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Role of Cell-cell Interactions and Palmitate on β -cells Function

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Abstract

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The islets of Langerhans secrete insulin in response to fluctuations of blood glucose level and efficient secretion requires extensive intra-islet communication. Secretory failure from islets is one of the hallmarks in progression of type 2 diabetes. Changes in islet structure and high levels of saturated free fatty acids may contribute to this failure. The aim of this thesis is to study the role of cell-cell interactions and palmitate on β -cells functions.

To address the role of cell-cell interactions on β -cells functions MIN6 cells were cultured as monolayers and as pseudoislets. Glucose stimulated insulin secretion was higher in pseudoislets compared to monolayers. Transcript levels of mitochondrial metabolism as well as glucose oxidation rate were higher in pseudoislets. Insulin receptor substrate-1 (IRS-1) phosphorylation was altered when cells were grown as pseudoislets. Protein expression levels related to glycolysis, cellular connections and translational regulations were up-regulated in pseudoislets. We propose the superior capacity of pseudoislets compared to monolayers depends on metabolism, cell coupling, gene translation, protein turnover and differential IRS-1 phosphorylation.

To address the role of palmitate on β -cells human islets were cultured in palmitate. Long term palmitate treatment decreased insulin secretion which is associated with up-regulation of suppressor of cytokine signaling-2 (SOCS2) and protein inhibitor of activated STAT-1 (PIAS1). Up-regulation of SOCS2 decreased phosphorylation of Akt at site T308, whereas PIAS1 decreased protein level of ATP-citrate lyase (ACLY) and ATP synthase subunit B (ATP5B). We propose long term palmitate treatment reduces phosphatidylinositol 3-kinase (PI3K) activity, attenuates formation of acetyl-CoA and decreases ATP synthesis which may aggravate β -cells dysfunction.

Keywords: Metabolism, PI3K, Pseudoislets, Human islets, SOCS, PIAS

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List of Papers

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

- I Functional differences between aggregated and dispersed insulin-producing cells (*Diabetologia*. 2013 Jul;56(7):1557-68).
- II Signaling in Insulin-Secreting MIN6 Pseudoislets and Monolayer Cells. (*J Proteome Res*. 2013 Dec 6;12(12):5954-62.)
- III* GLP-1 analogue recovers impaired insulin secretion from human islets treated with palmitate via down-regulation of SOCS2. *Manuscript*
- IV Role of PIAS1 in palmitate-mediated beta-cell dysfunction. *Manuscript*

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Abbreviations

[Ca ²⁺] _c	Cytoplasmic Ca ²⁺ concentration
ACC	Acetyl-CoA- carboxylase
ACLY	ATP citrate lyase
ADP	Adenosine diphosphate
ATP	Adenosine triphosphate
ATP5B	ATP synthase subunit beta
ELISA	Enzyme-linked immunosorbent assay
ER	Endoplasmic reticulum
ESI	Nano-electrospray ionization
EX	Exendin-4
FADH ₂	Reduced 1,5-dihydro-flavin adenine dinucleotide
FAS	Fatty acid synthase
FDR	False discovery rate
GLUT2	Glucose transporter 2
GSIS	Glucose stimulated insulin secretion
HFD	High fat diet
IL-1B	Interleukin-1 beta
IR	Insulin receptor
IRS	Insulin receptor substrate
KIC	Alpha-ketoisocaproic acid
LC-MS	Liquid chromatography mass spectrometry
LTQ FT	Linear ion trap Fourier Transform
MO	Monolayers
MS	Mass spectrometry
NADH	Reduced nicotinamide adenine dinucleotide
NF- κB	Nuclear factor-κB
PC	Pyruvate carboxylase
PDH	Pyruvate dehydrogenase
PDX1	Pancreatic and duodenal homeobox 1
PI	Pseudoislets
PI3K	Phosphatidylinositol 3-kinase
PIAS	Protein inhibitor of activated STAT
qPCR	Quantitative polymerase chain reaction
siRNA	Small interfering RNA
SOCS	Suppressor of cytokine signaling
TCA	Tricarboxylic acid cycle

Introduction

Diabetes mellitus is one of the oldest known human diseases [1], where excessive urination, which has a sweet taste because it contains sugar was described as a symptom. Ancient Persians, Indians and Egyptians also mentioned the same symptom. In those days people suffering from diabetes lead a poor life and eventually embraced a very painful death [1]. Scientists were for a long time unable to find the substance, which controls the blood glucose level. In 1869, Paul Langerhans defended his thesis presenting the micro-organs in the pancreas [2]. Later in 1893, Edouard Laguesse postulated the organs to produce secretion for digestion and he called them “islets of Langerhans” [3]. Not until 1921, three Canadian scientists isolated the substance responsible for controlling the blood glucose levels and named it “insulin” because it was isolated from the islets of Langerhans [4]. The islets of Langerhans contain mainly insulin-producing β -cells, which control the blood glucose level by secreting insulin in response to fluctuations in the blood glucose concentration [5]. Thus, β -cells are key to understanding the etiology of the two common forms of diabetes, type 1 and type 2 diabetes mellitus. Whereas loss of insulin secretion by autoimmune destruction of the β -cells is observed in type 1 diabetes, impaired insulin secretion in combination or not with insulin resistance are characteristics of type 2 diabetes mellitus [6-9]. The most common form of diabetes is type 2 diabetes mellitus. Both environmental factors and genetic causes have been identified as risk factors for type 2 diabetes [10-17]. The disease is connected with damage of small blood vessels resulting in neuropathy, retinopathy and nephropathy [18]. In this thesis we will focus on how cellular interactions and saturated free fatty acid palmitate plays role on β -cells functions.

Background

Islet anatomy

Islets of Langerhans are spherical micro-organs and constitute the endocrine portion of the pancreas. Each islet consists of approximately 2000 cells together with basement membranes, blood vessels, nerve fibers and connective tissue. The endocrine cells are essential for regulation of metabolism. The exocrine portion of the pancreas contains cells secreting digestive enzymes. The islet consists not only insulin-producing β -cells but also glucagon-producing α -cells and somatostatin-producing δ -cells are also present in the islet [19, 20]. Pancreatic islet cells have intimate contact with endothelial cells, which not only act as barrier but also transport oxygen and nutrients to the β -cell [21, 22]. It has been reported that islet endothelial cells secrete products promote β -cell proliferation, insulin content and insulin secretion [23, 24]. It is not only islet microvasculature structure but also the intra-islet cellular contacts that play an important role for proper functioning of the β -cells. Dispersed β -cells respond poorly to glucose compared to the intact islets [25-28]. These early studies reinforced the notion of cellular connection and cell-to-cell communication as important components for optimal β -cell function. Islets can be dissociated and re-aggregated to form so called “pseudoislets”, which maintain similar size and morphology as native islets [29, 30]. The pseudoislet model provides a useful tool to investigate the importance of cellular interactions. Pseudoislets can also aggregate from the MIN6 insulin secreting β -cell line. Similar to when primary islet cells re-aggregated into pseudoislets, aggregating MIN6 cells resulted in pseudoislets of similar size and morphology as primary islets [31]. Furthermore, enhanced insulin secretion was observed from these MIN6 pseudoislets in response to nutrient and non-nutrient stimuli compared to MIN6 cells grown in monolayers [31-35].

Glucose-stimulated insulin secretion

Glucose-stimulated insulin secretion (GSIS) occurs in a biphasic manner [36]. This pattern has been coupled to the glucose-induced rise in ATP production from glycolysis and mitochondrial oxidation. As a consequence the ATP/ADP ratio will increase, leading to the inhibition of K_{ATP} channel, depolarization,

opening of voltage-gated Ca^{2+} channels and increase in cytosolic Ca^{2+} concentration ($[\text{Ca}^{2+}]_c$) and insulin release [37-39]. This is known as first phase of insulin secretion. The second phase, which is the sustained phase of insulin secretion, is not dependent on K_{ATP} channel activity [40, 41]. The importance of mitochondrial molecules derived from glucose-induced anaplerosis of the second phase has been proposed [42]. GSIS requires glucose to be metabolized into pyruvate and subsequent entry into the tricarboxylic acid (TCA) cycle and oxidation to generate ATP. The importance of glycolytic ATP in GSIS in mouse islets was reported [43]. Pyruvate is produced from the glycolytic pathway and enters into the mitochondrial matrix either by pyruvate dehydrogenase (PDH) to form acetyl-CoA (oxidative pathway) or carboxylated by pyruvate carboxylase generating oxaloacetate for anaplerosis [42]. Both of the pathways have been shown to be important for proper GSIS as pyruvate enters into equal proportions [44-46]. The important anaplerotic pathways are pyruvate/malate, pyruvate/citrate and pyruvate/isocitrate, which were reported to play important roles in GSIS [47-51]. Although there is ample evidence demonstrating the importance of mitochondrial metabolism for fuel-induced insulin secretion, there is little evidence available how anaplerotic products may trigger or potentiate insulin secretion. NADPH and alpha-ketoglutarate and succinate, produced from the TCA cycle, have been proposed to act on exocytosis directly and as ligands for G protein-coupled receptors, respectively [51-53].

IRS-1 phosphorylation and insulin secretion

Insulin executes its function through insulin receptors (IRs) [54, 55], insulin like growth factor-1 receptors (IGF-1R) [56] and hybrid receptors between IR and IGF-1R [57, 58]. The presence of IRs was reported in β -cell lines (HIT-T15, INS1 and MIN6) and also in primary human and mouse islets [59-61]. When insulin binds the intrinsic tyrosine kinase activity of the receptor (IRK) is stimulated, which phosphorylates tyrosine residues of target proteins including the insulin receptor substrates (IRS-1 to -6) [62, 63]. Tyrosine phosphorylations of IRS proteins are important for proper propagation of insulin signal. Whereas tyrosine phosphorylation of the IRSs generally increases insulin signaling, serine/threonine phosphorylation negatively regulates insulin signaling and action [54]. In type 2 diabetic patients the later signaling has been shown to be up-regulated [64]. The activated insulin receptors propagate the signals through three major pathways: phosphatidylinositol 3-kinase (PI3K), mitogen activated protein kinase pathway and Cbl/CAP [62, 65]. Activation of PI3K can regulate β -cell proliferation, survival, gene transcription and insulin secretion [54, 66-71], although the activation of PI3K in insulin secretion has been debated [72-75].

Effect of free fatty acid on β -cells

Free fatty acids can be imported to the beta cell by spontaneous diffusion or via fatty acid transporter e.g. cluster of differentiation 36 (CD36) [89]. Acute exposure of fatty acid increases GSIS from β -cells [50, 90]. Several mechanisms have been proposed for this acute insulinotropic effect of fatty acids including changes in intracellular pool of long chain acyl-CoA:s which serves as coupling factors for exocytotic machinery [91], activating protein kinase C [92], altering ATP/ADP ratio [93], changing ion-channel activities [94], activating G-protein coupled receptor 40 (GPR40) [95, 96].

Although short term exposure of fatty acids induce insulin secretion but long term exposure has detrimental effect on β -cells function and survival [97, 98]. Different mechanisms by how long term palmitate exposure can effect β -cells have been proposed including increased apoptosis [99], ER stress activation [100], ceramide formation [101, 102], cytochrome C release [103], activation of PKC [104] and activation of calpain-10 [105], dissociation of Ca^{2+} channels and secretory granules [106] and signaling via GPR40 [107].

Suppressor of cytokine signaling

Cytokines are secreted glycoproteins that regulates various signaling pathways by interacting with different receptor complexes. Receptors of cytokine transmits their signal through different kinases such as JAK-STAT signaling pathway and dysregulation of this pathway leads to different autoimmune disease [108]. Suppressor of cytokine signaling (SOCS) proteins were identified as negative regulator of cytokine action [109]. To date, eight members of SOCS proteins have been identified: SOCS 1-7 and cytokine-inducible SH2 domain containing protein (CIS) [110]. SOCS proteins can inhibit cytokine signaling at least by two mechanisms either directly bind with JAK-kinase and inhibits its tyrosine kinase activity or binding with cytokine receptor to attenuate signaling [109]. In addition to inhibiting cytokine signaling SOCS proteins was shown to regulate beta cell function and insulin signaling. SOCS-1, SOCS-3 and SOCS-6 was reported to induce insulin resistance by inhibiting tyrosine kinase activity of insulin receptor. The above mentioned proteins can compete for the binding of insulin receptor substrates or can target the receptor substrates for degradation [111-114]. Increased expression of SOCS-1 and SOCS-3 has been reported in insulin sensitive tissues in rodents on high fat diet or murine models of obesity or humans with type 2 diabetes [111, 115, 116]. Indeed, acute inhibition of SOCS-1 and SOCS-3 expression in liver of *db/db* mice ameliorates some metabolic disorders and SOCS-1 deficient mice showed increased insulin sensitivity [117, 118]. Although the role of SOCS-1 and SOCS-3 are somewhat established in islets but the role of SOCS-2 in β -cells function is debated. SOCS-2 knockout mouse did not show any change

in GSIS, insulin tolerance [119] but the negative role of SOCS2 in insulin secretion was reported by several others where constitutively activated SOCS2 in mouse islets or adenoviral mediated overexpression of SOCS2 in MIN6 decreased GSIS and single nucleotide polymorphisms of SOCS2 was reported to be associated with T2D [120-122]. The constitutively activated SOCS2 decreased intracellular Ca^{+2} stores which is responsible for decreased GSIS as well as increase in proinsulin by decreasing both mRNA and protein level prohormone convertase 1/3 (PC1) [120].

Protein inhibitor of activated STATs

Cytokines regulate functions of immune cells by regulating transcriptional factors of the cells. Signal transducer and activator of transcription proteins (STATs), nuclear factor- κ B (NF- κ B) and SMA (small body size) -and MAD (mothers against decapentaplegic) -related proteins (SMADs) are three key families transcription factors regulated by cytokine signaling. The expression of STATs, NF- κ B and SMADs are tightly controlled at different levels and inappropriate regulation leads to different diseases including cancer and immune disorders [123]. Mammalian protein inhibitor of activated STATs (PIAS) were initially identified as negative regulator of STAT mediated signaling [124, 125]. The PIAS family consists of PIAS1, PIAS3, PIASx (PIAS2) and PIASy (PIAS4) [123]. PIAS proteins can either co-activate or co-repress more than 60 proteins and most of them are transcriptional regulators [123]. The negative regulation of PIAS occurs either by blocking the DNA binding activity of a transcription factor [125, 126] or by recruiting other co-factors such as histone deacetylase [127] or by promoting sumoylation of transcription factor as PIAS protein are recently implicated to have SUMO E3 ligase activity [128, 129]. There are very little information about role of PIAS protein in β -cells functions, in a recent study PIAS1 was showed to suppress the transcriptional activity of liver X receptor (LXR) [130] and LXR was reported to play an important role in insulin secretion by activating anapleotic pathway as well as by glycerolipid/free fatty acid cycling [131]. Another study reported PIASy suppress islet cell autoantigen 512 (ICA512) which was shown to regulate insulin gene transcription and secretion [132, 133].

Proteomics

Diabetes mellitus is a complex disease, which makes it necessary to determine complex changes in the expression levels to understand the mechanisms involved. With recent advances in mass spectrometry (MS) and bioinformatics, proteomic analysis can not only identify and quantify thousands of proteins but also give us an insight into differentially expressed proteins in diseased

state [76-81]. There are several quantitative MS-based approaches that have been used to study pancreatic islets, where proteins have been separated primarily by gel-based [82, 83] or liquid chromatography (LC) [76, 84] approaches and quantitation performed either by label-free [77, 79] or stable isotope labeling [78, 80]. In LC-MS, the sample is separated by LC, ionized by electrospray ionization (ESI) and deflected by magnetic fields in the MS/MS analysis. It has been observed that signal ESI intensity correlates with ion concentration [85]. The label-free approach used in the present study gives the opportunity to analyze prepared samples separately. In contrast, protein-labeling requires the samples to be combined and analyzed together. Label-free experiments require accurate normalization to compensate for possible deviations caused by run-to-run variations in the performance of LC and MS [86-88]. To further minimize methodological variability between label-free LC-MS analyses of different samples equal amount of protein in the samples, highly reproducible LC-MS and automated spectra analysis by computer algorithms are critical [87]. The highly reproducible nano-HPLC separation and high resolution 7 T hybrid Linear ion trap Fourier Transform (LTQ FT) mass spectrometer as well as powerful computational tools as Progenesis LC-MS data analysis pipeline used in the present study have greatly improved the reliability and accuracy of the analysis of complex biological samples. The identification, quantification and statistical analysis of numerous individual proteins in complex biological samples are required to determine changes in signaling pathways.

Aims

The general aim of the thesis is to understand the role of cell-cell interactions and palmitate on β -cells function. The specific aims for different papers were:

- I To evaluate the influence of cellular interactions on mitochondrial metabolism and IRS-1 signaling
- I To identify complex changes in pathway signaling in MIN6 pseudoislets and monolayers
- II To evaluate role of SOCS2 in insulin secretion
- III To evaluate role of PIAS1 on β -cells function

Material and Methods

Cell culture

Mouse insulinoma MIN6 monolayer cells were cultured in 250 ml tissue culture flasks (Becton Dickson Labware, Franklin Lakes, NJ, USA) at 37°C (95% O₂ and 5% CO₂) in Dulbecco's Modified Eagle medium (DMEM; Invitrogen, Paisley, UK) as described previously [134]. MIN6 pseudoislets were prepared by aggregating 3x10⁶ dispersed cells cultured in petri dishes made of non-adherent plastic (Becton Dickson Labware) for 3-5 days by using the same culture condition as for monolayers [31]. All experiments were performed between passages 20 to 30. Human islets were cultured for 4-8 days before experiments in CMRL medium containing 5.5 mmol/l glucose and supplemented with 10% fetal bovine serum, 1% L-glutamine, 100 units/ml penicillin and 100 µg/ml streptomycin. Islets from pancreases of C57Bl/6 mice were isolated using collagenase. The obtained mouse islets were cultured for 2 days in RPMI 1640 (Invitrogen) medium containing 11.1 mmol/l glucose supplemented with 10% fetal bovine serum, 100 units/ml penicillin and 100 µg/ml streptomycin. To prepare dispersed cells groups of 100 human and mouse islets were dispersed in 0.5% trypsin for 10-12 min and 3-5 min respectively and then treated with DNase I (Qiagen GmbH, Hilden, Germany) for 2 min. The resulting cell suspensions were placed in poly-L-lysine coated plates and cultured for 48 hours in the respective culture medium. The use of human and mouse islets was approved by the local ethical committees (Dnr 2010/006 for human islets and Dnr C106/11 for mouse islets).

Insulin secretion

Insulin secretion was measured from MIN6 monolayers, MIN6 pseudoislets as well as mouse and human islets. For static incubation experiments 10⁵ MIN6 cells were seeded into 12-well tissue culture plates (Becton Dickson Labware) and cultured for 3 days. The cultured monolayer cells or groups of 20 pseudoislets or human islets were pre-incubated for 60 min at 37°C in 1 ml KRBH buffer consisting of 130 mmol/l NaCl, 4.8 mmol/l KCl, 1.2 mmol/l MgSO₄, 1.2 mmol/l KH₂PO₄, 1.2 mmol/l CaCl₂, 5 mmol/l NaHCO₃ and 5 mmol/l HEPES, titrated to pH 7.4 with NaOH and supplemented with 1 mg/ml BSA and 2 mmol/l glucose. Subsequently, cells were incubated in 1 ml KRBH

buffer containing either 2 or 20 mmol/l glucose for 30 min at 37°C. Aliquots (200 µl) of medium were collected for determination of insulin secretion. Total protein was measured as described previously [134].

Insulin secretion was also measured dynamically from monolayer cells, groups of 20 pseudoislets, human and mouse islets by perfusing in the presence of 2 and 20 mmol/l glucose as described previously [135]. Individual pseudoislets and human islets were also perfused in 2 and 20 mmol/l glucose. For the dynamic insulin measurement, 5×10^4 MIN6 cells were attached to the central part of poly-L-lysine coated coverslips and cultured for 3 days. The samples were collected at 2 mmol/l glucose during either 10 min for individual islets or 20 min for other preparations. Subsequently, medium was exchanged to either 20 mmol/l glucose, 2 mmol/l pyruvate, 20 mmol/l alpha-ketoglutaric acid (KIC), 10 mmol/l KIC plus 10 mmol/l glutamine or 30 mmol/l KCl and sample collection continued for another 20 min. In some experiments, LY294002 (50 µmol/l) or wortmannin (100 nmol/l for monolayers, pseudoislets and mouse islets, 1 µmol/l for human islets) was introduced into the perfusion medium 30 min prior to sampling and present throughout the experiment. Insulin was measured by enzyme-linked immunosorbent assay (ELISA) as described previously [135].

Simultaneous measurements of insulin and cytoplasmic Ca^{2+} concentration

For simultaneous measurements of insulin and cytoplasmic Ca^{2+} concentration ($[\text{Ca}^{2+}]_c$), pseudoislets were loaded with 1 µM Fura-2 LR acetoxymethyl ester (TEFLabs, Inc., Austin, TX, USA) by incubating them for 60 min at 37°C in KRBH buffer containing 2 mmol/l glucose and 1 mg/ml BSA. After rinsing, groups of islets were allowed to attach to the central part of poly-L-lysine coated coverslips. The chamber was placed on the stage of an inverted microscope (Eclipse TE2000U, Nikon). The pseudoislets were perfused for 60 min with 2 mmol/l glucose in KRBH buffer supplemented with 1 mg/ml BSA at 37°C at a rate of 160 µl/min. $[\text{Ca}^{2+}]_c$ was recorded by dual wavelength fluorometry as previously described [136]. During the $[\text{Ca}^{2+}]_c$ recordings the perfusate was collected in 1 min intervals for subsequent insulin measurements. To measure the effect of PI3K inhibition the attached pseudoislets were perfused for 30 min in 2 mmol/l glucose. Wortmannin (100 nmol/l) or LY294002 (50 µmol/l) was subsequently added and perfusion continued for another 30 min before $[\text{Ca}^{2+}]_c$ was measured and fractions of perfusate was collected for insulin measurement in the continued presence of the inhibitors.

Western blot

For determination of levels of specific proteins western blotting was performed on MIN6 monolayers, MIN6 pseudoislets, human and mouse islets with some modifications as described previously [137]. Immunoblotting was conducted with antibodies against IRS-1, IRS-2, phosphorylated (p)-IRS-1 S636/639, p-IRS-1 S612, p-IRS-1 S307, p-Akt (S473), p-Akt (T308), p-S6 ribosomal protein (S235/236), total Akt, total S6 ribosomal protein, PDX1, SOCS1, SOCS2 FAS, ACC, Pfkf1, Pkm2, ATP-citrate synthase, actin (Cell Signaling, Danvers, MA, USA), glucokinase, GLUT2, Sfrs4, Psmf7, Aga, Rpl35a, ATP5B (Santa Cruz, CA, USA), PIAS1, Idh3g, Uba52 (Abcam, Cambridge, UK) and Snrpd1 (Proteintech Group Inc., Chicago, USA). The immuno-reactive bands were imaged with ChemiDoc™ XRS+ (Bio-Rad) and quantified with Image Lab™ software (Bio-Rad).

mRNA expression by real-time PCR

Total RNA was extracted from MIN6 monolayers, pseudoislets and human islets by using RNeasy Mini Kit (Qiagen GmbH). The reverse transcription reaction was performed with 1 µg total RNA using RT² First-Strand Kit (Qiagen GmbH). The obtained cDNA was processed for quantitative real-time reverse transcriptase PCR of 84 genes involved in mitochondrial electron transport chain, oxidative phosphorylation complexes, GPCR mediated signal transduction pathway and 12 house keeping genes including internal controls by using RT² Profiler PCR array kit (RT² Profiler™ PCR Array Mouse Mitochondrial Energy Metabolism, PAMM-008, GPCR pathwayFinder™ PCR array PAHS-071Z, Qiagen GmbH) and a Stratagene MX3000p real-time PCR system (La Jolla, CA, USA). The $\Delta\Delta C_t$ based fold were obtained by uploading the raw threshold cycle data to an integrated web based software package (RT² profiler PCR array data analysis version 3.5, Qiagen GmbH) for the PCR array system. Based on PCR array results some genes were validated by real-time PCR. The real-time PCR product was quantified by measuring SYBR Green (Agilent technologies, Santa Clara, USA) fluorescent dye incorporation with ROX dye reference and normalized to the housekeeping genes β -actin (*Actb*), glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*), hypoxanthine guanine phosphoribosyl transferase (*Hprt*) and Heat shock protein 90 alpha class B member 1 (*Hsp90ab1*).

Glucose oxidation rate

MIN6 monolayer cells and pseudoislets were harvested, in triplicate per observation, transferred to incubation vials containing Krebs-Ringer bicarbonate-HEPES buffer, supplemented with 20 mmol/l glucose containing (15.5 GBq/mol) D-[U-¹⁴C]glucose. The vials were incubated for 90 min at 37°C under an atmosphere of 95/5% O₂/CO₂, during slow shaking. The metabolism was arrested by adding 17 μmol/l antimycin A. Subsequently, the released labeled ¹⁴CO₂ was trapped in 250 μl hyamine hydroxide during an incubation at 37°C for 2 h, and the radioactivity measured by liquid scintillation. Cell numbers in aliquots of the harvested MIN6 monolayers were counted in a Becton Dickinson FACS caliber flow cytometer (Becton Dickinson, San Jose, CA, USA). As for the pseudoislets triplicate groups of 50 islets harvested in parallel to those incubated with labeled glucose were dispersed as described above, and the cell number counted in the flow cytometer. The glucose oxidation rates were subsequently expressed per number of cells.

Palmitate and exendin-4 preparation and treatment

Stock solutions of palmitate (Sigma) were prepared by dissolving the fatty acid in 50% ethanol to a final concentration of 100 mmol/l. The stock solution is then diluted in appropriate culture medium with 0.5% fatty acid free BSA (Boehringer Mannheim GmbH, Mannheim, Germany) to a molar ratio of 6:1. Exendin-4 (Sigma) was prepared to a stock solution of 10 mmol/l. Human islets were treated with or without 0.5 mmol/l palmitate in presence or absence of 10 nmol/l exendin-4 for 2 and 7 days. MIN6 pseudoislets were exposed to 0.5 mmol/l palmitate for 2 days.

siRNA transfection and palmitate treatment

MIN6 pseudoislets were transfected with scrambled or with SOCS2 siRNA for 24 hours in serum-free Opti-MEM (Invitrogen) medium. The transfection mixture contains 30 pmol/l siRNA and lipofectamine 2000 (Invitrogen). After transfection the pseudoislets were treated or not with 0.5 mmol/l palmitate for 48 hours.

Protein-protein interaction

For this experiments, 8 x 10⁶ MIN6 cells were cultured in 25 cm² flasks. After reaching 80% confluency cells were transfected with HaloTag-PIAS1 fusion construct (accession number NM_019663, catalog number EX-Mm07654-

M49) or HaloTag control vector (negative control) (Genecopoeia, Rockville, UK). After 24 hours transfection cells were harvested and kept at -80°C for 1 day. Cells were then lysed and proteins were pulled down according to manufacturer's protocol (Promega, Madison, USA). Briefly, cells were lysed in mammalian lysis buffer (Promega) and protease inhibitor cocktail (Promega) was added, incubated on ice for 15 min, homogenized using Dounce glass homogenizer and centrifuged. After centrifugation the supernatant was transferred to HaloLink resin (Promega), which was equilibrated with 1 x TBST in 0.1% IGEPAL-CA630 (Sigma). The proteins were allowed to bind in the resin for 2 hours at 4°C with gentle agitation. After washing 3 times the protein complex was eluted with buffer containing 8 mol/l urea and 150 mmol/l Tris at pH 8.5. The eluted proteins were separated by SDS gel electrophoresis and stained for 2 hours using Page blue staining solution according to manufacturer's protocol (Thermo Scientific, MA, USA). After separating the proteins, in-gel digestion procedure was performed essentially as described [138]. Briefly, the gel slices were cut into small (1 mm³) cubes, transferred into sample tubes and destained, washed and exposed to dithiothreitol (DTT) reduction and iodoacetamide (IAA) alkylation. Thereafter the proteins were digested by sequencing grade modified trypsin (Promega) at a concentration of 12.5 ng/μl in 25 mmol/l NH₄HCO₃ overnight at 37°C. The peptides were extracted by sonication in 60% acetonitrile (ACN) and 5% formic acid (FA). Finally, the extracted peptides were vacuum dried and resolved in 0.1% FA. Separation of the enzymatic peptides was performed on an EASY-nLC System (Thermo Scientific) using a 10 cm long C18-A2 column, ID 75 μm (Thermo Scientific). The mobile phase was composed of solvent A=0.1% FA and solvent B=0.1% FA, 99.9% ACN and the gradient used was 4-50% B in 60 min. Eluting peptides were electrosprayed on-line to a LTQ-Orbitrap Velos Pro ETD mass spectrometer (Thermo Scientific). One survey MS scan was acquired and followed by collision-induced dissociation (CID) MS/MS scans of 10 of the most intense ions. MS/MS spectra were searched using the MASCOT search engine (Matrix Science; version 2.2) embedded into Proteome Discoverer 1.4 (Thermo Scientific) against the Uniprot/Swiss-Prot database (release of 29-May-2013). The search parameters were: taxonomy: *Mus musculus*; enzyme: trypsin; fixed modifications: carbamidomethylation (C); dynamic modifications: oxidation (M) and deamidation (N, Q); peptide tolerance 10 ppm, MS/MS tolerance: 0.8 Da and maximum 2 missed cleavage sites. Proteins with at least two identified peptides with a probability of 0.95 or above were considered as correct identifications.

Co-immunoprecipitation

For the co-immunoprecipitation MIN6 cells were lysed and the Dynabeads approach used (Invitrogen). Briefly, the lysate was mixed with magnetic beads,

Dynabeads M-270 epoxy, at 4°C for 30 min. The Dynabeads were coupled with PIAS1 antibody by incubating the antibody with the beads for 24 hours at 4°C. After capturing the PIAS1 interacting partners the beads were washed for three times and eluted with elution buffer (Invitrogen). The eluted proteins were then electrophoresed and transferred to PVDF-membrane and incubated with antibody towards ATP-citrate lyase (ACLY; Cell Signaling), ATP synthase subunit 5 B (ATP5B; Santa Cruz, CA, USA) and PIAS1 (Cell Signaling). The immuno-reactive bands were imaged with ChemiDoc XRS⁺ (Bio-Rad).

Sample preparation for protein profiling

Cells were washed twice with PBS and lysed with a buffer containing 8 mol/l urea, 1% octyl beta-D-glucopyranoside in 10 mmol/l TrizmaBase, pH 8.0 for 20 min. After lysis samples were centrifuged at 10,000 rpm for 10 min to pellet any remaining debris. Buffer in the samples was exchanged to 50 mmol/l ammonium bicarbonate using PD SpinTrap G-25 columns (GE Healthcare, Life Sciences, Uppsala, Sweden) according to the manufacturer protocol. Total protein content was determined by the Bradford Protein Assay (Bio-Rad, Hercules, CA, USA). Samples were reduced by DTT (10 mmol/l, 56°C for 30 min), and alkylated by iodoacetamide (20 mmol/l, room temperature in darkness for 30 min). Before digestion with trypsin the Nanosep 3 Omega 3 kDa cut-off filters (Pall, Port Washington, NY, USA) were washed once with 100 µl 50% acetonitrile in 50 mmol/l NH₄HCO₃ by spinning them down. Samples were transferred onto the cut-off filters and spun down. The membranes were washed once with 100 µl 2% acetonitrile in 50 mmol/l NaHCO₃ and spun down and then with 100 µl 50% acetonitrile in 50 mmol/l NH₄HCO₃. Samples were diluted in 50 µl of NH₄HCO₃ and digested with trypsin at 37 °C for 18 hours. After digestion samples were spun down at 14,000 rpm for 30 min.

Protein expression profiles

Digested samples were re-dissolved in 0.1% trifluoroacetic acid to yield an approximate tryptic peptide concentration of 0.3 µg/µl prior to nanoLC-MS/MS analysis. The protein identification experiments were performed using a 7 T hybrid Linear ion trap Fourier Transform (LTQ-FT) mass spectrometer (ThermoFisher Scientific, Bremen, Germany) fitted with a nano-electrospray ionization (ESI) ion source (ThermoFischer Scientific). On-line nanoLC separations were performed using Agilent 1100 nanoflow system (Agilent Technologies, Waldbronn, Germany). The peptide separations were performed on in-house packed 15-cm fused silica emitters (75-µm inner diameter, 375-µm outer diameter). The emitters were packed with a methanol

slurry of reversed-phase, fully end-capped Reprosil-Pur C18-AQ 3 μm resin (Dr. Maisch GmbH, Ammerbuch-Entringen, Germany) using a pressurized packing device operated at 50-60 bars (Proxeon Biosystems, Odense, Denmark). The separations were performed at a flow of 200 nl/min with mobile phases A (water with 0.5% acetic acid) and B (89.5% acetonitrile, 10% water, and 0.5% acetic acid). A 100-min gradient from 2% B to 50% B followed by a washing step with 98% B for 5 min was used. Mass spectrometric analysis was performed using unattended data-dependent acquisition mode, in which the mass spectrometer automatically switched between acquiring a high resolution survey mass spectrum in the FTMS (resolving power 100,000 full width at half maximum) and consecutive low-resolution, collision-induced dissociation fragmentation of up to five of the most abundant ions in the ion trap.

Bioinformatics analysis

a) Label-Free Peptide and Protein Quantifications

The acquired spectra from 12 samples derived from pseudoislets (4 biological x 3 technical replicates) and 12 from monolayers (4 biological x 3 technical replicates) were uploaded to the Progenesis LC-MS Version 4.0 (Nonlinear Dynamics, Newcastle, England) after choosing Progenesis machine settings according to the respective acquisition instrument and analyzed the data as described previously.[139] Profile data of the MS scans were transformed to peak lists with respective peak m/z values, intensities, abundances (areas under the peaks), and m/z width. In the alignment step, we manually selected a reference run and the retention times of the other samples were aligned by manual and automatic alignment to a maximal overlay of all features. Features with only one charge or more than seven charges are masked at this point and excluded from further analyses. After alignment and feature exclusion, samples were divided into the appropriate groups (PI and MO), and raw abundances of the remaining features were normalized to allow correction for factors resulting from experimental variation.

Rank 1-10 MS/MS spectra were exported from the Progenesis software as Mascot generic file (mgf) and used for peptide identification with Mascot version 2.2 (Matrix Science, London, UK) in the International Protein Index (IPI) database version 3.68 mouse containing a total of 56,729 protein sequences. Search parameters used were: 10 ppm peptide mass tolerance and 0.6 Da fragment mass tolerance, 0 missed cleavage allowed, carbamidomethylation was set as fixed modification and methionine oxidation was set as variable modification. A Mascot-integrated decoy database search calculated a false discovery of $\leq 1\%$ when searching was performed on the concatenated mgf files with a significance threshold of $p \leq 0.01$ and the peptides resulted were re-imported into the Progenesis software. Calculations of the protein p value (one-way ANOVA) were then performed on the sum of the normalized abundances across all runs. All protein identifications are listed in supplemental Table 1.

b) Differential protein expression and pathway enrichment analysis

Based on the 12 technical replicates the mean normalized abundance for each protein was calculated for both the samples (PI, MO). From these mean normalized abundances the fold change ($\log_2(PI/MO)$) was calculated for each protein. The proteins with ANOVA values of $p \leq 0.05$ and additionally regulation of \log_2 (fold) value ≥ 1 or ≤ -1 were regarded as significant and were selected for further bioinformatics analysis. The pathway enrichment analysis was done using KEGG database by using bioCompendium tool developed at EMBL (available at <http://biocompendium.embl.de>). In this tool the Fisher's exact test was used to calculate the P-values for enriched pathways and the adjusted P-values were calculated by applying the Benjamini-Hochberg procedure in order to control the False Discovery Rate (FDR).

Data analysis

Results are presented as means \pm SEM. Statistical significance for the difference between two conditions was analyzed by the Student's *t*-test. Multiple comparisons between different groups were assessed using analysis of variance (ANOVA) followed by **Bonferroni's** post hoc test. $P < 0.05$ was considered statistically significant.

Results and discussion

Paper I

Functional differences between aggregated and dispersed insulin-producing cells (*Diabetologia*, 2013 Jul;56(7):1557-68).

The secretory capacity of MIN6 cells grown as pseudoislets in response to elevated glucose levels was compared with that of MIN6 cells grown as monolayers and with human islets. In static incubation pseudoislet insulin release was approximately 6-fold increased when the glucose concentration was raised from 2 to 20 mmol/l glucose. When MIN6 monolayer cells were exposed to the same glucose challenge a doubling of secretion was observed. The magnitude of the secretory response of human islets was similar to that of the MIN6 pseudoislets. Since mitochondrial anaplerosis plays an important role in insulin secretion [47-51] we investigated the role of pyruvate, KIC and KIC in combination with glutamine in insulin secretion from pseudoislets and compared to that generated from monolayer cells and human islets. The nutrient dependent stimuli induced a more vigorous response in pseudoislets compared to monolayers. This is in agreement with other reports, where MIN6 pseudoislets showed increased response to nutrient stimuli compared to monolayers [31, 140]. Since the mitochondrial substrates induced higher insulin secretion from pseudoislets compared to monolayers we hypothesized that expression levels of genes of mitochondrial metabolism were higher in pseudoislets compared to monolayer cells. To address the hypothesis, we investigated transcript levels of mitochondrial respiration and electron transport in pseudoislets and monolayer cells as well as glucose oxidation rate. A majority (76%) of the genes involved in Complex I (NADH-Coenzyme Q reductase), Complex II (Succinate-Coenzyme Q reductase), Complex III (Coenzyme Q-Cytochrome c reductase), Complex IV (Cytochrome C oxidase) and Complex V (ATP synthase) showed 1.4-fold or more increase in pseudoislets compared to monolayers. Genes that showed at least 2-fold in regulation in the array were also validated by qRT-PCR. Glucose oxidation rate in pseudoislets were (3.57 ± 0.63 (pmol glucose/cell x 90 min)) and monolayer cells were (1.09 ± 0.34 (pmol glucose/cell x 90 min)). Based on these results, we propose that enhanced mitochondrial metabolism contribute to the improved secretory capacity of cells in pseudoislets.

The IRS-1/PI3K pathway plays an important role in insulin secretion [61, 68, 69, 141]. Since islet anatomy plays a role in determining the insulin secretory capacity of the β -cell we measured IRS-1 in pseudoislets and monolayer cells. No difference was observed when total levels of IRS-1 were measured, however. In contrast, when levels of the inhibitory phosphorylation site at S636/639 of IRS-1 were determined, lower levels of p-IRS-1 were observed in pseudoislets and compared to monolayer cells. Likewise, when phosphorylation of p-IRS-1 at S636/639 was measured in intact and dispersed human and mouse islets, levels of IRS-1 phosphorylated at the inhibitory site were lower in intact islets compared to dispersed cells. To determine if the differences in p-IRS-1 at S636/639 were contributing to the observed differences in secretory patterns via effects on PI3K we measured insulin secretion in presence of PI3K inhibitor LY294002 and wortmannin. In support of a role of PI3K as a positive regulator of insulin secretion decreased GSIS was obtained from pseudoislets and human islets but not in monolayer cells when the inhibitors were applied. The notion for such a role of PI3K was further supported by reduced GSIS from mouse models lacking expression of PI3K regulatory subunits [71] or the catalytic subunit of the type 1B PI3K isoform [70]. To what extent the reduction in GSIS observed in exposure of PI3K inhibitors involved alterations in $[Ca^{2+}]_c$ was next investigated. In control pseudoislets rise in the glucose concentration from 2 to 20 mmol/l elicited a transient decrease in $[Ca^{2+}]_c$ followed by a marked increase that peaked within 2 min after the elevation of glucose. After the initial peak, $[Ca^{2+}]_c$ decreased to a plateau from which distinct oscillations ($\sim 2/\text{min}$) appeared. The $[Ca^{2+}]_c$ response was paralleled by a pronounced initial insulin secretory peak followed by oscillatory insulin levels. In pseudoislets pretreated with LY294002, a blunted GSIS was paralleled by a $[Ca^{2+}]_c$ response that was similar to control but with lower basal $[Ca^{2+}]_c$ and higher frequency of the glucose-induced oscillations. $[Ca^{2+}]_c$ was also recorded in pseudoislets pretreated with wortmannin. Although this drug markedly reduced insulin secretion it had no effect on $[Ca^{2+}]_c$. Together, these data indicate that the reduction in GSIS after PI3K inhibition is not explained by alterations in $[Ca^{2+}]_c$.

Paper II

Signaling in Insulin-Secreting MIN6 Pseudoislets and Monolayer Cells. (J Proteome Res. 2013 Dec 6;12(12):5954-62.)

In MIN6 pseudoislets and monolayer cells a total of 1792 proteins common to both pseudoislets and monolayers were identified. After discarding proteins with the abundances zero appearing in both pseudoislets and monolayers, 1576 proteins common to both biological conditions remained. For each protein the mean normalized abundance in pseudoislets and monolayer cells was determined. Based on the abundances obtained for pseudoislets and monolayer cells 488 proteins were differentially expressed with ANOVA values of $p \leq 0.05$ and additionally regulation of log₂ fold) value ≥ 1 or ≤ -1 . Out of the 488 proteins, 475 proteins IPI were matched with KEGG database. These 475 proteins were mapped to the bioCompendium tool and onto pathways using KEGG. Eleven pathways were significantly enriched with p value ≤ 0.05 . For each of the pathways, the identified proteins belonging to the pathways were listed, in total 221 proteins. No less than 98 of the 221 proteins belonging to the 11 significantly enriched pathways were found among the differentially expressed (one-fold up- or down-regulated; log₂) proteins. The 10 most up-regulated proteins when comparing pseudoislets and pseudoislets, based on the proteomic analysis, were validated by the orthogonal methodology western blotting. The proteins belonged to glycolysis, citrate cycle, lysosomal, ribosomal, spliceosomal and proteasomal pathways and thus belong to pathways enriched in the MIN6 pseudoislets and monolayer cells. When pyruvate kinase M2 isoform (Pkm2) and phosphofructokinase (Pfk1), members of the glycolytic pathway, were determined by western blotting they were both significantly up-regulated in pseudoislets. Proteins of the citrate pathway, ATP-citrate synthase (Acl) and isocitrate dehydrogenase 3 (Idh3g), were also markedly up-regulated in pseudoislets. N(4)-(beta-N-acetylglucosaminy1)-L-asparaginase (Aga), which belongs to the lysosomal pathway, was up-regulated more than 5-fold in pseudoislets compared to monolayers. Ribosomal protein L35a (Rpl35a), spliceosomal proteins small nuclear ribonucleoprotein D1 (Snrpd1) and serine/arginine-rich splicing factor 4 (Sfrs4), proteasomal protein preteosome 26s subunit (Psm7) were also up-regulated in pseudoislets compared to monolayers. In case of ribosomal protein ubiquitin A-52 residue ribosomal protein fusion product 1 (uba52), protein levels determined by western blotting were not different between pseudoislets and monolayers. The enriched pathways represent mechanisms including inter-cellular communication by direct cell-cell contact, exchange of small molecules through gap junctions and through paracrine effects, which have been suggested to contribute to explain the enhanced secretory function of intact islets [142-148].

Paper III

GLP-1 recovers impaired insulin secretion from human islets treated with palmitate via down-regulation of SOCS2. *Manuscript*

Secretory failure of the insulin-producing beta-cell is a crucial event in the progression of type 2 diabetes. One of the causes for such beta-cell failure is prolonged high levels of saturated free fatty acids (FFAs), which attenuate glucose-stimulated insulin secretion (GSIS) and increase apoptosis. Human islets were cultured for 2 and 7 days in the presence or absence of 0.5 mmol/l palmitate with or without 10 nmol/l exendin-4. Whereas islets treated with palmitate alone for 2 days showed almost 2-fold increase in GSIS compared to control, islets cultured for 7 days had significantly decreased insulin secretion. When exendin-4 was included during culture of islets exposed to palmitate, the perturbations of GSIS induced by the fatty acid were corrected. GSIS was significantly decreased after 2 days culture but increased after 7 days culture compared to islets exposed to palmitate alone. Mechanisms for the recovery of decreased GSIS from islets exposed to palmitate for 7 days by inclusion of exendin-4 were explored by measuring expression levels of 84 genes, which are members of GPCR signaling pathways. Palmitate-treatment altered expression of 36 genes by > 1.5 (up-regulation) or < 0.67 (down-regulation). Out of the 36 genes 9 were up-regulated and 27 were down-regulated. When human islets exposed to palmitate for 7 days were treated with exendin-4, 5 of the 9 up-regulated genes were down-regulated by < 0.67 . Among the 12 genes, which showed reversal of palmitate-induced differential expression by exendin-4, we validated SOCS1 and IL1-B. In addition we measured expression of SOCS2. Long-term palmitate treatment in human islets up-regulated transcripts level of SOCS1, SOCS2 and IL-1B and levels were decreased by inclusion of exendin-4. Next, we measured the protein levels of SOCS1 and SOCS2. SOCS1, which were not affected by exposure of human islets to palmitate or exendin-4 for 7 days. However, 7 days of palmitate treatment significantly increased SOCS2 protein level compared to control and addition of exendin-4 significantly decreased the protein level. To dissect the role of SOCS2 in modulation of insulin secretion by palmitate we measured PI3K activity by measuring Akt phosphorylation at site T308 as this site was previously correlated with PI3K activity [149, 150]. The observed increased SOCS2 protein level induced by palmitate treatment for 7 days was associated with decreased Akt T308 phosphorylation. Simultaneous exposure of the islets to exendin-4 reversed the process i.e. restored Akt phosphorylation, which was associated with decreased SOCS2 protein levels. In the present study we found that long-term palmitate treatment up-regulated mRNA expression of IL-1B, which is in agreement with other reports [151, 152]. As a consequence of inflammation suppressor of cytokine signaling (SOCS) proteins were up-regulated [153, 154]. Suppressor of cytokine signaling (SOCS) proteins were

reported to act as a negative regulator of cytokine action [109]. In this study our results suggest that long-term palmitate treatment can induce both SOCS1 and SOCS2 in mRNA levels while only SOCS2 was changed at protein level. In insulin-sensitive tissue from rodents fed high fat diet or human islets and beta-cell lines treated with high glucose or cytokines SOCS1, SOCS2 and SOCS3 levels were reported to be up-regulated both at mRNA and protein level [111, 115, 116, 155, 156]. Activation of SOCS proteins induced by cytokines can inhibit cytokine signaling by directly acting on Jak kinase to inhibit its tyrosine kinase activity or by binding to the cytokine receptor [109, 157-159]. In the present study it was demonstrated that when induction of SOCS2 was inhibited, the effects of palmitate were attenuated and GSIS restored. It may be concluded that SOCS2 in palmitate-treated human islets participates in signaling, which is detrimental to the beta-cell. The negative role of SOCS2 in insulin secretion was reported by several others, where constitutively activated SOCS2 in mouse, rat islets or adenoviral mediated overexpression of SOCS2 in MIN6 decreased GSIS [120-122]. A single nucleotide polymorphisms of SOCS2 was reported to be associated with T2DM [121]. The constitutively activated SOCS2 decreased intracellular Ca^{2+} stores, which were coupled to the decreased GSIS. Beside this, constitutively activated SOCS2 also increases pro-insulin by decreasing both mRNA and protein level pro-hormone convertase 1/3 [120]. Although islets from SOCS2^{-/-} mouse did not have decreased insulin secretion, but in this knock out mouse the compensatory role of other SOCS protein is unknown [119]. The induction of both SOCS1 and SOCS3 has negative effect of insulin signaling which occurs either by degrading IRS-1, IRS-2 [112] or by inhibiting tyrosine phosphorylation and thereby decreasing PI3K activity in beta-cells and adipose tissue [111, 155]. More importantly, we and others have reported the importance of PI3K activity in insulin secretion [68, 160-163]. Our results suggest that up-regulation of SOCS2 may have similar effect on PI3K activity as was reported for SOCS2 to interact with IGF-1R [164, 165]. Activation of the IGF-1R activates the PI3K cascade [166]. Our results suggest the potential role of SOCS2 in PI3K activity and GSIS.

Paper IV

Role of PIAS1 in palmitate-mediated beta-cell dysfunction. *Manuscript*

In paper IV we continued to further dissect the role of long of palmitate treatment in β -cells dysfunction. Levels of PIAS1 in 7 days palmitate-treated human islets were approximately doubled compared to islets not exposed to the fatty acid. To gain insight into the role of PIAS1 in islet function, PIAS1 interacting partners were pulled down. Among the PIAS1 interacting partners ATP citrate lyase (ACLY) and ATP synthase beta and alpha subunits (ATP5B

and ATP5A) were identified in the islet. The interaction between PIAS1 and the enzymes was validated by co-immunoprecipitation using MIN6 cells. The co-immunoprecipitation showed that PIAS1 interacted with ACLY and ATP5B proteins. Next, we measured levels of ACLY and ATP5B in PIAS1 over-expressing MIN6 cells. Both ACLY and ATP5B levels were decreased. Decreases in both protein levels of the enzymes were also observed in human islets when treated with palmitate for 7 days. Given the role of ACLY in lipogenesis we measured levels of ACC and FAS in palmitate-treated islets. Palmitate treatment decreased levels of ACC and FAS by 40% and 50%, respectively, compared to islets not exposed to the fatty acid. In the present study we demonstrated that long-term exposure of insulin-producing beta-cells to palmitate, which is associated with impaired insulin secretion, up-regulated PIAS1. PIAS1 has so far primarily been studied in cellular signaling including STAT1 and NF- κ B pathways in cytokine signaling [123, 167]. The potential role of the protein in lipid metabolism is only emerging [130, 131]. It was therefore intriguing to see that one of the PIAS1 interacting partners were ACLY. ACLY convert's citrate to acetyl-CoA and oxaloacetate and acetyl-CoA is an essential substrate for fatty acid synthesis, which is catalyzed by ACC [168]. Long-term palmitate treatment was reported to decrease level of ACLY as well as activity from human and mouse islets and also from islets from HFD mouse [169]. ACLY was also shown to play an important role in insulin secretion and apoptosis by reducing ER stress [169, 170].

PIAS1 was earlier reported to suppress the effect of LXR β [130] and LXRs were shown to control the expression of SREBP1c, FAS, ACC and SCD1 positively [171-174]. Both FAS and ACC was previously reported to play an important role insulin secretion, as decrease in ACC activity or knock down of FAS in INS-1 cells decreased GSIS followed by decrease in *de novo* fatty acid species [168, 175]. The importance of *de novo* fatty acid synthesis during GSIS was reported earlier [176, 177]. Our results showed that up-regulation of PIAS1 was followed by down-regulation of lipogenic genes ACC and FAS, which is consistent with other reports [130, 178-180]. ATP serves as the energy source for most molecular events [181]. In the beta cell impairment in ATP synthesis results in dysfunction with reduced GSIS [182, 183]. Overexpression or down-regulation of ATP5B increased or decreased ATP levels in INS-1 cells, respectively and GSIS was altered accordingly [184, 185]. ATP content was significantly lowered in livers of HFD induced T2DM mice underscoring the significant role of ATP5B in the synthesis of mitochondrial ATP [186, 187]. In this study PIAS1 was demonstrated to interact with ATP5B. Decreased levels of the ATP synthase subunit, which was found in palmitate-treated PIAS1 up-regulated islets, suggest that ATP synthesis is down-regulated and thereby reduced insulin secretion.

Conclusions

The main conclusions of this thesis are:

- Enhanced mitochondrial metabolism contribute to the improved secretory capacity from islets which has more cellular interactions.
- Cell-cell interactions alter IRS-1 phosphorylation which leads to enhanced insulin secretory capacity from islets.
- The enhanced secretory capacity from islets may depend on pathways regulating glucose metabolism, cell interaction and translational regulation.
- Long-term palmitate treatment up-regulates SOCS2, which may reduce PI3K activity thereby reducing GSIS.
- Long-term palmitate treatment of islets increased levels of PIAS1, which in turn decreased ACLY and ATP5B protein levels implicating decreased lipogenesis as well as ATP synthesis. PIAS1 levels may thus contribute to reduced GSIS and increased apoptosis.

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