ROMANIAN ACADEMY
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Summary of the Ph.D. thesis:

Molecular mechanism of insulin secretion in pancreatic β-cells

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ABBREVIATIONS LIST

BCA (Bicinhoninic acid)
BSA (Bovine serum albumin)
CHAPS 3-[(3-Cholamidopropyl)dimethylammonio]-1-propanesulfonate hydrate
CNX (Calnexin)
CRT(Calreticulin)
DIC (Differential interference contrast)
DMEM (Dulbecco's Modified Eagle's medium)
DMSO (Dimethylsulfoxide)
DNA (Deoxyribonucleic acid)
DTT (1,4 dithiothreitol)
E. coli (Escherichia coli)
EDEM (ER degradation-enhancing α-mannosidase-like protein)
ER (Endoplasmic Reticulum)
ERQC (Endoplasmic reticulum quality control)
FBS (Fetal bovin serum)
g (Gram)
GH47 (Glycosyl hydrolase 47)
GlcNAc (N-acetyl-D-glucosamine)
h (Hours)
HRP (Horseradish peroxidase)
IPTG (Isopropyl beta-D-thiogalactopyranoside)
KDEL (Lys-Asp-Glu-Leu sequence)
L (Litre)
LB (*Luria Bertani*)
M (Molar)
mM (Millimolar)
MW (Molecular weight)
NHK (Truncated form of alpha 1 antitrypsin)
NR (Non-reducing)
PAGE (Polyacrylamide gel electrophoresis)
PBS (Phosphate buffered saline)
PDI (Protein disulfide isomerase)
PEI (Polyethilenimine)
PFA (Para-formaldehyde)
R (Reducing)
RNA (Ribonucleic acid)
Rpm (Revolutions per minute)
SEM (Standard error of the mean)
SD (Standard deviation)
SDS- (Sodium dodecly sulfate)
siRNA (Small interfering RNA)
SRP (Signal recognition particle)

SRPR (SRP receptor)
Temed (Tetramethylethylenediamine)
U (Unit)
Ub (Ubiquitin)
α1AT (wt alpha 1 antitrypsin)

INTRODUCTION

Type 2 diabetes mellitus (T2DM) is a chronic metabolic disease that occurs as a results of the interaction between genetic and environmental conditions [1]. T2DM is characterized by insulin resistance and chronic hyperglycemic state that leads to deterioration of beta cell functions and reduction in pancreatic β-cells mass [2].

Insulin is a hormone responsible for glucose homeostasis, which is synthesized and secreted by the beta pancreatic cells of the islets of Langerhans within the pancreas. It is synthesized as a precursor, preproinsulin, on ribosomes of the rough endoplasmic reticulum (ER) and by a signal peptidase its 24 amino acides signal peptide is cleaved to generate proinsulin. Within the ER lumen, proinsulin folds by formation of three disulfide bonds. Proinsulin consists of a single chain molecule of 86 amino acids comprised of the insulin B domain (30 amino acids), and the insulin A domain (21 amino acids) linked together by a C peptide (fig. 1)

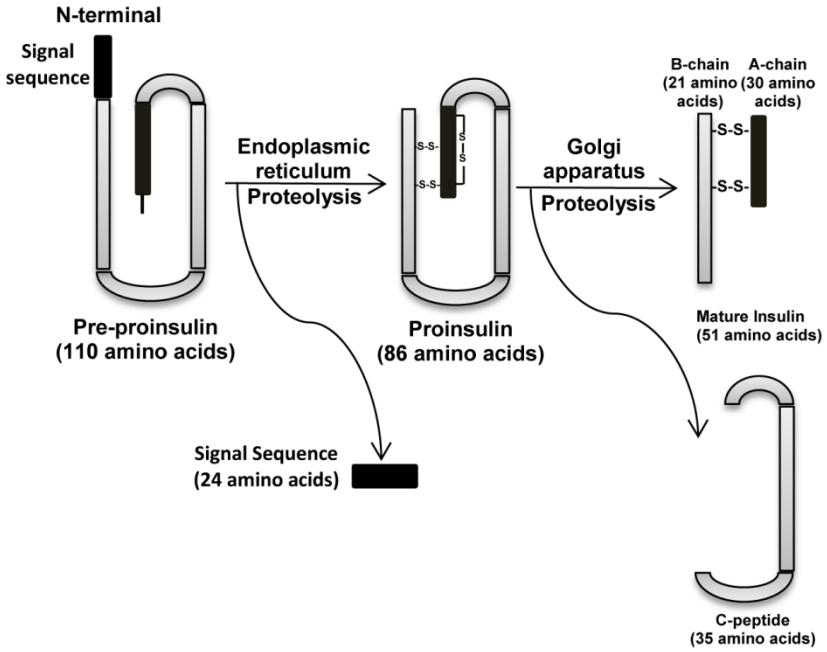

Figure 1. Biosynthesis and secretion of insulin in pancreatic beta cells. Preproinsulin is synthesized on the ribosomes of rough endoplasmic reticulum and is translocated into the ER lumen where it is converted to proinsulin upon removal of the signal peptide. Here the proinsulin is folded by formation of three disulfide bonds. Properly folded proinsulin is then transported to the Golgi apparatus from where it is packed into secretory vesicles. Inside the

secretory granules, proinsulin is cleaved into mature insulin and c-peptide and stored until the beta cells are stimulated to secrete insulin by exocytosis [3].

Then, properly folded proinsulin exits ER and is transported to the Golgi apparatus. Proinsulin molecules are sorted in the trans Golgi network into immature vesicles. In maturing vesicles proinsulin is cleaved by prohormone convertase PC1/3 and PC2 and carboxypeptidase E, which remove C-peptide, the B and A chains of mature insulin remaining attached by interchain disulfide bonds. Mature insulin is stored within secretory vesicles awaiting stimulation in order to release insulin. Glucose is the main secretagogue that modulates insulin release from beta cells. Glucose is transported in beta cells via GLUT-2 glucose transporters and after its phosphorylation by glucokinase it is metabolized to generate ATP. Increased ATP/ADP ratio causes closure of the ATP sensitive potassium K_{ATP} channels that allow potassium ions to accumulate in the cell, and the subsequently membrane depolarization increases calcium levels into the cell. High calcium concentration stimulates vesicle fusion with the plasma membrane to release their contents by exocytosis.

Glucose stimulated insulin secretion occurs as a biphasic model with a first phase of secretion involving docking of the readily releasable pool (RRP) at the plasma membrane and occurs within approx. 5-10 minutes after stimulation with glucose. The second phase of insulin secretion involves transportation of the reserve pool to the plasma membrane in order to be released by exocytosis.

Upon glucose stimulation, up to 50% of the total protein synthesised in these cells is represented by insulin, indicating that pancreatic beta cells possess a highly versatile endoplasmic reticulum (ER) and its synthesis and processing represents the main activity of the pancreatic beta cells [4].

The proinsulin folding occur within ER and since the protein translation is an error prone process, the rapidly increase of proinsulin synthesis can cause ER stress by accumulation of incompletely folded or misfolded proteins, This in turn, could activates the Unfolded Protein Response in order to maintain cellular homeostasis. Disturbed ER homeostasis and prolonged ER stress have implication in the development of many diseases as type 2 diabetes.

Therefore, these cells must possess highly efficient machineries for both folding and disposal of the terminally misfolded proteins. Disposal of misfolded proteins from ER involves protein complexes with role in recognition, retrotranslocation to cytosol and finally proteasomal degradation of the ubiquitin-tagged misfolded proteins. One of the mechanism by which incorrectly folded proteins are removed from the RE is endoplasmic reticulum

associated degradation pathway (ERAD). The mammalian ER degradation-enhancing α-mannosidase-like 1, 2 and 3 (EDEM1, 2 and 3) are mannosidases that have been implicated in the quality control of protein folding and ERAD substrate disposal. So far, most of the studies have been focused on the mechanism by which ERAD substrates are recognized by EDEM proteins and little is known about whether EDEM proteins have other functions. Since the pancreatic cells must respond rapidly to increased glucose concentration in blood by synthesizing and releasing a large amount of insulin, it was interesting to find out how ERAD machinery cope with such situations and whether and how insulin secretion is affected.

AIMS OF THE THESIS

The aim of this thesis was to investigate the role of ERAD pathway in proinsulin degradation and secretion in pancreatic β-cells. I specifically question the role of EDEM proteins in proinsulin traffic and secretion by examining the possibility that EDEM proteins might also play a role in these processes not only in the disposal of the misfolded proinsulin. For this, I focused my studies on two directions: investigation of the biochemical role of EDEM proteins in various aspects of insulin processing, stability and secretion in stably EDEM-overexpressing pancreatic β-cells lines and then, investigation of the physiological role of EDEM1 in a rat model, under normal and diabetic conditions.

RESULTS

The role of EDEM proteins in proinsulin traffic

To evaluate the role of EDEM proteins on beta cell function and proinsulin traffic I established INS-1E cell lines that stably expresses EDEM1 (INS-1E-EDEM1), EDEM2 (INS-1E-EDEM2) and EDEM3 (INS-1E-EDEM3), respectively, and I analyzed their effect on proinsulin content compared to INS-1E-Ctrl cell line that stably expresses the empty vector (pLPCX).

When EDEM1 was stably overexpressed in INS-1E cells it could be observed an increase of proinsulin content that was dependent on glucose concentration. By contrast, stably overexpression of EDEM2 had an opposite effect and decreased the proinsulin content

compared with control cells. Similar to the EDEM1, the EDEM3 overexpression also increased proinsulin content, but moderately. These results suggest that in the pancreatic beta cell lines INS-1E, the EDEM proteins are implicated in proinsulin traffic.

The effect of EDEM1 overexpression on biphasic insulin secretion

Having demonstrated that overexpression of EDEM1 protein increased synthesis and secretion of proinsulin in pancreatic beta cells I wanted to investigate the effects of EDEM1 overexpression on the first phase of insulin secretion in response to glucose. INS-1E-EDEM1 cells stimulated with three different glucose concentrations showed a significant increased of the first phase of insulin content and secretion compared to control cells (transduced with pLPCX vector)

Next, I wanted to know if overexpression of EDEM1 also influences the second phase of glucose stimulated insulin secretion. Interestingly, the insulin content and secretion was also increased in INS-1E-EDEM1 cells upon glucose stimulation, suggesting that EDEM1 overexpression also increases the second-phase of glucose stimulated insulin secretion compared with INS-1E-Ctrl cells.

Quantification of the relative intensity of the bands of the proinsulin have shown that expression of proinsulin was increased with 9.5% at low glucose stimulation, with 107% at medium glucose stimulation and with 94% at high glucose stimulation in INS-1E-EDEM1 cells compared with control cells. In addition, when the film was exposed for a longer period of time the insulin could be detected by Western Blotting in INS-1-EDEM1 cells but not in control cells.

Taken together, these results suggest that EDEM1 is involved in both phases of insulin secretion by increasing the beta cells secretory capacity in response to glucose.

The effects of EDEM1 overexpression on subcellular distribution of proinsulin

The effect of EDEM1 overexpression on proinsulin traffic was determined by confocal immunofluorescence staining. For this, co-immunofluorescence experiments were performed

for INS-1E-Ctrl and INS-1E-EDEM1 cells with antibodies against insulin and PDI (ER marker), respectively, and analyzed by confocal microscopy

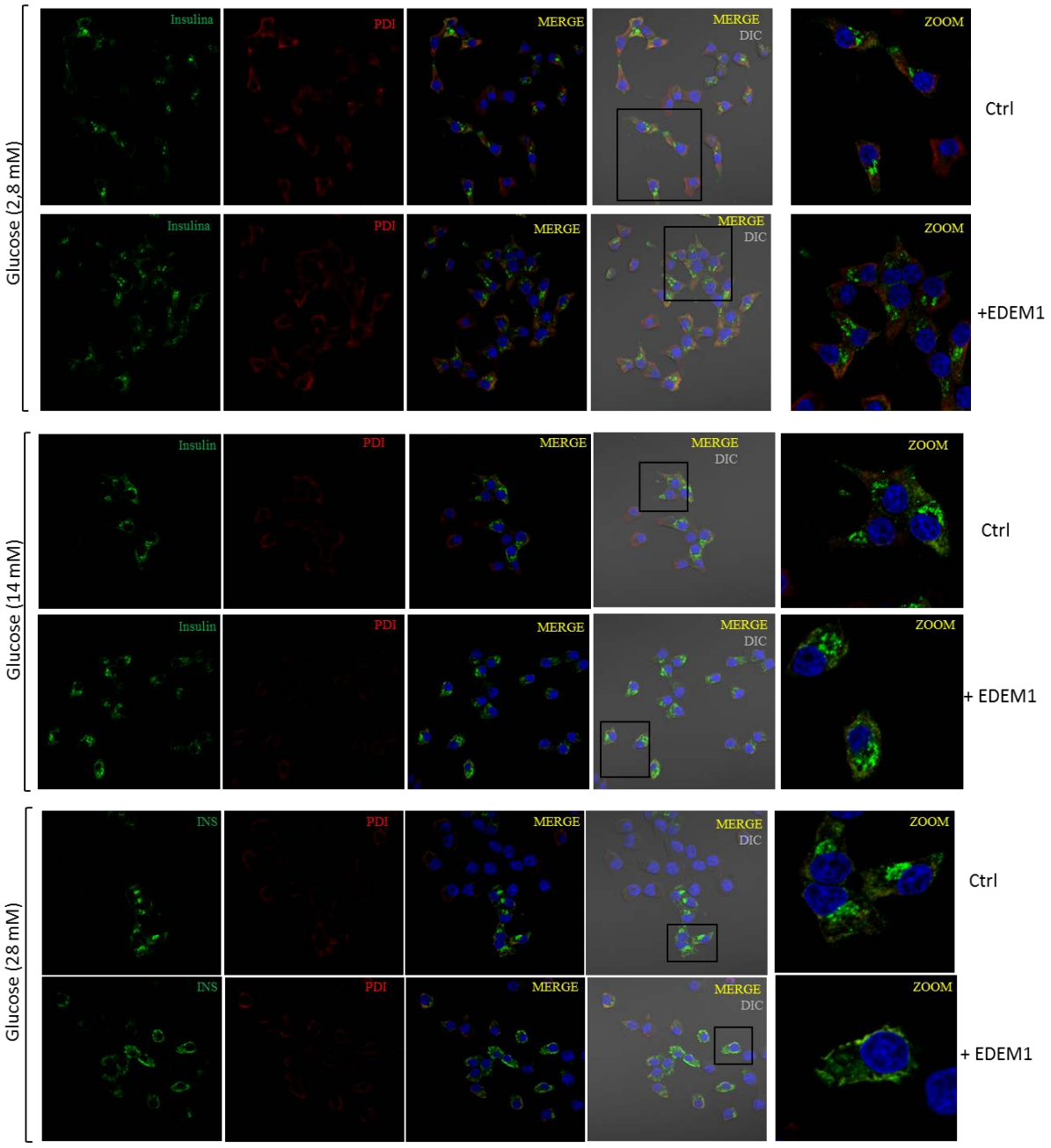

Figure 2. Subcellular distribution of proinsulin is modulated by EDEM1 overexpression in rat insulinoma cell line INS-1. INS-1E-Ctrl and INS-1E-EDEM1 cells were starved in medium without glucose and trated with various concentrations of glucose. Then, the cells were fixed and stained for proinsulin (green), PDI (ER marker) (red) and DNA (blue), respectively. Representative images for three individual experiments are shown.

Insulin staining in INS-1E-EDEM1 cells was higher than in control cells, suggesting the presence of a larger amount of insulin in these cells. Moreover, cells stimulated with high concentration of glucose showed proinsulin accumulation near plasma membrane suggesting that insulin is prepared to be secreted.

The effects of EDEM1 overexpression on proinsulin half life

To examine the stability of proinsulin, cells were treated with cicloheximide and the proinsulin fraction was measured at 20, 40 and 60 minutes.

Cycloheximide (CHX) assay revealed that proinsulin stability was significantly increased by EDEM1 overexpression. Moreover, EDEM1 increased the intracellular proinsulin as well as secreted proinsulin. Densitometry quantification of the proinsulin bands showed that proinsulin stability was increased by EDEM1 and its half-life was enhanced from approx. 20 min. to 40 min in the presence of EDEM1 protein.

EDEM1 is functional in INS-1E-EDEM1 cells

It is known from the literature that EDEM1 has a role in ERAD pathway, being involved in the recognition and targeting to proteasome for degradation of the misfolded proteins. To demonstrate that in INS-1E-EDEM1 cells, the EDEM1 overexpressed is functional I assessed its ability to accelerate degradation of NHK protein (Hong Kong null form of α1-antitrypsin), whose degradation is known to be EDEM1-dependent. As expected, overexpression of EDEM1 accelerated the degradation of NHK protein and furthermore, overexpression of EDEM1 also accelerated the degradation of endogenous wt α1AT. These results indicate that EDEM1 is functional in pancreatic beta INS-1E-EDEM1 cells and these cells possess an active ERAD pathway.

However, while overexpression of EDEM1 accelerated the degradation of NHK, the proinsulin content was increased, which is in agreement with previous results. These results strongly suggest that EDEM1 might be involved in other processes than ERAD pathway, such as insulin secretion.

Next, in order to inhibit the ERAD pathway and implicitly EDEM1 function, I treated cells with kifunensine and I analyzed the levels of proinsulin synthesis and secretion in the

presence or absence of EDEM1. I found that inhibition of mannosidases with kifunensine in pancreatic beta cells INS-1E-EDEM1 decreased both proinsulin content and secretion.

Assuming that inhibiting the mannosidase activity prevented EDEM1 to bind SEL1L protein, an important component of the ERAD complex, I can hypothesize that proinsulin traffic is dependent on the ERAD pathway. The above results which also showed that the level of SEL1L protein was increased in INS-1-EDEM1 cells are in agreement with this hypothesis.

Discussions

Here, I investigated the potential role of EDEM1, EDEM2 and EDEM3 proteins in the modulation of proinsulin trafficking. I found that in pancreatic beta cells, EDEM proteins are involved in proinsulin traffic.

EDEM1 is involved in both phases on glucose stimulated insulin secretion, by increasing the synthesis and secretion of proinsulin. I also found that proinsulin stability was increased by EDEM1 and its half-life almost doubled. Insulin immunostaining and visualization by confocal microscopy showed a significantly increase of proinsulin expression and number of granules docked at the plasma membrane, which are ready for release upon glucose stimulation.

PHYSIOLOGICAL ROLE OF EDEM1 IN DIABETIC RATS

The role of EDEM1 in glucose tolerance

To examine the physiological role of EDEM1 in animal model, first I induced diabetes in Wister rats by injecting a single dose of streptozotocin. Streptozotocin alter blood insulin level and increase glucose concentration. Changes as hyperglicemia and reduced blood insulin level reflect a degradation of the pancreatic beta cells. Fating blood glucose levels were tested daily and rats that developed diabetes were divided into two groups that were injected intraperitoneally with either pLPCX empty vector or pLPCX-EDEM1 retrovirus. The rats were sacrificed after 7 days of treatment.

Administration of the retroviral construct and glucose tolerance test were performed before treatment for diabetic group compared with healty group and after treatment for

diabetic group treated with pLPCX (STZ-pLCX), diabetic group treated with EDEM1 (STZ-EDEM1) and for healty group (-STZ). This analysis was performed to determine insulin response to elevated glucose concentration.

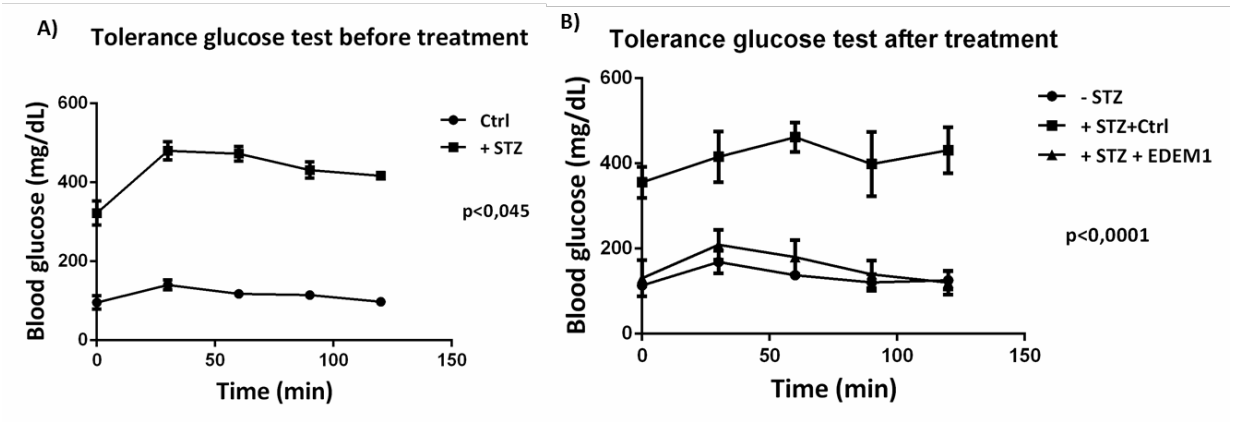

Figure 3. Glucose tolerance test before and after treatment with EDEM1 in diabetic rats compared with healthy animals. Glucose was administered to three rat groups. The glucose level was examined before glucose loading (A) and at 0, 30, 60, 90 and 120 minutes after glucose loading. The data are shown as the mean ± SEM.

As expected, -STZ group showed increased glucose tolerance and +STZ group showed decreased tolerance (Fig.3). Interestingly, after 7 days of pLPCX or PLPCX-EDEM1 treatment, the +STZ-EDEM1 group showed an increased glucose tolerance similar to the healthy rats group (Fig.3B). Instead, the +STZ-pLPCX rats group continued to show impaired glucose tolerance (Fig.3B).

These results suggest that EDEM1 increase glucose tolerance probably by restoring the function of pancreatic beta cells, increase the insulin secretion capacity and the response to high glucose concentrations.

Blood glucose levels in pLPCX/EDEM1 treated rats

To find if the retroviral tranduction affected the blood glucose concentration, the glucose concentration was daily measured in the three groups (fig. 4).

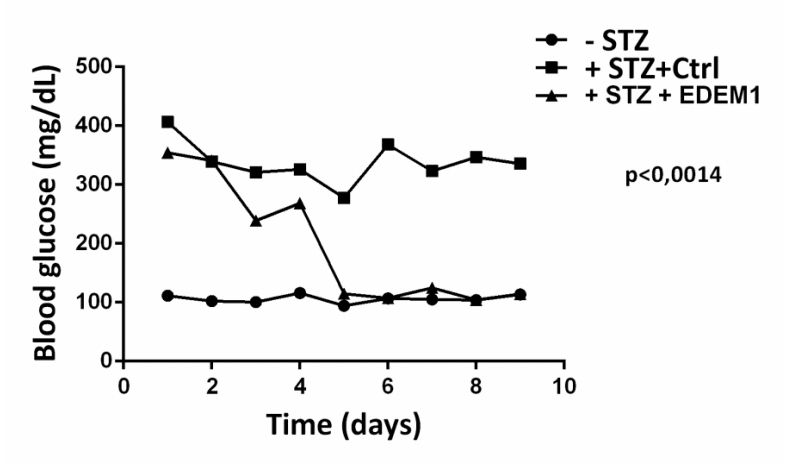

Figure 4. Blood glucose levels in rats treated with pLPCX or pLCX-EDEM1 after induction of diabetes. Fasting blood glucose levels were daily determined starting with the first day post-streptozotocin treatment until the last day of treatment. Values are expressed as mean ±SEM. P<0,0014 and compared to empty vector treated animals.

When I analyzed the blood glucose level I found that treatment with EDEM1 continuously decreased blood glucose level starting from the second day of treatment until the end of the experiment. At the end of the experiment blood glucose levels reached normal values similar to –STZ group (fig. 4). Instead, blood glucose levels of +STZ-pLPCX group were above 300mg/dl from the beginning until the end of the experiment.

The effect of EDEM1 on insulin in diabetic rats

Serum and pancreatic insulin levels were determined in rats post induction of diabetes and after 7 days of treatment with pLPCX or pLPCX-EDEM1 retroviral constructs, by ELISA and Western Blot assays, respectively.

A B C

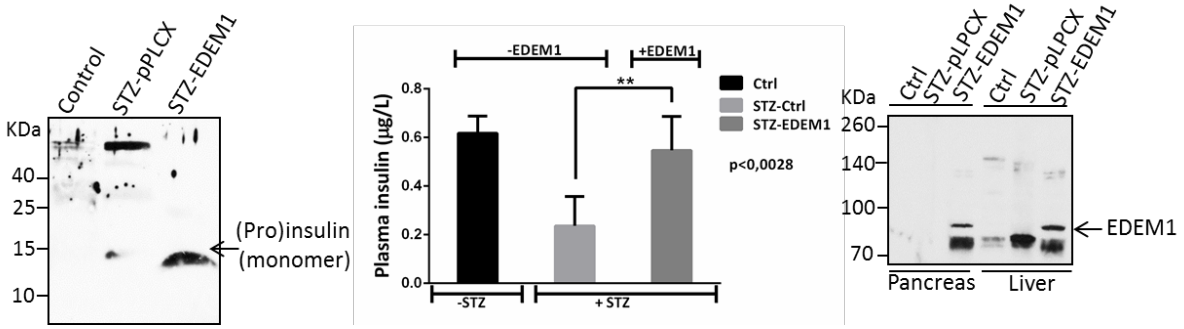

Figure 5. Serum and pancreas insulin levels in rats transduced with pLPCX and EDEM1. Diabetic and healthy animals were sacrificed after 7 days post treatment. Pancreas and liver were colected, lysed and analysed by Western Blotting using antibodies against proinsulin (A) and EDEM1 (C), respectively. Plasma insulin concentrations were determined before treatment using Elisa kit (B). The results are expressed as the mean ±SEM. Representative images from two individual experiments are shown.

Serum insulin levels determined by ELISA was higher in +STZ-EDEM1 group compared with +STZ-PLPCX group and similar to that of the –STZ group (Fig.5). Western blot analysis showed that transduction of EDEM1 in diabetic rats resulted in detection of monomeric proinsulin, whereas transduction with control retroviral (empty vector-pLPCX) resulted in detection of high molecular weight forms of proinsulin (Fig.5).

The organ transduction of pLPCX/pLPCX-EDEM1 vectors was examined by Western blotting. EDEM1 expression was detected in pancreas and liver from rats treated with the pLPCX-EDEM1, confirming the efficieny of the procedure for retroviral mediated transduction in rats (Fig. 5).

Discussions

In the previous chapter I have shown that EDEM1 overexpression increased insulin synthesis and secretion. In the study presented in this chapter I investigated the physiological role of EDEM1 in diabetic animal model.

Glucose tolerance test showed that diabetic rats after treatment with EDEM1 exhibited increased glucose tolerance suggesting that their pancreas possesses a pool of insulin and is able to respond to glucose stimulation. Serum insulin levels in rats transduced with EDEM1 retroviral vector was incresed compared with that in control group. Western blotting analysis showed that in diabetic rats transduced with EDEM1 protein the proinsulin was mainly detected as monomer compared to the control rats in which the largest quantity of proinsulin was detected as oligomers form and only a small amount was detected as monomer.

GENERAL CONCLUSIONS

- ✓ The stable cell lines that I developped from parental INS-1E cells succesfuly overexpressed EDEM1, EDEM2 and EDEM3 proteins, respectively, and these proteins were involved in proinsulin traffic in INS-1E pancreatic beta cells.
- ✓ Overexpression of EDEM1 enhanced proinsulin synthesis and secretion on both phases of glucose stimulated insulin secretion.
- ✓ Analysis of immunofluorescence samples revealed that in EDEM1 overexpressing cells it was an increased number of granules docked at the membrane which are ready for release upon glucose stimulation.
- ✓ Immunoprecipitation and pulse-chase experiments showed that overexpression of EDEM1 enhanced both proinsulin half-life and proinsulin secretion.
- ✓ EDEM1 overexpression in pancreatic beta INS-1E cells upregulated the expression of some ERAD components.
- ✓ Overexpression of EDEM1 accelerated the degradation of NHK (misfolded form of α1-antitripsin), indicating that EDEM1 is functional in pancreatic beta INS-1E cells.
- ✓ Retroviral transduction of EDEM1 increased serum insulin, glucose tolerance and decreased blood glucose in diabetic rats.
- ✓ Taken together, all these results suggest that EDEM1 might be involved in other processes then ERAD pathway, such as insulin synthesis and secretion and that manipulation of EDEM1 could be a useful therapeutic target in diabetes.

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