenings (Daniel), “pepp-talk” throughout my thesis work and telling me the importance of having a

family (Lena). Valuable methodology help and also great “pepp-talk” for my future post-doc position (Juliette).

Barbara Leibiger, Christopher Barker, Christina Bark, Dominic-Luc Webb, Tilo Moede, Caroline Jaques, Alejandro Caicedo, Raffaella Poggioli, Stephan Speier, Jelena Petrovic, Pilar Vaca-Sanchez, Vladimir Sharoyko, Christopher Illies, Jia Yu, Luo-Sheng Li and Irina Zaitseva for making the lab to what it is! Thanks for all advice throughout my thesis work.

My family **Joacim, Elsie-Marie, Mahmoud** and **Tony** who have supported me throughout my thesis work and were/are always there for me. You mean so much to me, more than words can say.

My grandmother **Yvonne** and my late grandfather **Gottfried** for all our nice times together and for always being proud of me. I wish, grandpa, that you could see this thesis.

My best friend **Marina Sjöström** for always making me think on the bright side with her amazing personality. Thanks for all our wine evenings and the dominance on the dance floors! One could not have a better friend than you.

Katarina Fredriksson, Jhansi Kota and Szilvia Nilsson for our nice friendship, support and for knowing you. We have had so many nice and funny times together and we share hobbies and interests that are very important to me.

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