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Diabetogenic Effects of Glucocorticoids – Role of Protein Phosphatase 5 and GLP-1 Receptor Activation

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ABSTRACT

Obesity, the metabolic syndrome and type 2 diabetes are inextricably linked together, and all increase with rapid pace globally. Glucocorticoid (GC) excess is associated with diabetogenic effects, including insulin resistance and glucose intolerance, and a role for GCs has also been proposed in the development and pathology of the metabolic syndrome. Studies have indicated that patients with glucose intolerance or the metabolic syndrome have elevated levels of cortisol and in addition, the metabolic syndrome shares many phenotypical characteristics and pathologies with cortisol excess. This thesis aimed at studying diabetogenic effects of GCs and the role of protein phosphatase 5 (PP5) and GLP-1 receptor activation in a setting of GC excess in mice *in vivo*. Apoptosis of insulin producing pancreatic β -cells is believed to play an important role in the pathologic development from insulin resistance and disturbed glucose tolerance to overt diabetes. Mechanisms underlying GC-mediated direct cytotoxic effects on β -cells were studied *in vitro* with a special emphasis on the involvement of PP5.

For studies *in vivo*, mice were exposed to GCs for five weeks via their drinking water and characterized in terms of glucose and lipid handling. The results revealed several features mimicking the metabolic syndrome in humans. Mice became obese with ectopic fat deposition and dyslipidemia, they became hyperglycemic and hyperinsulinemic with insulin resistance and they developed hypertension. Treatment with the GLP-1 receptor agonist liraglutide slowed progression towards obesity and ectopic fat deposition and improved glucose control. Mice with a global knock-out of PP5 (*Ppp5c*^{-/-}) were in part protected against GC-induced hyperglycemia and hyperinsulinemia. *Ppp5c*^{-/-} mice receiving vehicle exhibited better glucose handling than *Ppp5c*^{+/+} cognates during glucose tolerance test. The observed effects of the model were reversed after GC removal. Furthermore, the studies revealed insights into β -cell adaptation to the increased insulin demand, in this setting of GC-induced insulin resistance. Increased islet volume due to cell proliferation, increased β -cell and α -cell mass, increased insulin secretory capacity and islet chaperone expression were found in GC-treated mice.

The *in vitro* study revealed that insulinoma cells and isolated pancreatic islets succumbed to detrimental direct effects of the synthetic GC dexamethasone via increased apoptosis. Dexamethasone activated mitogen-activated protein kinase (MAPK) signaling as evident by enhanced phosphorylation of p38 MAPK and c-Jun N-terminal kinase (JNK). Inhibition of p38 MAPK attenuated dexamethasone-induced apoptosis and decreased phosphorylation of the GC receptor. In contrast, inhibition of JNK augmented the cytotoxic effect of dexamethasone. Downregulation or lack of PP5 made cells and islets more susceptible to the apoptotic GC-effects with concomitant activation of p38 MAPK, suggesting that MAPKs and PP5 work in concert to regulate the cytotoxic effects of GCs in these cells.

In conclusion, the model mimicking the metabolic syndrome in humans could be valuable for studying mechanisms behind development of the metabolic syndrome and diabetes, as well as the multifaceted relations between GC excess and disease. Furthermore, liraglutide can be beneficial for patients at risk of developing metabolic complications in the setting of GC surplus. Lack of PP5 was protective against the deleterious effects of GCs *in vivo*, however, in the *in vitro* situation with direct effects of GCs on β -cells, a lack of PP5 was instead detrimental, leading to the conclusion that the actions of PP5 might be double edged and contradictory in different tissues.

LIST OF PUBLICATIONS

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

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Diabetology & Metabolic Syndrome 6:3
- III. **Liselotte Fransson**, Petra Wolbert, Richard E. Honkanen, Åke Sjöholm, Henrik Ortsäter. Genetic disruption of protein phosphatase 5 protects against corticosterone-induced hyperglycemia and hyperinsulinemia in mice.
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- IV. **Liselotte Fransson**, Victoria Rosengren, Titu Kumar Saha, Nina Grankvist, Tohidul Islam, Richard E. Honkanen, Åke Sjöholm, Henrik Ortsäter (2014) Mitogen-activated protein kinases and protein phosphatase 5 mediate glucocorticoid-induced cytotoxicity in pancreatic islets and β -cells.
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LIST OF ABBREVIATIONS

[Ca ²⁺] _i	Free cytoplasmic calcium concentration
11 β -HSD1	11 β -hydroxysteroid dehydrogenase type 1
11 β -HSD2	11 β -hydroxysteroid dehydrogenase type 2
ACTH	Adrenocorticotrophic hormone
ADP	Adenosine diphosphate
ASK-1	Apoptosis signal-regulating kinase 1
ATM	Ataxia telangiectasia mutated
ATP	Adenosine triphosphate
BSA	Bovine serum albumin
Calr	Calreticulin
cAMP	Cyclic adenosine monophosphate
Canx	Calnexin
CBG	Corticosteroid-binding globulin
CC3	Cleaved caspase 3
Cdc	Cell division cycle protein
CDKN1A	Cyclin-dependent kinase inhibitor 1
CoA	Coenzyme A
CRH	Corticotropin-releasing hormone
DAG	Diacylglycerol
DMEM	Dulbecco's Modified Eagle Medium
DNA-PK	DNA-dependent protein kinase
DPP-4	Dipeptidyl peptidase-4
ECL	Enhanced chemiluminescence
eIF2 α	Eukaryotic translation initiation factor 2 α
ELISA	Enzyme-linked immunosorbent assay
Epac	Exchange protein activated by cAMP
ER	Endoplasmic reticulum
ERK	Extracellular signal-regulated kinase
FBS	Fetal bovine serum
GC	Glucocorticoid
GIP	Glucose-dependent insulinotropic peptide
GLP-1	Glucagon-like peptide-1
GLP-1R	GLP-1 receptor
GLUT	Glucose transporter
GR	Glucocorticoid receptor
GSIS	Glucose-stimulated insulin secretion
HRI	Heme-regulated inhibitor
Hsp	Heat-shock protein
IPGTT	Intraperitoneal glucose tolerance test
IPinsTT	Intraperitoneal insulin tolerance test
IR	Insulin receptor
IRS	Insulin receptor substrate
JNK	c-Jun <i>N</i> -terminal kinase
MAPK	Mitogen-activated protein kinase

MAP2K	MAPK kinase
MAP3K	MAPK kinase kinase
MR	Mineralocorticoid receptor
mRNA	Messenger RNA
NEFA	Non-esterified fatty acids
PBS	Phosphate buffered saline
PC	Pyruvate carboxylase
PCR	Polymerase chain reaction
PDI	Protein disulfide-isomerase
Pdx1	Pancreatic and duodenal homeobox 1
PERK	PKR-like ER localized eIF2 α kinase
PFA	Paraformaldehyde
PKA	Protein kinase A
PP5	Serine/threonine protein phosphatase 5
PPP	Phosphoprotein phosphatase
qPCR	Quantitative PCR
ROS	Reactive oxygen species
RPMI	Roswell Park Memorial Institute (cell culture medium)
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SEM	Standard error of the mean
SGK1	Serum/glucocorticoid regulated kinase 1
siRNA	Short interfering RNA
STIP1	Stress-induced phosphoprotein 1
SVA	Statens veterinärmedicinska anstalt
T1DM	Type 1 diabetes mellitus
T2DM	Type 2 diabetes mellitus
TPR	Tetratricopeptide repeat
TXNIP	Thioredoxin-interacting protein
UPR	Unfolded protein response
VDCC	Voltage-dependent Ca ²⁺ channels
WHO	World Health Organization

1 INTRODUCTION

1.1 DIABETES AND THE METABOLIC SYNDROME

Diabetes is said to be one of the first diseases ever described. The oldest record of the disorder is found on Egyptian papyrus scrolls dating back to about 1500 BC. These hieroglyphs give a clinical image of a patient with pronounced lack of insulin – a condition known today as type 1 diabetes mellitus (T1DM). Before the discovery of insulin this was a deadly condition, affecting young people with marked weight loss, increased thirst and frequent urination, preceding certain premature death. The symptoms of the patients led to the name of the disease, diabetes mellitus, with diabetes meaning “to pass through” referring to the excessive discharge of urine, and mellitus meaning “honey-sweet” coming from the fact that the high blood sugar in these patients leads to excretion of glucose via the urine, giving it a sweet taste noticed by ancient physicians [1, 2].

The first patient ever receiving insulin injections as a treatment for T1DM was Leonard Thompson in 1922. This young boy would most likely have died within weeks without treatment, but was saved due to the insulin injections and lived for an additional 13 years with continuous treatment. The discovery that diabetic patients could be medicated with injections of insulin made Frederick Banting and John J. R. Macleod Nobel Prize laureates in 1923. This would however not have been possible without the discoveries of the pancreatic islets by Paul Langerhans in 1869, the development of the technique with pancreatic duct ligation, leading to degeneration of the exocrine pancreas, described by Claude Bernard, as well as the finding that surgical removal of the pancreas gave dogs diabetes, discovered by Oscar Minkowski in 1889. Oscar Minkowski understood that the pancreas secreted something that regulated blood glucose levels, but it was not until Frederick Banting and Charles Best used his and Bernard’s knowledge and extracted insulin from the endocrine pancreas that the big breakthrough was a fact. Banting and Best injected the extraction into pancreatectomized diabetic dogs and they found that this improved their condition. The extraction was named insulin after the Greek word for islet, *insula*, and a treatment for T1DM patients was soon a fact [1, 2].

The majority of patients suffering from diabetes today are, however, not T1DM patients on insulin therapy, but patients having another form of the disease, namely type 2 diabetes mellitus (T2DM). About 90% of diabetic patients nowadays have T2DM. The major difference between T1DM and T2DM is that T1DM is an autoimmune disease with loss of insulin-producing pancreatic β -cells leading to insulin deficiency whilst T2DM is a complex spectrum of complications where patients, at least early in the disease, can have very high insulin levels but, due to loss of the insulin response, still experience hyperglycemia [1, 3, 4]. The prevalence of T2DM is increasing fast globally and the disease that in earlier days mostly affected the elderly population is now an actuality and a growing problem also in children and adolescents [5]. According to the World Health Organization (WHO) about 347 million people world-wide have diabetes today and diabetes deaths will double between 2005 and 2030 [6, 7]. This global diabetes epidemic goes hand in hand with rapid increases in overweight, obesity and physical inactivity, not only in the developed world, but more and more evident also in developing countries [7-12].

Concurrent with the increased prevalence of obesity, another factor involved in the diabetes epidemic is advancing globally – namely the metabolic syndrome. The metabolic syndrome is a cluster of abnormalities including insulin resistance, hyperglycemia, abdominal obesity, dyslipidemia and hypertension, together giving an increased risk for T2DM and cardiovascular events [13-15]. A link between hypertension and T2DM had been discussed already in the 1920s but it was G. M. Reaven that first described the metabolic syndrome with its concurrent components, and proposed insulin resistance as a common etiological factor [16]. Reaven called the collection of disorders “syndrome X”, but it was later renamed metabolic syndrome.

This thesis will discuss T2DM and the metabolic syndrome in the setting of glucocorticoid (GC) excess, as well as the role of protein phosphatase 5 (PP5) and glucagon-like peptide-1 (GLP-1) receptor activation for alleviation of the diabetogenic effects induced by GCs. In order to understand the pathology behind the metabolic syndrome and T2DM it is important to know the normal physiological state concerning insulin signaling, regulation of blood glucose levels and the insulin-secreting pancreatic β -cell. To introduce my work I will therefore first describe how blood glucose homeostasis is maintained in health, followed by pathology of insulin resistance and T2DM, β -cell death and the metabolic syndrome, explained in more detail. In the end of my introduction I will present GC effects, PP5 and GLP-1.

1.1.1 The insulin-secreting pancreatic β -cell

The pancreatic β -cell is one of the specialized hormone-secreting cells situated in the islets of Langerhans. These pancreatic islets, or regions of cell clusters, are small micro-organs scattered through the pancreatic gland. The pancreas, located in the abdominal cavity, is a mixed exocrine and endocrine organ producing digestive enzymes as well as hormones involved in metabolic regulation. The endocrine, hormone-producing, part, made up of the islets of Langerhans, represents 1-2% of the pancreas mass. The islets consist of α -cells producing glucagon (Fig. 1), β -cells producing insulin (Fig. 1), δ -cells producing somatostatin, PP-cells producing pancreatic polypeptide and the more recently discovered ϵ -cells producing ghrelin [17].

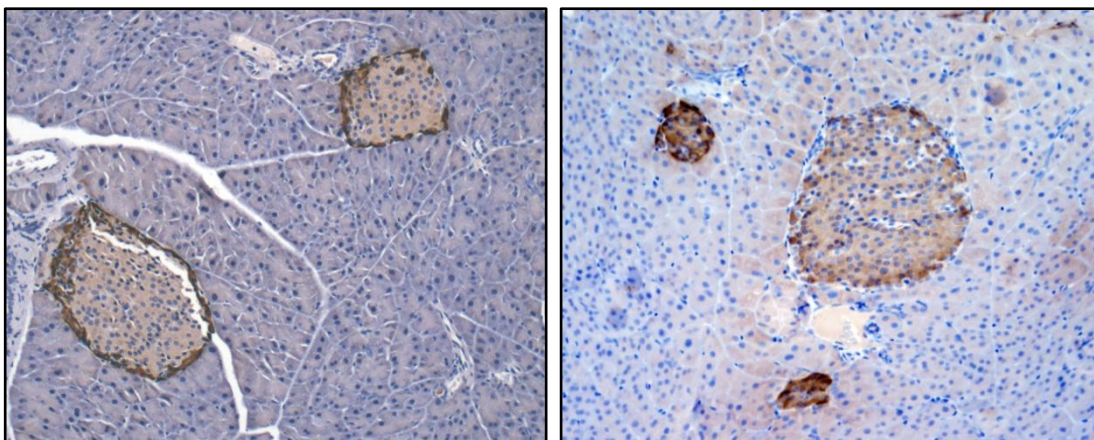


Figure 1. Mouse islets immunostained for glucagon (left) and insulin (right).

These endocrine cells cooperate to maintain glucose homeostasis where the function of glucagon is to raise blood glucose levels, opposite to that of insulin which promotes glucose lowering activities. Somatostatin acts to inhibit the secretion of other hormones including insulin and glucagon and pancreatic polypeptide affects both endocrine and exocrine release.

The majority of the cells in human pancreatic islets, between 50-80%, are insulin-producing β -cells [18-20]. Their embryonic origin, the same as for the other islet cells, is endocrine precursor cells that differentiate from a subset of epithelial progenitor cells in the pancreatic ducts. These cells aggregate in clusters and make up the pancreatic islets during the first trimester of embryonic development [21, 22]. Appearance of pancreatic β -cells is found already at week 9 post conception in human embryos and the β -cell mass gradually increases via differentiation and proliferation throughout the prenatal period [23].

Expansion of pancreatic β -cell mass also occurs postnatally and in adulthood, primarily by self-duplication [24-26] but also by neogenesis [27]. β -cells display considerable plasticity and can increase in both number and size in response to an increased demand for insulin, as found with chronic hyperglycemia and insulin resistance [28-32].

1.1.2 Insulin synthesis and release

Insulin is the only hormone in the body capable of directly lowering blood glucose levels. The top priority task for the pancreatic β -cells is to sense nutrients, neurotransmitters and hormones in the circulating blood, as well as neuronal stimuli, and integrate these signals into an appropriate insulin secretory rate in order to maintain euglycemia [33]. A prerequisite for insulin secretion is the biosynthesis of the hormone. The rate of biosynthesis needs to be regulated in order to compensate for the variations in secretory rate. The synthesis of insulin includes transcription of the gene to mRNA, translation of insulin mRNA into preproinsulin, processing of preproinsulin via proinsulin into insulin and C-peptide and induction of additional components of the secretory pathway to support processing, transport and exocytosis of insulin granules. The endoplasmic reticulum (ER) is the cell organelle playing an essential role for these processes. A functional ER is crucial to all eukaryotic cells but especially important in a professional hormone secreting cell as the pancreatic β -cell. In eukaryotic cells, the ER provides a contained environment for the synthesis and modification of membrane proteins and proteins destined for secretion. To assist the protein folding process, the ER contains high levels of protein chaperones including the glucose-regulated proteins, Grp78 and Grp94, calnexin, calreticulin, protein disulphide isomerase and Erp72 [34-37].

Synthesized and processed insulin is stored as hexamers in crystalline form within secretory granules close to the β -cell plasma membrane (the reserve pool) or docked with the membrane (the readily releasable pool) [38]. Upon stimulation these membrane vesicles fuse with the plasma membrane and insulin is released to the extracellular space. After a meal the blood glucose rises, which is sensed by the pancreatic β -cells, leading to glucose-stimulated insulin secretion (GSIS). GSIS includes a triggering and an amplifying pathway [39]. The triggering pathway starts with the entry of glucose into the pancreatic β -cell via glucose transporter (GLUT) 1

(GLUT 2 in rodents) and phosphorylation of glucose to glucose 6-phosphate by glucokinase, which is the rate limiting step in glycolysis [1, 40, 41]. During glycolysis glucose is, via a chain of enzyme-catalyzed reactions and intermediates, converted to pyruvate. Pyruvate enters the mitochondria and the citric acid cycle either as acetyl coenzyme A (acetyl CoA) via the action of pyruvate dehydrogenase or as oxaloacetate via the anaplerotic enzyme pyruvate carboxylase (PC) [42]. Of note, production of lactate is virtually absent in primary pancreatic β -cells due to low expression levels of lactate dehydrogenase [43]. Glycolytical and mitochondrial glucose metabolism eventually results in increased production of adenosine triphosphate (ATP) and thus an increase of the ATP to adenosine diphosphate (ADP) ratio [44]. This rise in ATP/ADP ratio leads to closure of the ATP-sensitive K^+ (K_{ATP}) channels in the plasma membrane, causing membrane depolarization [45-47] and subsequent opening of voltage-dependent Ca^{2+} channels (VDCC), influx of extracellular Ca^{2+} into the cell and thus increased levels of cytoplasmic free Ca^{2+} ($[Ca^{2+}]_i$). The increase of $[Ca^{2+}]_i$ triggers fusion of the secretory granules with the plasma membrane and subsequent insulin exocytosis [48-50].

In addition to the triggering effect on insulin secretion, glucose metabolism also generates signaling molecules that amplifies insulin secretion without acting on K_{ATP} channels [51, 52]. This amplifying pathway potentiates the stimulatory effect of elevated $[Ca^{2+}]_i$ and has been found to be impaired in animal models of T2DM [53, 54]. The amplifying pathway is, as the triggering pathway, dependent on glucose metabolism [55, 56] but does not require an additional increase of $[Ca^{2+}]_i$ [38, 57].

The insulin release has a first and a second phase. The first phase begins only minutes after glucose stimulation but is transient, lasting about 5-10 minutes. If hyperglycemia still prevails, a slower but sustained second phase of insulin secretion emerge [58]. The first phase originates from the readily releasable pool of insulin granules. It enhances glucose uptake and is believed to suppress hepatic glucose production [59]. Loss of first phase insulin secretion is an early pathogenic event in T2DM [1, 60]. While first phase insulin release has been considered to be the result of the triggering pathway, the second phase has been attributed to the amplifying pathway. However recent data has indicated that the amplifying pathway is operative also during first phase insulin release, showing that the boundaries between triggering and amplifying pathways are not clear cut during biphasic insulin secretion [39, 58, 61, 62].

Insulin secretion occurs in a pulsatile, or oscillatory, manner [63-65]. Pulsatile insulin delivery has greater hypoglycemic effect than continuous delivery [66]. Loss of the insulin oscillations is found in T2DM patients, as well as their close relatives, and might contribute to insulin resistance [67-69]. The oscillations in insulin release occur synchronous with oscillations in $[Ca^{2+}]_i$ [48] but insulin oscillations persist also during stable low or elevated $[Ca^{2+}]_i$ [70, 71]. The underlying mechanisms controlling oscillatory insulin secretion remains elusive but its synchrony with fluctuations in metabolic parameters such as oxygen consumption and ATP production, suggest cellular metabolism to be the pacemaker and that Ca^{2+} has an amplifying effect [72-75]. The pulsatile secretion of insulin is coordinated not only between β -cells within an islet but also between islets. Gap junctions couples β -cells electrically and this, together with diffusible factors such as ATP, is essential for the synchronization of insulin release between β -cells within an islet. In addition, intrapancreatic nerves and diffusible factors seem most important spreading the pulsatility between different islets [74, 76].

1.1.3 Insulin signaling and regulation of blood glucose levels

The main purpose of the insulin secreted by the pancreatic β -cells is to maintain blood glucose homeostasis by suppressing hepatic glucose production (gluconeogenesis) [59], and by promoting glucose uptake in insulin-sensitive tissue. Insulin stimulates glucose uptake in skeletal muscle and adipose tissue by increasing translocation of GLUT4 to the plasma membrane [77, 78]. Furthermore, insulin initiates a signaling cascade promoting lipogenesis and lipid uptake and inhibiting lipolysis in adipose tissue, as well as stimulating glycogen synthesis in skeletal muscle [79].

Insulin promotes its action by signaling via the transmembrane tyrosine kinase insulin receptor (IR). The IR activated by insulin undergoes a conformational change leading to autophosphorylation of the tyrosine kinase domains and recruitment of a battery of regulatory and signaling molecules including insulin receptor substrate (IRS), phosphatidylinositol 3 kinase (PI3K) and AKT (protein kinase B). IRS proteins act complementary but also show tissue-specific differences in mediating insulin action, with IRS-1 playing a prominent role in skeletal muscle and IRS-2 being the most important IRS in the liver [80]. The recruitment and subsequent phosphorylation of IRS followed by PI3K docking and interaction as well as phosphorylation of AKT are all important steps in the insulin signaling pathway and constitutes the starting points for the insulin regulated metabolic processes in the cells [1, 81, 82].

1.1.4 Insulin resistance and type 2 diabetes

As mentioned above, T2DM is increasing rapidly world-wide affecting many millions of people. The disease is characterized by a relative lack of insulin leading to hyperglycemia. There are different criteria for the diagnosis of T2DM but the WHO classification of the disease is a fasting plasma glucose level above 7 mmol/L together with symptoms of hyperglycemia or, if there are no other symptoms, two fasting plasma glucose measurements above 7 mmol/L at different occasions. An oral glucose challenge can also be used where the plasma glucose should not exceed 11.1 mmol/L two hours after a 75 g glucose drink. A person with fasting plasma glucose above 6 but below 7 mmol/L is considered to have impaired fasting glycemia and a value between 8 and 11 mmol/L after a glucose challenge represents impaired glucose tolerance. These later conditions are revealing an increased risk of developing T2DM [1, 6].

The increase of T2DM goes hand in hand with increasing obesity. A hallmark of T2DM is insulin resistance which leads to a loss of the insulin response in the tissue, subsequent hyperglycemia and hyperinsulinemia. Obesity, especially abdominal obesity, is strongly associated with an increased risk of developing insulin resistance as well as T2DM [79, 83-85] but still, most obese people do not develop the disease [86]. The adipocytes are responsible for storage of energy as fat and with excessive energy intake and/or physical inactivity they have an impressive ability to grow in size and number to cope with the energy surplus. However if the adipocyte tissue is dysfunctional or not able to further expand, the lipid overflow will also be stored ectopically, in undesired sites in organs such as liver, heart and skeletal muscle, kidney and also pancreas. This ectopic fat storage inside organs, together with increased inflammation and increased levels of circulating non-esterified fatty acids (NEFA) released from adipocytes, are key factors for insulin resistance [84, 85, 87, 88]. Increased fatty acid metabolites such as diacylglycerol (DAG) or fatty acyl CoA in

muscle and liver, originating from increased NEFA uptake or decreased β -oxidation, can negatively influence correct IRS phosphorylation, leading to a diminished ability for insulin signaling [83, 87]. Abdominal fat cells are also more metabolically active than subcutaneous fat cells and in obesity, production of many adipokines and proinflammatory cytokines is increased, having negative impact on insulin sensitivity [89]. However, circulating concentrations of adiponectin, which is an adipokine with insulin-sensitizing and anti-inflammatory properties, are inversely associated with abdominal obesity [90].

Although dyslipidemia and insulin resistance are common findings in obese patients with an increased risk of T2DM, as long as the pancreatic β -cells can compensate for the insulin resistance by releasing more insulin, normoglycemia is maintained. However, impaired β -cell function and insulin release critically changes the conditions [91]. It has been shown in *post-mortem* studies that patients with T2DM have reduced β -cell mass, and β -cell death via apoptosis and/or decreased ability for β -cell replication or regeneration are the common explanations for this phenomenon [92-97]. Mechanisms underlying β -cell apoptosis will be further explained in the next subsection.

Clinical manifest glucose intolerance is estimated to be diagnosable ten or more years after onset of the disease [98]. However, even at this stage, a change in life-style with increased physical activity and decreased caloric intake can make substantial differences in glucose and insulin levels as well as weight loss [99]. When life-style changes are not enough there are also a number of different medications available for patients in need. Metformin is a first-line drug of choice, especially in obese T2DM patients, and it functions by decreasing liver gluconeogenesis, increasing insulin sensitivity in skeletal muscle and delaying intestinal glucose absorption [100]. A class of drugs called sulfonylureas is also used in T2DM treatment and their mode of action is to increase β -cell sensitivity to glucose by closure of the K_{ATP} channels and thus increase of insulin secretion. These drugs might, however, have negative effects on β -cell survival and mass due to constant secretion activity and possible exhaustion of β -cells [101]. The glitazone pioglitazone is also used in T2DM treatment and acts by activating PPAR- γ and thereby increasing insulin sensitivity in liver, skeletal muscle and fat cells [102]. Some glitazones have, however, been withdrawn from the market due to presumed severe side effects. Insulin in various forms and regimens is also used in later stages of T2DM, alone or in combination with oral drugs. The GLP-1 receptor agonists, such as liraglutide, and the dipeptidyl peptidase-4 (DPP-4) inhibitors are incretin-based drugs belonging to a newer generation of treatment for T2DM. They will be discussed further in later headings. The newest class of antidiabetic agents approved is the SGLT-2 inhibitors, one of which (dapagliflozin) is on the market in Europe [103]. SGLT-2 inhibitors act by inhibiting glucose re-absorption in kidney tubuli, thereby promoting urinary glucose excretion.

1.1.5 β -cell apoptosis

Programmed cell death, or apoptosis, is a normal physiological event during embryonic development [23]. In combination with replication and/or neogenesis, apoptosis also regulates β -cell plasticity in adult life. However, β -cell apoptosis is also linked to the pathological events seen in T1DM and T2DM [91]. In both cases a loss of β -cell mass

leads to lack of sufficient insulin needed to maintain euglycemia. Since human β -cell mass cannot be measured *in vivo*, evaluation of β -cell mass in patients relies on autopsy studies. Such studies performed on pancreatic glands retrieved from T1DM patients has indicated that β -cell loss ranges from 70 to 100% [93, 104]. In T2DM patients these numbers have ranged from 0 to 65%, depending on the studies [93]. In both T1DM and T2DM patients, an increase in β -cell apoptosis has been found [95, 104-106]. It has been estimated that by the time T2DM is clinically diagnosed, some 50% of functional β -cell mass has already been lost [107]. Hence, finding ways to protect β -cells from apoptotic demise, and thus modify the progressive course of T2DM, is one of the most urgent issues to be addressed in modern diabetology.

The cause of β -cell apoptosis in the development of T2DM is multifactorial and both genetic predisposition, inflammation and other environmental factors play a role. Metabolic stressors such as high glucose in combination with saturated fatty acids can be detrimental for β -cell function and survival, an effect referred to as glucolipotoxicity [108-110]. Different explanations exist regarding the underlying causes of the toxicity, with ER stress and oxidative stress being two of the most favored.

1.1.5.1 ER stress and β -cell apoptosis

ER stress is a complex process with many pathways, and it can lead to apoptosis especially in cells with major secreting tasks like pancreatic β -cells. In diabetes research, the link between ER stress and β -cell apoptosis has gained more and more interest since it was shown that ER stress markers are up-regulated in both human islets from T2DM patients [111, 112] and in islets from animal models of obesity and diabetes [113].

Agents or conditions that compromise ER protein folding capacity or an overload of newly synthesized polypeptides, as in a situation with high insulin demands, can lead to accumulation of unfolded or misfolded proteins in the ER. This in turn activates the unfolded protein response (UPR) [114]. The UPR is a collection of cellular events aiming to alleviate ER stress by decreasing overall protein synthesis through phosphorylation of the eukaryotic translation initiation factor 2 α (eIF2 α) [115], through enhanced protein folding capacity by increased expression of chaperones and through activation of mechanisms for protein degradation [116]. UPR activity is controlled by three pathways each powered by a specific transducer: the RNA-activated protein kinase-like ER localized eIF2 α kinase (PERK), the inositol-requiring enzyme 1 α (IRE1 α) and the activating transcription factor (ATF) 6. Under conditions of severe ER stress, or when the response has been compromised, the cell may be incapable of maintaining ER homeostasis and in such cases the detrimental effects of ER disruption can, via the UPR pathways, lead to apoptosis [117].

In pancreatic β -cells, exposure to both cytokines [118] and saturated fatty acids [119-121] activates parts of the ER stress response that is followed by an increased rate of apoptosis. While mild ER stress sensitizes β -cells to the apoptotic effects of cytokines via increase of proapoptotic proteins [122], and cytokines themselves induce ER stress by down regulating Ca²⁺ pumps in the ER membrane [123], the mechanism by which fatty acids can induce ER stress in pancreatic β -cells is currently not well known. Furthermore, both high and low glucose can activate the UPR in pancreatic β -cells [124, 125] and in accordance with the pro-survival function

of the UPR, several studies have indicated a necessity of especially PERK signaling for preserved β -cell function, and that an inability to activate PERK signaling and phosphorylation of eIF2 α results in reduced function and death of pancreatic β -cells [126-130].

1.1.5.2 Oxidative stress and β -cell apoptosis

Besides ER stress, oxidative stress induced by reactive oxygen species (ROS) has been linked to β -cell apoptosis, via glucotoxicity and secretory dysfunction [131]. ROS are derived from mitochondria during glucose metabolism and have been found to be both positive and negative signaling molecules for GSIS as well as destructive and pro-apoptotic in β -cells [132-134]. Hyperglycemia combined with hyperlipidemia increases ROS further and might be part of the explanation for glucolipotoxicity [135, 136]. Pancreatic β -cells have a relatively low levels of antioxidant defense molecules [137, 138] and might therefore be conspicuously vulnerable to the deleterious effects of ROS [139, 140]. Antioxidant treatment has been proposed as possible means to slow the adverse processes of excessive ROS production and thus slow β -cell apoptosis [139, 140]. The mechanism behind the apoptotic effects of oxidative stress and ROS is the DNA damage response, which can either lead to DNA repair via checkpoint signaling and cell-cycle arrest or to apoptosis [141, 142].

1.1.5.3 β -cell apoptosis signaling via MAPK

An evolutionary well conserved signaling cascade that functions as a positive regulator of apoptosis is the mitogen-activated protein kinase (MAPK) signaling pathway [143, 144]. The MAPKs regulate a number of cellular functions and disturbances in their signaling networks are coupled to the pathogenesis of several diseases, including different forms of cancer [145, 146], insulin resistance and diabetes [147, 148] and neurodegenerative disorders [149]. Three pathways represent the core of the MAPK signaling: the extracellular signal-regulated kinase (ERK), c-Jun *N*-terminal kinase (JNK) and p38 MAPK pathways. ERK is primarily activated by growth factors, mitogens and proinflammatory cytokines, whereas JNK and p38 MAPK mainly are activated by environmental stresses such as UV radiation and oxidative stress, hypoxia, DNA-damaging agents and cytokines [150]. The MAPKs are regulated and activated through a phosphorylation cascade beginning with a MAPK kinase kinase (MAP3K) that phosphorylates and activates a downstream MAPK kinase (MAP2K) which in turn phosphorylates and activates a downstream MAPK. The MAPK in turn phosphorylates a substrate that in general is either another protein kinase or a transcription factor [144]. Phosphorylation events by kinases and dephosphorylation, induced by protein phosphatases, are key elements in the regulation of MAPK signaling. In general, activation of ERK affects cell proliferation and differentiation, whereas activation of p38 MAPK and JNK affects cell proliferation, differentiation, immune response and apoptosis. In pancreatic β -cells it has been shown that low glucose levels lead to ROS production and ROS and H₂O₂ in turn activate MAPKs and promote apoptosis [151, 152].

1.1.6 The metabolic syndrome

The metabolic syndrome is a collection of abnormalities that include hyperglycemia, abdominal obesity, dyslipidemia and hypertension [13-15]. The components of the metabolic syndrome are also associated with endothelial dysfunction, atherosclerosis, vascular morbidity and mortality [153, 154]. The pathogenesis of the metabolic syndrome is multifactorial where caloric surplus, due to western diet and sedentary lifestyle, is coupled with genetic factors and these together interact to produce the syndrome. As mentioned above, the prevalence increases dramatically and goes hand in hand with the increase of T2DM [155, 156]. It is estimated that as much as one fourth of the world's adult population has the metabolic syndrome [157, 158] and since the metabolic syndrome is an explicit risk factor for T2DM, the increase of the metabolic syndrome is directly connected to the increase of T2DM.

Studies have indicated that patients suffering from the metabolic syndrome have elevated levels of cortisol [159-164] as do patients with glucose intolerance [165]. However, cortisol elevations in these conditions are far from as apparent as cortisol levels seen in patients with overproduction of endogenous cortisol, as in Cushing's disease or Cushing's syndrome. Even so, a disturbed regulation of the cortisol secretion has been reported in patients with the metabolic syndrome [154], and individuals with abdominal obesity have been found to have increased urinary free cortisol [166], loss of diurnal cortisol variation [167] as well as abnormal feed-back suppression of cortisol production in response to higher doses of the synthetic GC dexamethasone [168]. The metabolic syndrome also shares many phenotypical characteristics and pathologies with cortisol excess and in line with that, a hypothesis has been formulated that cortisol plays a role also in the development and pathology of the metabolic syndrome [154, 169-171].

1.2 GLUCOCORTICOIDS – SECRETION AND FUNCTION

Glucocorticoid (GC) hormones, like cortisol in humans and corticosterone in rodents, are a class of steroid hormones with 21-carbon structures consisting of four cycloalkane rings joined to each other. They act by binding to the glucocorticoid receptors (GR), which are present in almost all cells, as well as to the mineralocorticoid receptors (MR) [172]. GCs are primarily responsible for regulating carbohydrate metabolism [173, 174], thus the suiting name glucocorticoids. In principle, GCs mobilize glucose to the systemic circulation by affecting different target tissues. GCs are also part of the regulatory network in the immune system that inhibits several inflammatory pathways and blocks immune activity, and GC-based drugs are therefore effective anti-inflammatory and immunosuppressive medications against allergies, asthma and autoimmune diseases as well as effective in decreasing the risk of transplant rejection after allotransplantations [175]. However, GCs have a number of targets and the widely prescribed GC-based drugs can unfortunately give rise to several severe side-effects related to metabolic disturbances as well as a wide range of other pathological states [172, 176, 177].

1.2.1 Regulation of glucocorticoid secretion

GCs are produced from cholesterol (Fig. 2) and released from the zona fasciculata of the adrenal gland under the control of a prototypic neuroendocrine feedback system, the hypothalamic-pituitary-adrenal (HPA) axis. GCs are stress hormones and secreted in response to a single stimulator, adrenocorticotrophic hormone (ACTH), which is released from the anterior pituitary [178]. ACTH is itself secreted mainly under control of the hypothalamic peptide corticotropin-releasing hormone (CRH). Both CRH and ACTH release is negatively influenced by GC secretion, leading to a negative feedback loop where GCs inhibit their own release. The central nervous system is thus the commander-in-chief of GC responses, providing an excellent example of close integration between the nervous and endocrine systems (neuroendocrine) [179-181]. Cortisol levels vary in diurnal cycles with the lowest levels between 10 in the evening and 4 in the morning. Cortisol levels then rise before awakening and continue to increase 30-40 minutes following wake up. During the day, the cortisol levels slowly decrease again, apart from a peak after lunch, and levels stay low during evening and night until initial rise in the early morning [182-184]. Disturbances in the HPA axis and in the diurnal pattern of GC levels, as well as hyper- and hypocortisolism, have been associated with a number of diseases and pathological states such as Cushing's syndrome and disease, Addison's disease, irritable bowel syndrome, posttraumatic stress disorder, pain and fatigue syndromes and, as introduced above, the metabolic syndrome [84, 162, 163, 180, 185, 186].

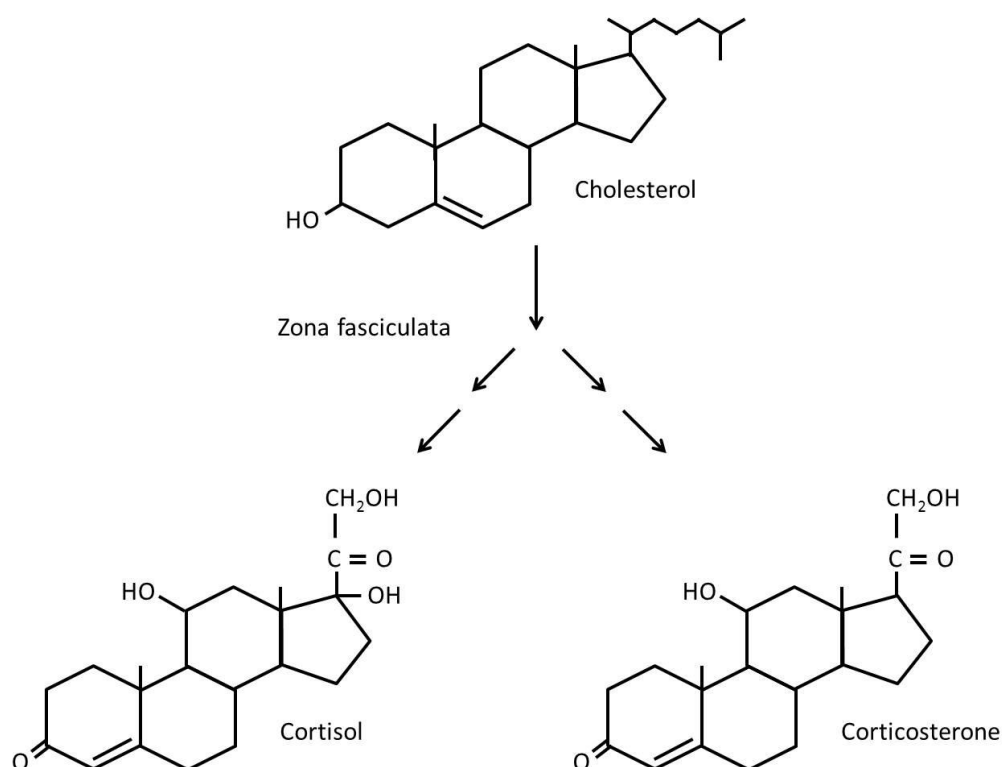


Figure 2. Synthesis of cortisol and corticosterone from cholesterol in the adrenal cortex.

1.2.2 Glucocorticoid action in different tissues

In the liver, cortisol induces *de novo* glucose synthesis via increased expression of phosphoenolpyruvate carboxykinase (PEPCK), an important catalytic enzyme regulating a rate limiting step of hepatic gluconeogenesis [187]. Cortisol also potentiates the action of other hyperglycemic hormones, such as glucagon, catecholamines and growth hormone, on glycogenolysis. Together this leads to increased release of glucose from hepatocytes [180, 188]. Furthermore, GC exposure leads to increased triglyceride synthesis and decreased fatty acid oxidation in the liver, thus increasing the accumulation of liver lipids [189-191]. Cortisol also prevents the uptake and use of glucose in adipose tissue and skeletal muscle by interfering with insulin signaling and insulin stimulated translocation of GLUT4 to the plasma membrane [192-195]. The effect of cortisol on glycemia is further enhanced through the increased breakdown of triglycerides in peripheral adipose tissue, which provide energy and substrates for gluconeogenesis. In contrast, cortisol promotes hypertrophy and differentiation in abdominal adipocytes, promoting abdominal obesity and further insulin resistance [84, 180]. Furthermore, being a catabolic hormone, cortisol stimulates wasting of muscle tissue via reduction of protein synthesis and via degradation of protein and release of amino acids [172, 196]. The increased rate of protein metabolism leads to increased urinary nitrogen excretion and the induction of urea cycle enzymes [197, 198].

The modulatory effects of cortisol on the immune response and inflammation are due to a GC-induced downregulation of a large number of pro-inflammatory cytokines such as IL-1 β , TNF α , IL-6, IL-8, IL-12 and IL-18, as well as suppression of synthesis of chemokines, prostaglandins and leukotrienes. GCs inhibit phospholipase A2 activity leading to a reduction of arachidonic acid release from phospholipids and a consequent decrease of eicosanoid synthesis. However, GCs also enhance secretion of certain pro-inflammatory cytokines counteracting the immunosuppressive effects, thus emphasizing the importance of a balance in the innate immune response system. GCs also suppress the expression of inducible nitric oxide synthase (iNOS), which leads to a decreased release of nitric oxide from endothelial cells and thus inhibition of endothelial-mediated inflammatory reactions. Moreover, GCs affect the adaptive immunity by decreasing the ability of dendritic cells to present antigens and elicit a T cell response, by inhibiting upregulation of major histocompatibility complex (MHC) class II as well as stimulatory molecules. Furthermore, GCs influence B cell proliferation and T cell survival as well as release of IgE antibodies [199-201].

Another feature of GC action is during embryonic development where endogenous GCs are important for the regulation of embryo implantation, growth and development of the fetus and placenta as well as production of lung surfactants to promote maturation of the lungs in later pregnancy [202].

Regulation of GC action is not only dependent on GC release and the HPA axis but also on tissue dependent intracellular enzymes catalyzing the conversion of glucocorticoids from the non-active form, such as cortisone in humans and 11-dehydrocorticosterone in rodents, to the active form, cortisol in humans and corticosterone in rodents, and vice versa. 11 β -hydroxysteroid dehydrogenase type 1 (11 β -HSD1) is widely expressed in many tissues and is responsible for the activation of cortisone to cortisol in liver [203], adipose tissue [204], skeletal muscle [205], pancreas [206], brain [207], blood vessels [208] and immune cells [209, 210].

11 β -hydroxysteroid dehydrogenase type 2 (11 β -HSD2), on the other hand, works in the opposite direction and protects the aldosterone sensitive tissues such as kidneys, sweat glands and colon from illicit activation of the MR by glucocorticoids [207], and the placenta from GC passage to the fetal circulation [202].

1.2.3 Adverse effects of glucocorticoid excess

As mentioned above, GCs have a number of targets and unfortunately several adverse effects related to metabolic disturbances are seen in patients on long-term treatment with GC-based medications as well as during hypercortisolism as in Cushing's disease, a medical condition caused in most cases by an ACTH-secreting pituitary adenoma. The adverse effects include muscle atrophy, increased central fat deposition, dyslipidemia, insulin resistance and glucose intolerance as well as β -cell dysfunction [172, 176, 177, 211]. It is also a known phenomenon in the clinic that diabetic patients risk a worsening of their glycemic control when they receive GC-based medications. In patients with endogenous over-production of GCs, as in Cushing's disease and syndrome, the incidence of insulin resistance, glucose intolerance and diabetes can be as high as 40% [212, 213] and hypercortisolemic states share many features with the metabolic syndrome [154]. As mentioned above, studies have indicated that patients suffering from the metabolic syndrome show elevated levels of cortisol [159-164] as do patients with glucose intolerance [165]. The diabetogenic properties of GCs can in part be explained by the metabolic effects that serve to increase blood glucose levels, as introduced previously. These effects lead to hyperinsulinemia and β -cell adaptation to meet the increased insulin demand [214, 215]. However, GCs also exert direct cytotoxic effects on isolated pancreatic islets and insulinoma cell lines [216-218], which will be elaborated on further under later headings. This can lead to failure of β -cell adaptation and subsequent augmentation of the impaired glucose homeostasis [219-223]. The negative effects of GCs can often be transient and revert after drug removal [224] but in susceptible individuals, a special form of diabetes – steroid-induced diabetes – can occur after long-term GC treatment [225, 226]. The prevalence of steroid-induced diabetes has been found to be between 10 and 20% depending on dose and administration of GC medications. Age and body mass index (BMI) are also risk factors associated with the disease [227].

Other adverse effects of GC excess include skin atrophy and delayed wound healing, osteoporosis, muscle atrophy, glaucoma and cataract, central nervous system complications such as disturbances in mood, behavior, cognition and memory, menstrual irregularity, hypertension, increased risk of infection and re-activation of viruses, peptic ulcer and pancreatitis [172, 181, 213, 228, 229]. The effect of GCs on hypertension is partly due to the GC-induced activation of the MR, leading to increased sodium and water reabsorption in the kidneys, and partly due to actual GR activation in tissues involved in blood pressure regulation, including kidney, brain and the vasculature [230]. It can also occur secondarily to insulin resistance, as one physiological effect of insulin is to promote vasorelaxation [231].

1.3 GLUCOCORTICOID RECEPTOR REGULATION

Most GCs are transported in the blood bound to corticosteroid-binding globulin (CBG). GCs exert their function in cells of target tissues by dissociation from CBG and diffusion through the plasma membrane into the cytoplasm, and subsequently binding to intracellular receptors, either the GR or the MR [232-234]. The GR has a structure that is characteristic for members of the nuclear receptor superfamily, with individual domains important for different functions. The *N*-terminal part contains a transcriptional activation domain, the central zinc-finger region includes the DNA-binding domain that determines target gene specificity and the *C*-terminal domain that includes the ligand binding part as well as an additional transcriptional regulatory section [234, 235]. The receptor forms large protein heterocomplexes, together with heat-shock protein (Hsp) 90, Hsp70, p23 and tetratricopeptide repeat (TPR) proteins, which affect the signaling and activity of the receptor by phosphorylation and association or dissociation. One particular TPR protein associated with the GR is serine/threonine protein phosphatase 5 (PP5) which will be further introduced later.

The heterocomplexes are essential for steroid action since the chaperones maintain the GR in a folded and thus competent state. The non-active GR-complex resides in the cytosol but upon ligand binding it undergoes conformational changes and becomes phosphorylated, which trigger translocation of the GR from the cytosol to the nucleus via the nuclear pore. This shuttling can either include a dissociation of the Hsp90 heterocomplex and GR before nuclear entry of GR by itself, or that the GR-Hsp90 heterocomplex jointly enters the nuclear pore and dissociation occurs once inside the nucleus [236-239]. The ligand-bound GR inside the nucleus homodimerizes and exerts its effects by transcriptional activation or repression. This is performed by direct high-affinity binding of the GR to specific glucocorticoid-responsive elements (GRE) on the promoter or intragenic regions of GC target genes, by binding to other DNA-bound transcription factors such as NF- κ B, or by binding directly to DNA and interact with neighboring DNA-bound transcription factors [236, 240]. Examples of genes that are upregulated by GC activity are serum/glucocorticoid regulated kinase 1 (SGK1) [241], cyclin-dependent kinase inhibitor 1/p21 (CDKN1A) [235], glucocorticoid-induced leucine zipper (GILZ) [242], MAPK phosphatase 1 [243] and tristetrarprolin (TTP) [244]. After the genomic activities the receptor is shuttled back to the cytosol from the nucleus, a process that is substantially slower than the rapid translocation in the opposite direction [238].

Genomic transcriptional effects are not the only possible activating route for GC signaling via the GR. Apart from the classic GR signaling pathway described above, GR also signal in a non-genomic manner. Ligand-bound GR can interact directly with membrane associated receptors and second messengers and thereby promote anti-inflammatory and suppress inflammatory pathways [175, 240, 245].

1.3.1 Glucocorticoid receptor phosphorylation and activation

The activation status of numerous kinases and transcription factors depends on the process of reversible phosphorylation induced by kinases and phosphatases. The GR is not an exception and the receptor can be phosphorylated at multiple sites, leading to modulation of the transcriptional activity within different cell types [246-252]. Up to eight different phosphorylation sites have been identified on the GR [248, 253].

Phosphorylation of serine residues 203, 211, 226 and 404 in human form (corresponding to serines 212, 220, 234 and 412 in the mouse form) is particularly associated with GR transcriptional activity [254]. Cellular sensitivity to GCs is likely to be determined by the GR phosphorylation status and different patterns of GR phosphorylation can thus lead to differences in the transcriptional activity of the receptor. Phosphorylation of the GR is in general induced by the ligand binding. The kinases responsible for GR phosphorylation have been found to be primarily cyclin dependent kinase (CDK) complexes and MAPKs [248, 255].

Phosphorylation on serine 203, which is induced by CDKs or ERK MAPK, has been found in the basal non-active state of the GR but is further enhanced upon hormone interaction. Phosphorylation on serine 203 results in loss of translocation of the GR from the cytosol to the nucleus, and thus inhibition of GR transcriptional activity. Phosphorylation on serine 211, by CDKs or p38 MAPK, leads to a conformational change and a transcriptionally more active GR, in most cell types. Phosphorylation on serine 226, induced by JNK [256], is found in the basal state, as with serine 203, but is further enhanced by ligand binding. Serine 226 phosphorylation of the GR leads to a less active GR signaling due to increased export of GR from nucleus to the cytosol. Phosphorylation of serine 404, by glycogen synthase kinase 3 (GSK-3), induces a conformational change of the GR which leads to altered co-factor recruitment and a modified transcriptional response resulting in attenuated GC signaling [247, 248, 251, 253, 257]. Dephosphorylation of the receptor is performed by protein phosphatases such as PP1, PP2A and PP5 [258-260].

1.3.2 Glucocorticoid action in pancreatic β -cells

GC treatment in humans and rodents has in studies resulted in insulin resistance and hyperinsulinemia, but with maintained normal fasting blood glucose levels and glucose tolerance. Thus the disposition index, the product of insulin secretion and peripheral insulin sensitivity, has remained constant in healthy subjects on GC therapy [214, 261-265]. A plausible explanation for this is the compensatory β -cell efficacy, hypertrophy and proliferation that increases insulin secretory capacity [215, 224, 266, 267].

However, apart from the systemic effects of GC signaling that impact glycemia, GCs also exert direct cytotoxic effects on isolated islets of Langerhans and insulinoma cell lines [216-218, 268]. This has been proposed as a contributing factor to failure of β -cell adaptation, and thus disrupted glucose homeostasis, in susceptible individuals upon GC treatment [219-223]. The underlying mechanisms for GC-induced β -cell toxicity are however not fully characterized, and the effects of GCs on β -cell function have shown to be largely dependent on dosage and duration of exposure [269]. Short term direct effects of GCs *in vitro* has included hypersecretion and improved GSIS [270] as well as decreased GSIS [271-273]. Longer *in vitro* exposure does on the other hand directly negatively impact the pancreatic β -cells, resulting in compromised insulin production [269], insulin secretion [206, 271, 274] and β -cell viability [217]. Furthermore, mice overexpressing GR in the pancreatic β -cells develop β -cell failure [275, 276] and diabetes [277]. Impaired β -cell function has also been observed after glucocorticoid treatment in humans [278, 279], and patients with Cushing's syndrome from GC medications and animal models of steroid-induced diabetes are associated

with loss of GSIS. Immunosuppressive GC treatment has also been shown to adversely affect islet transplantation outcome [280].

In rat β -cells, GC exposure cause GLUT 2 degradation, leading to decreased glucose sensing [281]. GCs have also shown to decrease expression of glucokinase and thus negatively affect glucose metabolism [282]. In mice, GCs can increase glucose cycling by augmenting the activity of glucose-6-phosphatase, the enzyme that hydrolyzes glucose-6-phosphate to a phosphate group and free glucose [275]. By these events, GCs may reduce glucose uptake and phosphorylation and thus decrease ATP synthesis and influx of Ca^{2+} .

Additional mechanisms might also contribute to the adverse effects of GCs and the GC toxicity in β -cells. The insulin gene contains multiple transcriptional elements responsive to GCs, and GCs can down-regulate insulin gene expression via transcriptional repression [283, 284]. In addition, the expression of pancreatic and duodenal homeobox 1 (Pdx1), a transcription factor important for β -cell development, function and survival, is negatively influenced by GCs [285, 286]. Since GCs have a direct pro-apoptotic effect in β -cells *in vitro* [216-218], ER stress has been proposed as a possible mechanism negatively affecting the β -cells [287]. Likewise, MAPKs with p38 MAPK activation and subsequent thioredoxin-interacting protein (TXNIP) upregulation might be involved in GC-induced β -cell apoptosis [218]. In other cell types, MAPK activity has been found to affect the outcome of GC exposure and GC-induced apoptosis [252, 288, 289] .

1.4 SERINE/THREONINE PROTEIN PHOSPHATASE 5 (PP5)

1.4.1 Kinases and phosphatases

Regulation of proteins by the means of phosphorylation is a fundamental mechanism in the control of numerous cellular processes and signaling events, and the most common form of reversible protein posttranslational modification. Phosphorylation of a protein can be either activating or inhibiting as well as lead to a conformational change or a change in surface property, influencing recognition of other proteins. It is also a powerful means for signaling amplification. Protein kinases are the enzymes responsible for the phosphorylation process, which is normally performed by adding a phosphate group onto a serine, threonine or tyrosine residue [290, 291]. Phosphorylation events of important targets need proper regulation and can therefore be rapidly counteracted by dephosphorylations. Dephosphorylation events are catalyzed by different protein phosphatases, and the phosphorylation level in a cell is the product of a finely tuned dynamic balance between the actions of protein kinases and protein phosphatases [292-294].

The most abundant class of protein phosphatases is the ser/thr phosphoprotein phosphatase (PPP) family consisting of PP1, PP2A, PP2B/calcineurin, PP4, PP5, PP6 and PP7, including their different isoforms [290, 295]. Protein phosphatases are much fewer than protein kinases, the ratio being about 1:15, but are still not as well studied. This is mainly due to the fact that it is very difficult to distinguish between the effects performed by the various phosphatases. Inhibitory compounds can often target and affect many different phosphatases, whereas kinase inhibitors or activators are generally more specific [290, 296]. Okadaic acid, calyculin A, microcystin, cantharidin, tautomycin and fostriecin are all chemicals with potent inhibitory effects on the activity

of PP1, PP2A, PP2B, PP4, PP5 and PP6, with okadaic acid being the most used in studies [297]. Since inhibitors of phosphatase activity are unspecific, other strategies to study the effects of a certain phosphatase is the use of RNA interference or animal knock-outs. The phosphatases themselves are also much more promiscuous and substrate unspecific than the numerous kinases. Whereas kinases might have only one or a few targets, protein phosphatases can have plentiful substrates depending on location in the cell and interactions with regulatory subunits and other factors [296, 298].

1.4.2 Roles of PP5

Most members of the PPP family have a number of isoforms encoded by different genes, however the ser/thr protein phosphatase PP5 is encoded by one single gene, and its catalytic, regulatory and targeting TPR domains are contained within the same polypeptide chain [299, 300]. The N-terminal TPR domains are unique for PP5 among its family members and are used for protein-protein interactions. In solution they block substrate access by folding over the catalytic site, giving PP5 an autoinhibitory conformation. During normal conditions PP5 is mainly in this inactive state with a very low basal activity, making up less than 1% of total measurable phosphatase activity. However, upon interaction with target proteins via the TPR domains, PP5 goes through a conformational change allowing the substrate access to the catalytic site [301-303]. PP5 is highly conserved among species and expressed in most, if not all, mammalian cells. It is still, however, not much known about the roles of PP5 in normal biology and in pathology, and only a few substrates of PP5 have been identified, including GR [246, 304], apoptosis signal-regulating kinase 1 (ASK-1) [305], ataxia telangiectasia mutated (ATM) [306] and DNA-dependent protein kinase (DNA-PK) [307]. Substrates for PP5 dephosphorylation identified in the test tube might however not always accurately reflect a certain role for PP5 *in vivo* and in many contexts it is not clear whether PP5 acts directly on a substrate or on upstream targets, and acts unaided or is activated as part of a complex with other protein phosphatases. By using the yeast two-hybrid method, PP5 has been found to be associated with a number of proteins involved in signaling networks, including the Hsp90 and GR complex [259], the Hsp90 and estrogen receptor complex [308], the Hsp90 and heme-regulated inhibitor (HRI) complex [309], the Hsp90 and stress-induced phosphoprotein 1 (STIP1) complex [310], Rac GTPase [311], Raf-1 MAP3K [312], $G\alpha_{12}$ and $G\alpha_{13}$ subunits of heterotrimeric G proteins, possibly via Hsp90 interaction [313], the A subunit of PP2A [314], cryptochrome 2 [315] and cell division cycle protein (Cdc)16, Cdc27 and the Hsp90 Cdc37 complex [316, 317]. As mentioned previously, the known inhibitors for PP5 are not specific but also affect many other phosphatases. PP5 *in vitro* activators include polyunsaturated fatty acids such as arachidonic and linoleic acids [318-320]. Furthermore, PP5 has been suggested to play an important role in different cancers and has been found to be upregulated in human tumor tissue [321]. In breast cancer cells, estradiol has been shown to increase the expression of PP5 which in turn promotes proliferation and enhances the growth rate of estrogen-dependent tumors [322-324]. Additionally, the transcription factor hypoxia-inducible factor-1 (HIF-1) increases PP5 in human hepatoma, lung carcinoma and breast carcinoma cells [325].

1.4.2.1 PP5 and reactive oxygen species

As mentioned previously, MAPK signaling requires sequential phosphorylation of a number of downstream kinases. The phosphatases responsible for the dephosphorylation of MAPKs thus represent critical elements in the control of the MAPK signal transduction. PP5 can bind to the MAP3K ASK-1, during conditions of oxidative stress in the test tube and in cell lines, and thereby decrease phosphorylation and activity of ASK-1. ASK-1 activates JNK and p38 MAPK by phosphorylation and subsequent, this phosphorylation as well as ASK-1 induced apoptosis, has shown to be inhibited by PP5 [305]. Furthermore, during hypoxic conditions ASK-1 and JNK are activated in human cancer cell lines, and PP5 can also in this setting inhibit ASK-1. Prolonged hypoxia also leads to increased expression of PP5 via HIF-1 transcription factor, probably functioning as a pro-survival approach in low oxygen environments, counter acting the hypoxia-induced ASK-1 signaling response that promotes apoptosis [325]. Similarly, in neuronal cells ROS has shown to induce MAPK activation and apoptosis, but also to suppress PP5 and PP2A expression. Overexpression of PP5 and PP2A in these cells prevents ROS-induced MAPK activation and apoptosis [326]. In pancreatic β -cells, PP5 has also been shown to affect the outcome of the response to ROS and palmitate. The negative effects of ROS and palmitate on these cells, including JNK phosphorylation and apoptosis, are potentiated by a lack of PP5 [151], leading to the assumption that PP5, on a molecular level, acts as a suppressor of apoptosis via inhibition of MAPK signaling in pancreatic β -cells.

1.4.2.2 PP5 and glucocorticoid receptor regulation

As described previously, the effect of GCs is dependent on the activation of GR and that is in turn dependent on phosphorylation [247]. PP5 has been shown to associate with the GR-chaperone heterocomplex [259] and to influence both GR activation and phosphorylation in various cell types. In human lung carcinoma cells PP5 has been shown to stimulate proliferation via inhibition of the GC-mediated cell cycle arrest pathways [260], in airway smooth muscle cells PP5 prevents phosphorylation of GR and consequently negatively affects GC responsiveness in asthma patients [246], in breast carcinoma cells estrogen inhibits GC-induced suppression of cell proliferation via PP5-mediated dephosphorylation of the GR [304] and in mouse embryonic fibroblasts it has been shown that PP5 can affect lipid accumulation and metabolism as well as adipocyte differentiation, via dephosphorylation and subsequent loss of activation of the GR [327]. In concordance with this, mice with a global knock-out of PP5 [328] are partly protected against high-fat diet-induced weight gain [329]. Due to the above effects of PP5 on GR activation in the various cell types, it has been suggested that PP5 might play a role in the activation of the GR and outcome of the response to GC signaling also in pancreatic β -cells [151].

1.5 THE INCRETIN SYSTEM AND GLP-1

Glucagon-like peptide-1 (GLP-1) is a small peptide hormone that is synthesized and released mainly from the enteroendocrine L-cells situated in the intestinal tract. The proglucagon gene gives rise to an mRNA that due to tissue-specific posttranslational

processing results in glucagon in the pancreatic α -cells, GLP-1 and glucagon in the central nervous system and GLP-1 and GLP-2 in the intestinal L-cells [330]. More recently, it has also been shown that GLP-1 can be produced in islets [331, 332]. GLP-1 enters the circulation in two equipotent forms, GLP-1₇₋₃₇ and GLP-1₇₋₃₆amide, the latter being the more abundant form [330, 333, 334]. GLP-1 is released in a nutrient-dependent manner and the L-cells respond to sugar, proteins and fats [335]. The GLP-1 secretion is pulsatile with oscillations of intracellular Ca^{2+} concentrations. Both hormonal and neuronal stimulation of L-cells enhance GLP-1 secretion and cholinergic control also affect the pulsatility release [336-338]. The circulating levels of biologically active GLP-1 rises within minutes after food intake, but then fall rapidly due to renal clearance and degradation by dipeptidyl peptidase-4 (DPP-4), giving GLP-1 a circulating half-life of only a few minutes [330, 339]. GLP-1 and glucose-dependent insulintropic peptide (GIP), the latter secreted from enteroendocrine K cells, both belong to the incretin family of hormones. Incretins stimulate a decrease of blood glucose levels, following nutrient absorption, by affecting multiple targets in the signal regulation of metabolic homeostasis [340].

1.5.1 Effects of GLP-1 receptor activation

GLP-1 exerts its effect by binding to the GLP-1 receptor (GLP-1R), which has shown to be present in pancreatic islets, kidney, immune cells, intestine, and peripheral and central nervous systems. Whether it is also present in adipose tissue, liver and heart remains uncertain and might be species dependent [340, 341]. GLP-1 has numerous effects in many different tissues, including the so called incretin effect. The incretin effect, induced by incretins in response to food intake, is a potent augmenting effect on insulin secretion from pancreatic β -cells at increased blood glucose levels [333, 342]. Activation of GLP-1R in pancreatic β -cells stimulates increase of cyclic adenosine monophosphate (cAMP) and activates downstream pathways coupled to protein kinase A (PKA) and exchange protein activated by cAMP 2 (Epac2), which are involved in a wide variety of intracellular events including ion channel activity with closure of K_{ATP} channels, elevations of $[\text{Ca}^{2+}]_i$ levels and increased exocytosis of insulin containing secretory granules. These effects of GLP-1 signaling all contribute to enhancement of GSIS [333, 343-346]. GLP-1R activation in pancreatic α -cells inhibits glucagon secretion at hyperglycemia, further promoting a decrease of the blood glucose levels. Apart from the incretin effect, it has been shown in animal models and human islets *ex vivo* that GLP-1R activation also inhibits β -cell apoptosis and enhances β -cell neogenesis and proliferation as well as increases the expression of insulin and genes important for β -cell function [347-349].

Other actions of GLP-1, not directly related to the pancreas, include regulation of short- and long-term energy homeostasis in the so called gut-brain axis. GLP-1R activation reduces appetite, food intake and weight gain in lean, obese and T2DM subjects. Both peripheral GLP-1 from intestinal L-cells and GLP-1 from the central nervous system are believed to be key regulators involved in this feeding control. GLP-1R activation also slows down gastric emptying, either via a direct mechanism in the gut or via the gut-brain axis and neuronal control that may contribute to increased satiety and delayed carbohydrate uptake. Furthermore, GLP-1R activation in the central

nervous system in rodents acts neuroprotective after stroke and in neurological disorders such as Alzheimer's and Parkinson's disease [350-353].

1.5.2 Incretin agonists and DPP-4 inhibitors

T2DM patients have been found to have a loss of the incretin effect and this is believed to play an important role in the insulin insufficiency and hyperglycemia of the disease. Even though a down regulation of both GLP-1 and GIP receptors have been found in pancreatic islets at hyperglycemic conditions [354], GLP-1 remains capable of stimulating insulin secretion in T2DM patients [355, 356]. A loss of response to GIP is, however, more pronounced and therefore the lack of incretin effect in T2DM patients is believed to be mainly due to negative effects on GIP activity [357]. There are also studies reporting a decreased post-prandial GLP-1 secretion in T2DM subjects, but other reports show opposing results, concluding that even though reduction of GLP-1 levels have been found in individual study participants or groups of T2DM patients it is not a universal characteristic representative for all T2DM patients [358].

Due to the positive effects of GLP-1 on feeding control and glycemia and since T2DM patients still respond to GLP-1 activity and secrete more insulin and improve β -cell function, incretin mimetics, or GLP-1 analogues, have emerged as new therapeutic options for T2DM patients. Exenatide and liraglutide are both incretin mimetics used in the clinic, with the advantage of having circulating half-lives of several hours after subcutaneous injection, instead of a few minutes as with injection of native GLP-1 [359, 360]. Exenatide is a synthetic version of exendin-4, a naturally occurring peptide first found in the venom of the Gila monster (*Heloderma suspectum*). This peptide is more DPP-4 resistant and has a 53% sequence homology with native GLP-1. Liraglutide on the other hand is more similar to GLP-1 with 97% sequence homology and is metabolized by DPP-4. Still liraglutide has substantially longer plasma half-life than Exenatide [361]. Both Exenatide and liraglutide risk giving rise to side effects, where nausea and vomiting are the most common reasons for withdrawal from therapy [359, 361]. DPP-4 inhibitors are another class of drugs with the action of preventing the rapid fall of native plasma GLP-1 after a meal. Vildagliptin, Sitagliptin, Saxagliptin and Linagliptin are examples of DPP-4 inhibitors and since they inhibit the action of DPP-4 they prevent the enzymatic degradation of both GLP-1 and GIP, promoting an enhanced incretin effect. Even though the effect of incretin mimetics and DPP-4 inhibitors in theory would be similar, differences exist between the therapy options. DPP-4 inhibitors are suggested to have a more favorable tolerability profile with less side effects than GLP-1 analogues; however, DPP-4 inhibitors do not suppress appetite or promote weight loss, and GLP-1 analogues give a superior glycemic control [357, 359].

2 AIMS OF THE THESIS

The general aim of this thesis was to investigate diabetogenic effects of glucocorticoids, and the role of protein phosphatase 5 and GLP-1 receptor activation in this context.

The specific aims were to:

- Further characterize a potential mouse model for the metabolic syndrome, in terms of corticosterone-induced effects on glucose control, lipid handling and pancreatic islet adaptation to insulin resistance.
- Investigate potential beneficial effects of the GLP-1 receptor agonist liraglutide in the above setting of corticosterone excess in mice.
- Characterize the role of protein phosphatase 5 in the above setting of corticosterone excess in mice.
- Elucidate pathways that regulate glucocorticoid action in pancreatic β -cells and the role of protein phosphatase 5 in this context.

3 MATERIALS AND METHODS

3.1 ANIMALS AND TREATMENT

3.1.1 The C57BL/6 mice

The C57BL/6 mouse is a widely used inbred mouse strain. We used the C57BL/6J originating from the Jackson Laboratory [362]. Male mice were bought from Nova (Sollentuna, Sweden) and used in study I and II at the age of 8 to 10 weeks at experiment start. Mice were fed *ad libitum* and housed in 12 hours of light/12 hours of darkness cycles. In study IV, pancreatic islets from C57BL/6J mice were also used. The studies were performed according to the guidelines of Karolinska Institutet and approved by the Stockholm South animal ethics committee (S201-10 with supplements S118-11 and S75-12 for study I, S49-12 for study II and S201-10 for study IV).

3.1.2 The PP5 knock-out mice

The PP5 knock-out mice were used in study III and study IV. The mice were generated on the C57BL/6 background and mice with a global knock-out of exon 1 of the PP5 gene (*Ppp5c*^{-/-}) were compared to wild-type cognates (*Ppp5c*^{+/+}) as controls [328]. The breeding of *Ppp5c*^{-/-} mice generated viable and fertile offspring; however, *Ppp5c*^{-/-} mice were underrepresented, yielding 19% contrasting to 25% expected by Mendelian inheritance. *Ppp5c*^{+/+} and *Ppp5c*^{-/-} were kept and backcrossed in our local breed and used in generations N8 and N9. Determinations of the different genotypes were made on genomic DNA from ear labeling punches. The DNA was isolated using GeneJET Genomic DNA Purification Kit (Thermo Scientific, Waltham, MA, USA) and amplified using polymerase chain reaction (PCR) with specific primers. PCR products were separated according to size, and thus wild-type or knock-out alleles, using electrophoresis with 1% agarose gel. Mice were fed *ad libitum* and housed in 12 hours of light/12 hours of darkness cycles. In study III female and male mice were used at the age of 8 weeks at experiment start and in study IV pancreatic islets from male mice were used. The studies were performed according to the guidelines of Karolinska Institutet and approved by the Stockholm South animal ethics committee (S201-10 with supplement S75-12 for study III and S201-10 for study IV).

3.1.3 Corticosterone and liraglutide treatment

3.1.3.1 Corticosterone treatment

C57BL/6J mice in study I and II and *Ppp5c*^{+/+} and *Ppp5c*^{-/-} in study III were randomly assigned to either receive corticosterone (25 or 100 µg/ml, (Sigma, St Louis, MO, USA)) or vehicle (1% ethanol) via their drinking water for five weeks. This corticosterone regimen is known to produce a robust mouse model with metabolic syndrome features [169]. A subgroup of mice in study I was also followed-up in a recovery study, where, after the five weeks of treatment, the GCs were discontinued

and all mice received vehicle for an additional three weeks, giving a total study period of eight weeks.

3.1.3.2 Liraglutide treatment

In study II, mice were randomly assigned to be given once daily subcutaneous injections (between 9:00 and 10:00 am) of liraglutide (Novo Nordisk A/S, Bagsvaerd, Denmark) or PBS in the same manner. To improve liraglutide tolerability, the drug was given in escalating doses starting at 0.15 mg/kg body weight with a daily increment of 0.025 mg/kg body weight until the final dose of 0.3 mg/kg body weight was reached [363]. This dose was maintained during the remaining part of the study period.

3.1.4 Monitoring and tests

3.1.4.1 Weekly monitoring

Food intake, body weight and blood glucose levels in random-fed mice were monitored weekly, using a hand-held glucometer (One-Touch Ultra 2; LifeScan, Milpitas, CA, USA) and blood was collected for serum insulin level evaluations with ELISA according to the manufacturer's instructions (Mercodia, Uppsala, Sweden). In study II, the blood samples for determination of glucose and insulin concentrations were taken in the morning in non-fasted animals prior to administration of liraglutide or PBS. In study I and III blood for serum insulin levels were collected from six hours fasted animals.

3.1.4.2 Insulin and glucose tolerance tests

Intraperitoneal insulin tolerance test (IPinsTT) and intraperitoneal glucose tolerance test (IPGTT) were performed during the fourth and fifth week of treatment, respectively, and IPinsTT was also performed during the eighth week for the mice in the recovery part of study I. For IPinsTT and IPGTT, mice were fasted one or six hours, respectively, and then injected IP with human recombinant insulin (Humalog, Eli Lilly, Indianapolis, IN, USA) (1 or 2 IU/kg body weight) or glucose (2 or 1.5 g/kg body weight). Blood glucose was monitored at multiple times 0 to 60 (IPinsTT) or 120 (IPGTT) minutes after injection. In a short version of IPinsTT in study I, mice were injected with insulin (1 IU/kg body weight) or PBS and sacrificed after 15 minutes. Organs were then snap-frozen and later subjected to Western blot analysis of levels of phosphorylated AKT1/2/3. In study II, on the day of the tolerance tests, liraglutide was injected as usual in the morning, six hours before the tests. During the IPGTT in study II, blood was also collected before and at 15 and 30 minutes post glucose injection for determination of serum C-peptide levels, which were evaluated using ELISA according to the manufacturer's instructions (Alpco Diagnostics, Salem, NH, USA).

3.1.4.3 Mean arterial pressure measurements

In study I, a group of mice was subjected to blood pressure measurements after the study period. Mice were sedated (2% Isoflurane, Apoteket, Sweden) and catheters were surgically placed in the jugular vein for constant saline infusion (10 ml/hour/kg) and the carotid artery for mean arterial pressure measuring (PowerLab, ADInstruments,

Spechbach, Germany). Mean arterial pressure was sampled during a 30 minute period after surgical methods and resting period.

3.1.4.4 *Sacrifice*

After the treatment period, mice were sacrificed by CO₂ exposure, blood was sampled by heart puncture for serum measurements and different organs were collected, weighed and snap-frozen. Pancreatic glands were dissected and either used immediately for islet isolation, for evaluation of total insulin content or fixated in 4% phosphate-buffered paraformaldehyde for 48 hours, paraffin-embedded and sectioned for immunohistochemistry.

3.1.5 Staining and measurements

3.1.5.1 *Oil-red-O*

Frozen fragments of liver and *M. femoralis* were embedded in NEG-50 (Thermo Scientific, Waltham, MA, USA). Cryosections (12 µm) were obtained from different parts of the tissues and stained for neutral lipids using oil-red-O as previously described [364]. Quantification of lipid droplets was also done in accordance with previous description [364].

3.1.5.2 *Immunostaining and quantitative measurements in pancreas*

3.1.5.2.1 Study I

Vehicle and GC (100 µg/ml) treated mice were injected IP with 50 mg/kg body weight BrdU for the seven last days of treatment. Paraffin-embedded pancreatic glands, 4 or 5 from each group, were sectioned longitudinally (5 µm sections), stained for insulin or BrdU and analyzed as previously described [151]. For each pancreatic gland, the islet volume and BrdU incorporation were determined from all islets identified in nine sections from different parts of the gland. Islet volumes were estimated with the nucleofector method [26] with the exception that the whole section area was examined, using a computerized setup for stereology (newCAST software, Visiopharm, Hoersholm, Denmark) and presented as mean islet volume. For BrdU incorporation (as a measure of DNA synthesis), BrdU-positive cells were counted and presented as BrdU-positive cells divided by islet volume.

3.1.5.2.2 Study II

Pancreatic glands, 5 or 6 from each group, were excised and processed according to a previous description [365] except that 4% phosphate-buffered paraformaldehyde (PFA) was used as fixative solution. Determination of α -cell and β -cell mass was done by point counting morphometry on each pancreas section immunostained for either glucagon or insulin, as previously described [32]. Each section was systematically scored with a grid of 130 points (final magnification $\times 100$) using the image processing and analysis software - ImageJ (freely available at <http://rsbweb.nih.gov/ij/>). The numbers of intercepts over β -cells, endocrine non- β -cells and exocrine pancreatic tissue were counted. The β -cell relative volume was calculated by dividing the intercepts over β -cells by the intercepts over the total pancreatic tissue; the β -cell mass was then

estimated by multiplying the β -cell relative volume by the total pancreas weight. The same methodology was applied for counting α -cell mass in sections immunostained for glucagon. Averaged islet-cell proliferation was obtained by counting total islet cells stained for insulin and Ki67 using the same software cited above. All islets found in each pancreas section were sampled ($1,714 \pm 218$ β -cell nuclei per group). The rate of islet cell proliferation was expressed as the proportion of Ki67-positive cells of total islet cells [32].

3.1.5.3 Total insulin content

Splenic parts of pancreatic glands were obtained and wet weighed at sacrifice. Acid ethanol (0.18 M HCl in 95% ethanol) was added, pancreatic glands were homogenized by sonication and insulin was extracted over night at 4°C. Samples were clarified by centrifugation and used for insulin content evaluation using insulin ELISA according to the manufacturer's instructions (Mercodia, Uppsala, Sweden).

3.1.5.4 Serum parameter measurements

In study I, II and III blood was collected from heart puncture at sacrifice and serum samples were analyzed regarding levels of non-esterified fatty acids (NEFA), total cholesterol and triglycerides (analyzed at Karolinska University Laboratory at Södersjukhuset, Stockholm and Center for Inherited Metabolic Diseases, Karolinska University Hospital, Solna). In study I, C-peptide levels in serum from heart puncture were evaluated using ELISA according to the manufacturer's instructions (Alpco Diagnostics, Salem, NH, USA). In study II, corticosterone and corticotropin (ACTH) levels in serum from heart puncture were evaluated using EIA (corticosterone EIA, Enzo Life Sciences, Lausen, Switzerland) and ELISA (mouse corticotropin ELISA, Wuhan EIAab Science, Wuhan, China) respectively, according to the manufacturer's instructions.

3.2 ISOLATED PANCREATIC ISLETS AND INSULINOMA CELL LINES

3.2.1 Pancreatic islets

3.2.1.1 Islet isolation

Islets isolated from 3 to 6 months old male C57Bl/6J mice (study I and IV), *ob/ob* mice (local breed) (study IV) or *Ppp5c*^{+/+} and *Ppp5c*^{-/-} (study IV) were used. Mice were sacrificed by exposure to CO₂ followed by decapitation or heart puncture. The pancreatic glands were excised and islets were isolated by collagenase digestion. Islets used for chaperone expression in study I were lysed directly after isolation and subjected to protein or mRNA evaluations. Islets to be used for GSIS in study I or treated with GCs in study IV were placed in RPMI-1640 culture medium (SVA, Uppsala, Sweden) containing 11 mM glucose and supplemented with 10% (w/v) fetal bovine serum (FBS) (Sigma-Aldrich, St. Louis, MO, USA), 2 mM L-glutamine (SVA, Uppsala, Sweden), 6 mg/ml penicillin G and 5 mg/ml streptomycin sulfate (Invitrogen, Carlsbad, CA, USA) for an overnight recovery at 37°C in 5% CO₂. Islets were then transferred to new medium (as described above) supplemented with treatment additions.

3.2.1.2 Glucose-stimulated insulin secretion

After recovery, some islets were subjected to GSIS with 30 minutes pre-incubation in Calcium 5 (Ca5) buffer (containing 25 mM HEPES, 125 mM NaCl, 5.9 mM KCl, 1.2 mM MgCl₂, 1.26 mM CaCl₂ and 0.1% BSA) with 2 mM glucose at 37°C followed by 60 minutes incubation in Ca5 buffer with either 2 mM or 20 mM glucose at 37°C. Buffers were collected for insulin analysis with ELISA according to the manufacturer's instructions (Mercodia, Uppsala, Sweden). Islets were then washed with PBS, sonicated and lysed and DNA was purified and proteins removed with the use of phenol-chloroform extraction. DNA concentrations were measured with spectrophotometry (Picodrop Microliter UV/Vis Spectrophotometer; Picodrop Ltd., Hinxton, United Kingdom) and GSIS data were normalized to islet DNA content.

3.2.2 MIN6 cells

3.2.2.1 Culture and treatment

Mouse insulinoma, MIN6, cells [366], derived from mouse pancreatic β -cells, were maintained in DMEM containing 1 mM sodium pyruvate and 25 mM glucose (Invitrogen, Carlsbad, CA, USA), and supplemented with 15% (w/v) FBS, 6 mg/ml penicillin G and 5 mg/ml streptomycin sulfate (Invitrogen, Carlsbad, CA, USA), 2 mM L-glutamine (SVA, Uppsala, Sweden) and 50 μ M β -mercaptoethanol (Sigma-Aldrich, St. Louis, MO, USA) at 37°C and 5% CO₂. Cells were used between passages 22 and 30.

3.2.2.2 PP5 suppression with siRNA

Short interfering RNAs (siRNAs), targeting three different regions of mouse *Ppp5c* mRNA (NM_011155), or a corresponding scrambled (negative control) siRNA (Santa Cruz Biotechnology, Santa Cruz, CA, USA), were used to suppress PP5 expression in MIN6 cells. Cell transfection was aided by electroporation, using a Nucleofector[®] II (Lonza, Cologne, Germany) with Solution V and program G-16 and 100 pmol of the indicated siRNA for 4 x 10⁶ cells per transfection. Proper transfection and subsequent suppression of *Ppp5c* mRNA was verified for each experiment.

3.3 IN VITRO TECHNIQUES

3.3.1 Reagents

Reagents of analytical grade and deionized water were used. Dexamethasone, prednisolone, triamcinolone, betamethasone and RU486 came from Sigma-Aldrich (St. Louis, MO, USA). SB203580 and SP600125 were obtained from Calbiochem (La Jolla, CA, USA) and collagenase was from Roche (Roche Diagnostics GmbH, Mannheim, Germany).

3.3.2 Assessment of cell viability and apoptosis

Cell viability was assessed by a Cytotoxicity Detection Kit^{Plus} (Roche Diagnostics GmbH, Mannheim, Germany). After exposure, cells were washed twice with PBS and then lysed in PBS supplemented with 1% Triton X-100. In this setting, the amount of lactate dehydrogenase released after cell lysis correlates with the amount of living cells after treatment. Apoptosis was detected by measuring cytoplasmic DNA-histone nucleosomes generated during apoptotic DNA fragmentation using the Cell Death Detection Kit ELISA^{PLUS} (Roche Diagnostics GmbH, Mannheim, Germany), according to the manufacturer's instructions.

3.3.3 SDS-PAGE and Western blot

Protein samples from MIN6 cells, isolated islets or other tissues were prepared for Western blot analysis by washing with PBS followed by sonication (for islets and tissues) and cell lysis on ice with a buffer containing 20 mM Trizma base, 150 mM NaCl, 0.1% SDS, 1% Triton X-100, 0.25% Na-Deoxycholate, 2 mM EDTA, 1 mM Na₃VO₄, 50 mM NaF and 1% protease inhibitory cocktail (Sigma P-8340) for 15-30 minutes. After lysis, the preparations were collected and centrifuged at 14,000g for 10-15 minutes at 4°C. Supernatants were transferred to new tubes and the total protein concentrations were determined by the DC protein assay, according to the manufacturer's instructions (Bio-Rad Laboratories, Hercules, CA, USA). Samples were subjected to SDS-PAGE and thereafter Western blot with polyvinylidene difluoride (PVDF) membranes. Immunoblot analyses were performed using antibodies recognizing calnexin (CANX), protein disulfide-isomerase (PDI/P4HB), Grp78/HSPA5, phosphorylated GR at position serine 220 and total GR (Abcam, Cambridge, UK), Calreticulin (ERp60/CALR), Grp94/HSP90B1, ERp72/PDIA4, the phosphorylated and total forms of PERK, eIF2 α , p38 MAPK and JNK and the cleaved form of caspase 3 (CC3) (Cell Signaling Technology, Danvers, MA, USA), CHOP/DDIT3, PP5 and the phosphorylated and total forms of AKT1/2/3 (Santa Cruz Biotechnology, Santa Cruz, CA, USA). Immunoreactive bands were detected using Enhanced chemiluminescence (ECL) (GE Healthcare, Fairfield, CT, USA) and imaged and quantified using Molecular Imager ChemiDoc XRS with Quantity One Software v. 4.6.5 (Bio-Rad Laboratories, Hercules, CA, USA). After imaging, the membranes were stained with Coomassie Brilliant Blue (Bio-Rad Laboratories, Hercules, CA, USA) for total protein normalization.

3.3.4 cDNA synthesis and quantitative PCR

Total RNA was extracted from isolated islets or MIN6 cells, using AurumTM Total RNA Mini kit, and reversely transcribed into complementary DNA (cDNA) with iScriptTM cDNA Synthesis kit according to the manufacturer's instructions (Bio-Rad Laboratories, Hercules, CA, USA). The gene expression levels of mRNAs were measured by SYBR Green-based quantitative PCR (qPCR) (SYBR Green Master mix, Thermo Scientific, Waltham, MA, USA) using the cDNA and mouse-specific primers for Calr, Canx, Pdia1/P4hb, ERp72/Pdia4, Grp94/Hsp90b1, Grp78/Hspa5, Chop/Ddit3, Sgk1, Cdkn1a/p21 and Gadd34/Ppp1r15a mRNAs. Actb (β -actin) mRNA was used as

housekeeping gene for normalization. The following formula was used for quantification: target amount = $2^{-\Delta\Delta C_t}$, where $\Delta\Delta C_t = (C_{t_{\text{target gene}}} - C_{t_{\text{Actb}}})_{\text{Sample}} - (C_{t_{\text{target gene}}} - C_{t_{\text{Actb}}})_{\text{Control}}$ [367]. See Table 1 for primer sequences.

Table 1. Primer sequences

Primer targets, mouse	Forward primer	Reverse primer
<i>Calr</i>	5'-ATGCTAAGAAGCCTGAGGAC-3'	5'-TTCAGGGTTGTCAATTTCTG-3'
<i>Canx</i>	5'-TGTGATCCTCTTCTGCTGTT-3'	5'-CTTCTTCCCTTCCCTTCTTCA-3'
<i>Pdia1/P4hb</i>	5'-GCGCATACTTGAGTTCTTTG-3'	5'-GCTCATCTGACTCTGGTTTG-3'
<i>ERp72/Pdia4</i>	5'-CAGAAGCATCAGCCATTAAG-3'	5'-AATCAAAGCTGAAGTCCACA-3'
<i>Grp94/Hsp90b1</i>	5'-AGCTGAAGTGAACAGGATGA-3'	5'-TGCATTTTCATCAGTTAGGG-3'
<i>Grp78/Hspa5</i>	5'-TGGCACTATTGCTGGACTGA-3'	5'-CCCAGGTCAAACACAAGGAT-3'
<i>Chop/Ddit3</i>	5'-TCTGTCTCTCCGGAAGTGTA-3'	5'-CTGGTCTACCCCTCAGTCCTC-3'
<i>Sgk1</i>	5'-AGCCATCCTGAAGAAGAAAG-3'	5'-CCCTCTGGAGATGGTAGAAC-3'
<i>Cdkn1a/p21</i>	5'-ATATACGCTGCCTGCCCTCT-3'	5'-AAGGGCCCTACCGTCCTACT-3'
<i>Gadd34/Ppp1r15a</i>	5'-GGTGGTCCAGCTGAGAATGA-3'	5'-TCTTCCGTGGCTTGATGTTT-3'
<i>Actb</i>	5'-GACGTTGACATCCGTAAAGA-3'	5'-GCCAGAGCAGTAATCTCCTT-3'

3.4 STATISTICAL ANALYSIS

Data are presented as mean \pm SEM. The Student's *t*-test, one-way ANOVA followed by Bonferroni *post-hoc* test, or Kruskal-Wallis nonparametric test followed by Dunn's *post-hoc* test, was used as appropriate to identify differences between groups, using GraphPad Prism 5.0 software. A value of $p < 0.05$ was considered statistically significant.

4 RESULTS AND DISCUSSION

4.1 CORTICOSTERONE EXPOSURE IN MICE – A MODEL OF THE METABOLIC SYNDROME AND GC EXCESS

Prevalence of obesity, the metabolic syndrome and T2DM increase very fast globally and the need for understanding of the underlying causes for disease development is crucial. Different animal models are useful in studying both the negative effects of obesity and the development of T2DM. Examples of rodent animal models used include the leptin-deficient *ob/ob* [368] and the leptin receptor-deficient *db/db* [369] mice that both develop obesity and a diabetes-like syndrome of hyperglycemia, glucose intolerance and disturbed plasma insulin levels [370, 371]. High-fat feeding of C57BL/6 mice also gives rise to a phenotype desirable for studying obesity, pre-diabetes and diabetes [372]. The obese Zucker diabetic fatty (ZDF) rat [373, 374] and the non-obese Goto-Kakizaki (GK) rat [375, 376] are other examples of animal models that are used in studies and that develop T2DM.

The overall topic for this thesis deals with the diabetogenic effects of GCs, and study I investigates a mouse model first described by Karatsoreos *et al* [169], which - due to GC exposure via the drinking water - presents many features resembling the metabolic syndrome as seen in humans. In study II, this model is used to investigate possible protective effects of GLP-1R activation with liraglutide and in study III mice lacking PP5 are treated with GCs in the same manner, evaluating possible effects of PP5 knock-out. Study IV is an *in vitro* study examining pathways underlying direct GC action in pancreatic β -cells as well as effects of decreased or lost PP5 expression in an insulinoma cell line and in isolated pancreatic islets.

4.1.1 Lipid handling and effects of liraglutide and PP5 knock-out

Study I shows that the mice exposed to GCs via their drinking water for five weeks gain significantly more weight (Fig. 3) and have much larger omental, perirenal, subcutaneous and epididymal fat deposits than vehicle-treated mice. This is a dose-dependent consequence of the GCs since a higher GC dose gives rise to a more pronounced effect on obesity than a lower GC dose. The mice also exhibit dyslipidemia as evident by elevated NEFA, cholesterol and triglyceride levels in serum. Increased levels of circulating NEFA, released from adipocytes, are key factors for insulin resistance [84, 85, 87, 88] and high cholesterol and triglyceride levels are strongly associated with cardiovascular risks, especially in a setting with other features of the metabolic syndrome [15]. Moreover, the mice treated with corticosterone show ectopic fat distribution with highly increased amounts of fatty droplets in liver tissue and *M. femoralis*, and the mice become hypertensive. Taken together, these effects reveal an obese and dyslipidemic phenotype with hypertension and fat inclusions in insulin responsive organs; typical features in human abdominal obesity and clinical characteristics in patients with the metabolic syndrome [13].

Study II reveals that the above characteristics are partly counteracted by the treatment with liraglutide. Body weight gain was postponed, epididymal fat tissue weight was significantly smaller and ectopic fat in liver were less distinguished in the mice treated with GCs combined with liraglutide (GC/Lira) as compared to the mice treated with GCs and PBS (GC/PBS). These are all parameters constituting the lipid profile and lipid handling, and even though liraglutide treatment did not fully counteract the negative effects of GCs on dyslipidemia the study revealed that liraglutide postponed the appearance of obesity and decreased ectopic fat distribution.



Figure 3. C57BL/6J mice treated with vehicle (left) or corticosterone (right) in study I.

The results from study III are less clear in terms of the possible protective effects of PP5 knock-out and also GC effects in the model. As mice with a global knock-out of PP5 [328] previously have been shown to be partly protected against the negative effects of a high-fat diet [329] these mice might also be protected against the adverse effects of GCs. The study investigated the effects of GCs on both male and female wild-type *Ppp5c*^{+/+} mice and their cognates lacking PP5, *Ppp5c*^{-/-}, however, the weight gain and obesity found in study I and II due to GC treatment could surprisingly not be reproduced in study III. There was no significant difference in body weight gain between treatments or genotypes in male mice. The data seem to indicate a loss of weight in the beginning of the corticosterone exposure, but this weight loss is only partly recovered during the study period. This discrepancy between the results in the GC-model with C57BL/6 mice in study I and II and the wild-type *Ppp5c*^{+/+} mice on the C57BL/6 background in study III might be explained by individual differences in sensitivity. But interestingly, also pancreatic islets isolated from *Ppp5c*^{+/+} mice in study IV were less sensitive to the apoptotic effects of the synthetic GC dexamethasone compared to islets isolated from normal C57BL/6 mice, which will be discussed under later headings. Moreover, since the mice used in study III were backcrossed eight or nine generations (N8 and N9) they are not considered fully congenic and even though the differences are small this might affect the outcome of these mice when exposed to corticosterone, as compared to study I and II with normal C57BL/6 mice. On the other

hand, the female wild-type mice in study III gained significantly more weight when exposed to corticosterone than did the vehicle-treated animals, consistent with the earlier findings. Interestingly, the female mice lacking PP5 were partly protected against the GC-induced weight gain. This is in line with the previous findings where mice lacking PP5 were protected against the high fat-feeding induced weight gain [329]. Additionally, monitoring of the *Ppp5c*^{+/+} and *Ppp5c*^{-/-} mice in-house breeding has previously revealed that *Ppp5c*^{-/-} mice have a tendency to be smaller than *Ppp5c*^{+/+} mice, a feature that becomes significant in larger cohorts at 2 months of age in male mice and at 5 months of age in female mice. However, there was no statistically significant difference in body weight between genotypes in any of the genders at the start of study IV.

Apart from the weight gain, fat tissue weight and dyslipidemia were also investigated in the *Ppp5c*^{+/+} and *Ppp5c*^{-/-} mice exposed to corticosterone. Even though no difference in weight gain was found in the male mice due to the GC treatment, substantial increases in fat tissue weight was evident in both male and female mice. Fat tissue weight was evaluated in relation to body weight, indicating that even though the male mice did not gain more weight with the corticosterone treatment, they still became more obese and assembled more fat tissue in all fat deposits evaluated. Furthermore, dyslipidemia was evident in the corticosterone-treated mice. However, no difference in fat tissue weight or dyslipidemia was found when comparing genotypes. This data stands in contrast to the results from the study with these mice on the high-fat diet, where the *Ppp5c*^{-/-} mice accumulated less fat than *Ppp5c*^{+/+} cognates [329]. Considering the multiple and potent consequences of GC exposure however, the effects of a high-fat diet and of treatment with corticosterone cannot be expected to result in the same phenotype. Even though lack of PP5 is protective in the high-fat diet setting it does not affect the outcome of corticosterone-induced fat accumulation in either male or female mice.

4.1.2 Glycemia and effects of liraglutide and PP5 knock-out

Insulin resistance and hyperglycemia are properties of the metabolic syndrome and GC excess [13]. These parameters were monitored in studies I, II and III. In GC-treated mice, fasting serum insulin levels were elevated already after one week of treatment and remained high during the study period, indicating development of insulin resistance and compensatory insulin secretion. Moreover, insulin resistance was corroborated in the tissues of GC-exposed mice, as injected insulin failed to decrease blood glucose levels during IPinsTT, compared to vehicle-treated mice. This was further confirmed by examining insulin-induced phosphorylation of AKT. In liver, skeletal muscle and heart muscle tissues from mice exposed to GCs, insulin were unable to induce phosphorylation of AKT, which is required for the translocation of GLUT4 to the plasma membrane and thus important for the insulin response [81]. During the treatment period, random-fed blood glucose levels were significantly increased in the mice exposed to GCs, despite the elevated insulin levels. The mice were also glucose intolerant as blood glucose levels during IPGTT rose to significantly higher levels in GC-treated mice compared to vehicle-treated mice. The negative effects of GC exposure on glycemia and insulin resistance can be transient if the GC administration is discontinued [224]. In study I, a subgroup of mice that had received GC-treatment for

five weeks were thereafter given vehicle for a continuation of three weeks. The results clearly show that also in this model of GC excess the negative GC effects were rapidly reversible. Fasting serum insulin and random-fed blood glucose were both completely normalized at study week six, that is already one week after GC removal. Furthermore, IPinsTT after the three week recovery period revealed a normal insulin response in the same mice that three weeks earlier presented significant insulin resistance.

Moreover, these glucometabolic parameters were partly affected by liraglutide treatment in study II. The GC/Lira mice had lower insulin and blood glucose levels during the study period than did GC/PBS mice, and also lower glucose levels during IPGTT, indicating a better glucose tolerance. Increased C-peptide levels in GC/Lira mice during the IPGTT also indicate a possible improvement of β -cell responsiveness to glucose by the treatment. However, at the end of the study period, at week five, liraglutide was no longer able to prevent hyperglycemia. Additionally, treatment with liraglutide did not improve insulin sensitivity in the GC-exposed mice, as evaluated with IPinsTT. Even though liraglutide fell short in providing full protection against the GC-induced glucose intolerance, the study clearly showed that liraglutide postponed the appearance of obesity, hyperglycemia and hyperinsulinemia and furthermore improved glucose clearing during IPGTT.

Mice lacking PP5 have previously been shown to have reduced fasting glycemia and improved glucose tolerance during IPGTT, with normal insulin sensitivity, when given standard diet [151], and to better maintain insulin sensitivity when given high-fat diet [329]. When examining these parameters in study III, we found that the corticosterone treatment gave rise to hyperglycemia and hyperinsulinemia, as expected, but that male *Ppp5c*^{-/-} mice were fully protected against the corticosterone-induced increase in random-fed blood glucose during the study period, indicating a complete protective effect of PP5 knock-out on glucocorticoid-induced hyperglycemia. As mentioned, fasting serum insulin was also increased during the corticosterone treatment and *Ppp5c*^{-/-} mice displayed a less marked hyperinsulinemia than their *Ppp5c*^{+/+} controls. Studies with PP5-deficient cells in culture have proposed that PP5 has a role in lipid metabolism [327] and thus the protective effect of PP5 knock-out in the high-fat diet study might be due to the lack of fat accumulation. However, that cannot explain the protective effect of PP5 knock-out in this setting with corticosterone exposure, since there was no difference in body weight gain, fat tissue weight or dyslipidemia between genotypes in male mice, but still the mice showed to be completely and partly protected against the corticosterone-induced hyperglycemia and hyperinsulinemia, respectively.

Surprisingly, when it came to glucose handling after an IPGTT glucose injection, the expected glucose intolerance due to the corticosterone treatment was absent. Our IPGTT data are difficult to interpret because both *Ppp5c*^{+/+} and *Ppp5c*^{-/-} mice on GC treatment seemed to clear glucose more effectively than vehicle-treated *Ppp5c*^{+/+} mice, albeit the difference was not statistically significant. These results, as well as the lack of weight gain discussed above, are strikingly different from the situation in normal C57BL/6 mice in study I and II. Still, a significant difference during the glucose challenge was found in vehicle-treated animals when comparing genotypes, showing a better glucose handling ability in *Ppp5c*^{-/-} mice. Since the *Ppp5c*^{-/-} mice - as mentioned earlier - have been shown to eliminate glucose faster during glucose challenge [151], the reason for the protective effect of PP5 knock-out might lie in the glucose handling capability in the tissues. This can, however, not be explained by a difference in insulin sensitivity in corticosterone-treated *Ppp5c*^{-/-} since there was no difference in glucose

handling after insulin injection at IPinsTT between the genotypes in study III, either in vehicle- or corticosterone-treated mice. It might though be explained by an increased capability of the pancreatic islets to secrete insulin in *Ppp5c*^{-/-} mice. GSIS has indeed been shown to be elevated in MIN6 cells with reduced expression of PP5 [151], and in a setting with the same level of insulin resistance as in *Ppp5c*^{+/+} mice and *Ppp5c*^{-/-} mice exposed to corticosterone in study III, an enhanced capability to release insulin would be protective against hyperglycemia to a certain extent. Hyperinsulinemia though, as evaluated weekly during the study period, was not as evident in *Ppp5c*^{-/-} mice as in *Ppp5c*^{+/+} mice on corticosterone treatment, leading to the conclusion that less insulin is released from the pancreatic islets in the *Ppp5c*^{-/-} mice, and not more. That is most likely also the case at the time point measured; however, since that is serum insulin after six hours of fasting it does not qualify as insulin released during GSIS *in vivo*. The insulin levels released if measured in serum from random-fed mice might be completely different, perhaps with much higher insulin levels in *Ppp5c*^{-/-} mice, normalizing the otherwise impending hyperglycemia.

Taken together, as shown in studies I, II and III regarding glycemia, this model with GC administration via the drinking water shows typical features of the metabolic syndrome, T2DM and GC excess, revealing hyperglycemia, hyperinsulinemia, glucose intolerance and insulin resistance. Liraglutide treatment fell short in providing full protection against the GC-induced glucose intolerance, but study II clearly showed that liraglutide postponed the appearance of hyperglycemia and hyperinsulinemia and also improved glucose clearing during IPGTT. In study III, wild-type mice exposed to corticosterone exhibited hyperglycemia and hyperinsulinemia. Importantly, both these diabetogenic effects of the steroid were more or less prevented in *Ppp5c*^{-/-} mice, indicating that inhibition of PP5 expression is advantageous for glucose control during periods of GC exposure. In fact, genetic deletion of PP5 was more effective than liraglutide in protecting against the corticosterone-induced impairment of glucose homeostasis, lending support to the idea that inhibition of PP5 could be an attractive strategy to maintain glucose homeostasis in the face of insulin resistance. This protective effect might possibly be due to an increased capability of insulin release from the pancreatic islets of *Ppp5c*^{-/-} mice, but further studies, examining glucose-stimulated insulin release in this setting, needs to be performed for this hypothesis to be confirmed or rejected.

4.1.3 Compensatory islet cell adaptations to GC exposure *in vivo*

Pancreatic β -cells have a substantial potential to adjust to increased demands, as in settings with loss of the insulin response in tissues. On the cellular level, β -cells can adapt their insulin production and secretory capacity [377, 378]. In addition, expansion of the β -cell mass by proliferation occurs during conditions of insulin resistance [30-32]. In line with this, study I shows increased GSIS from islets isolated from mice treated with corticosterone. Furthermore, mRNA and protein levels of important ER chaperones were upregulated in islets from GC-exposed mice, suggesting that the ER chaperone expression adapt to meet the increased demand for insulin production. When examining cell proliferation and β -cell mass in the present *in vivo* model with GC excess and elevated insulin secretion, study I showed increased BrdU incorporation and study II showed increased numbers of Ki-67-positive cells in islets from mice treated

with corticosterone. This indicates that the islets in the model have increased cell proliferation, adapting to the augmented demand, leading to expansion of the β -cell mass. Indeed, pancreatic insulin content and islet volume in study I (Fig. 4) and islet density in study II were increased in response to corticosterone treatment. This expansion in islet tissue was due to an increase in both α -cell mass and β -cell mass, as shown in study II.

Mice treated with corticosterone in combination with liraglutide, in study II, had as mentioned the same level of impairment regarding insulin sensitivity, as indicated by their similar capacity to clear glucose after an insulin injection at IPinsTT. The mice also displayed the same increase in β -cell mass, indicating that the β -cell mass is primarily a reflection of the reduction in insulin sensitivity, which is not predominantly targeted by liraglutide. Also, since liraglutide had no effect on β -cell mass or insulin sensitivity, but improved glucose clearance in response to a glucose challenge at IPGTT, the effect of liraglutide is best explained by short-term effects on insulin secretion capacity. Indeed, examination of C-peptide secretion during IPGTT revealed that GC/lira-treated mice had elevated C-peptide levels as a response to glucose, compared to the GC/PBS group. Thus, the positive effects of liraglutide in this setting might be due to direct effects on the insulin-producing β -cells, promoting augmentation of the insulin exocytosis at hyperglycemia, rather than promoting proliferation of β -cells or affecting insulin sensitivity in peripheral tissues.

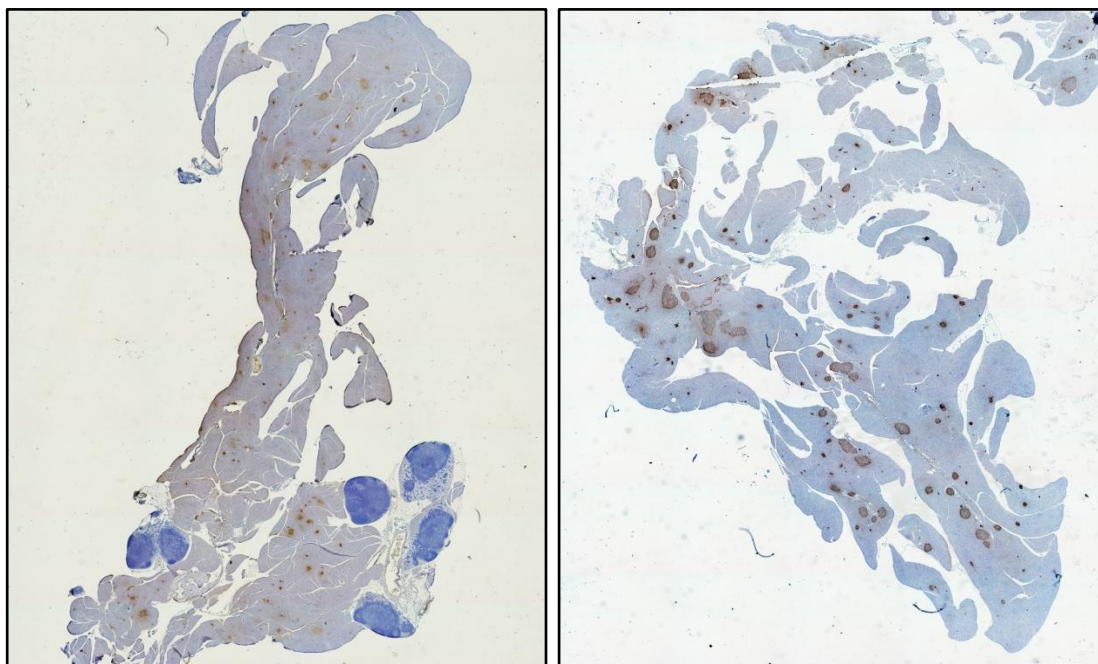


Figure 4. Immunohistochemical pancreas sections stained for insulin from a mouse treated with vehicle (left) or corticosterone (right).

Although refuted [379], a recent study has examined pancreata in human *post mortem* specimens from T2DM patients, subject or not to incretin therapy [380]. The study reports on an increase of pancreatic mass including both exocrine and endocrine tissue, with elevated β -cell and α -cell area and mass. The authors were concerned that

the findings of marked α -cell hyperplasia together with glucagon-expressing microadenomas demonstrate a risk for development of glucagon-expressing neuroendocrine tumors in patients on incretin based therapy. As mentioned above, an expansion of α -cell mass due to corticosterone exposure was also found in study II, whereas no significant difference was found between GC/lira and GC/PBS groups or vehicle/lira and vehicle/PBS groups. Thus, in the setting of GC excess in mice presented in study II, the five week liraglutide treatment did not promote α -cell mass expansion, neither when given alone nor when used together with corticosterone. In fact, liraglutide seemed to reduce the induction of α -cell mass seen in the GC-exposed mice, although this finding did not reach statistical significance.

Since GCs have direct cytotoxic effects on islets and pancreatic β -cells *in vitro* [216-218, 268] apoptosis and ER-stress markers were also examined in the model in study I (data not presented). However, no significant statistical difference between corticosterone- or vehicle-treated mice was found when examining levels of phosphorylated PERK or eIF2 α in isolated pancreatic islets. Furthermore, cleaved caspase 3, as marker for apoptosis, was evaluated in isolated islets and in immunohistochemical sections of pancreata from study I. Although positive immunostaining of activated caspase 3 was detected in the exocrine tissue, essentially no cells stained positive for cleaved caspase 3 could be localized to islets. Similarly, in lysates of isolated islets, Western blot experiments failed to reveal any accumulation of cleaved caspase 3, indicating that very few, if any, islet cells were undergoing apoptosis at the time of analysis. Due to the marked islet-cell proliferation, β -cell mass expansion and high insulin levels during the corticosterone-treatment, as well as a the lack of detection of the apoptotic marker, a loss of insulin-producing β -cells due to apoptosis is not a likely scenario in the model at this stage of the treatment. However, it cannot be excluded that a further continuation of the corticosterone-treatment has a possibility to induce β -cell disruption and apoptosis, thus leading to a loss of the insulin hypersecretion and additional deterioration of the phenotype leading to T2DM.

4.2 THE ROLE OF PP5 IN GLUCOCORTICOID-INDUCED DIRECT EFFECTS ON β -CELLS *IN VITRO*

The effects of GCs are dependent on activation of the GR and that is in turn dependent on phosphorylation [247]. PP5 has been shown to influence GR phosphorylation status and action in various cell types [246, 259, 260, 304, 327]. Furthermore, the negative effects of palmitate and ROS on insulin-producing MIN6 cells are augmented by a lack of PP5, via a mechanism including MAPK activation [151]. This has led to an assumption that PP5, on a molecular level, might be a suppressor of apoptosis in pancreatic β -cells, acting via MAPK. Also, since GCs are known to be directly cytotoxic to β -cells [216-218] and since in other cell types it has been shown that MAPKs are involved in the response to GC exposure [252, 381], the mechanisms behind the GC-induced cytotoxicity in pancreatic β -cells might involve MAPK activation. Taken these assumptions together, study IV aimed at elucidating pathways that regulate GC action in pancreatic β -cells, investigating the role of MAPK activation and PP5 during GC exposure. Since direct effects on β -cells and islets are difficult to

study during *in vivo* conditions where the systemic metabolic consequences of GC exposure interfere with the direct GC-mediated effects, this study used isolated pancreatic islets and insulin-producing MIN6 cells that were exposed to GCs in culture. Isolated mouse islets lacking PP5 or MIN6 cells with decreased levels of PP5 were treated with the synthetic GC dexamethasone, with or without MAPK inhibitors. Previous findings showing that GCs are cytotoxic to β -cells [216-218] were reproduced in study IV, as both islets and MIN6 cells succumbed to apoptosis after GC exposure, an effect that was exerted via the GR since it could be prevented by a GR antagonist. Although evidence of β -cell apoptosis after *in vivo* exposure to GCs is lacking and no induction of apoptosis was found - with the duration employed - in pancreatic islets of mice from study I, impaired β -cell function has been observed after prednisolone treatment in humans [279]. Additionally, patients with impaired β -cell function (low insulin responders) are predisposed to becoming overtly diabetic during GC therapy [223].

The underlying mechanisms evoking GC-induced cytotoxic effects on β -cells are not well understood but a role for ROS has been proposed [218, 382]. This is based on findings that dexamethasone can induce ROS production in RINm5F insulinoma cells and that overexpression of catalase in these cells prevents the cytotoxic effects induced by dexamethasone [382]. Furthermore, dexamethasone has been shown to promote the expression of TXNIP, a negative regulator of the antioxidant protein thioredoxin [218]. TXNIP in turn promotes apoptosis in β -cells [383] and GC-induced induction of TXNIP expression in pancreatic β -cells is dependent on p38 MAPK activation [218]. These observations are in alignment with the findings presented in study IV, where activation of MAPK signaling is evidenced by enhanced phosphorylation of ASK-1 and its downstream targets p38 MAPK and JNK following dexamethasone exposure. However, the effects of GCs on MAPK activity are complex. The results from study IV undoubtedly show that exposing MIN6 cells and isolated pancreatic islets to dexamethasone activates the MAPK signaling pathway, supporting previous observations [218, 384]. Still, it is known from studies in other tissues that dexamethasone can attenuate p38 MAPK and JNK phosphorylation induced by cytokines [385], glucose starvation [249] or UV light exposure [386]. It has also been shown that the GR antagonist RU486 can induce apoptosis through activation of p38 MAPK [381].

ER-stress in the PERK pathway was also investigated in study IV and in contrast to dexamethasone-induced activation of MAPK signaling, the integrated stress response via PERK signaling was not promoted by dexamethasone in this setting. In fact, study IV shows decreased phosphorylation of eIF2 α , as has also previously been noted [287], and decreased mRNA levels of Chop/Ddit3 and Gadd34/Ppp1r15a. This is in contrast to the results obtained in study I where no difference was found in phosphorylation of eIF2 α in islets from GC-treated mice, further emphasizing the difference between *in vivo* and *in vitro* studies.

The results presented in study IV also provide insights into the regulation of GC action in pancreatic β -cells. Phosphorylation of the GR can provide both positive and negative regulatory inputs with respect to GR transcriptional activation. Serine 220 of the rodent GR is a substrate for p38 MAPK activity and changing serine 220 to alanine 220 profoundly diminishes GR-driven gene transcription and apoptosis in lymphoid cells and hepatocytes, as does pharmacological inhibition of p38 MAPK [288, 387]. Study IV shows that a p38 MAPK inhibitor reduces dexamethasone-induced

phosphorylation of the GR at serine 220 and subsequently attenuates dexamethasone-induced cytotoxicity, aligning well with reduced GR activity. However, the reduction of dexamethasone-induced apoptosis is not complete, revealing that p38 MAPK activation is not the only mechanism behind the GC-induced apoptosis in insulin-producing cells. Investigation of the effects of JNK inhibition was also performed in study IV. As previously mentioned, JNK is responsible for phosphorylation of serine 234 of the GR, leading to a loss of GR action [256]. The results show that inhibition of JNK leads to augmentation of the dexamethasone-induced apoptosis, consistent with the concept that JNK action negatively influences GR activity. Moreover, inhibiting GR activity by RU486 completely blocked the augmenting effect of the JNK inhibitor on the GC-induced apoptosis.

Finally, GR activity is not only regulated by kinases but also by phosphatases. Treatment of cells with the phosphatase inhibitor okadaic acid results in hyperphosphorylation of the GR, retaining of the receptor in the nucleus and, consequently, transcriptional activation in mammalian cells [258, 388]. *In vitro* experiments with A549 cells have showed that suppression of PP5 with the use of antisense oligonucleotides increases GR transcriptional activity both in the absence and presence of GCs [260]. In airway smooth muscle cells, PP5 has recently been shown to decrease phosphorylation of GR at serine 220/211 and thereby reduce the cell sensitivity to GC treatment [246]. Results from study IV with MIN6 cells with reduced levels of PP5 expression and islets lacking PP5 show that the PP5 disruption increases the susceptibility to dexamethasone-induced apoptosis. Additionally, dexamethasone-induced p38 MAPK phosphorylation is increased in the cells with reduced PP5 levels. These results indicate that PP5 serves to inhibit direct cytotoxic effects of GCs in β -cells, supporting the notion that endogenous phosphatase activity of the GR, and thus GR dephosphorylation, is catalyzed by PP5 [389]. Importantly, the protective effects against GC toxicity induced by the p38 MAPK inhibitor is in the same range as the augmenting effects of PP5 inhibition, which does not prove but supports the conclusion that p38 MAPK and PP5 are operating via the same mechanism.

Taken together, the results from study IV show that GC treatment of β -cells induces p38 MAPK and JNK phosphorylation that work in opposite to regulate the cytotoxic effects exerted by GCs. The study also points to the conclusion that PP5 acts as a mediator of β -cell cytoprotection during GC exposure in culture. Still, in contrast to these seemingly harmful consequences of disrupted PP5 activity investigated in β -cells *in vitro*, the *in vivo* experiments in study III, show that *Ppp5c*^{-/-} mice are protected against hyperglycemia and hyperinsulinemia induced by corticosterone. Further studies are undoubtedly needed to pin down the actions of PP5 in various organs, and the putative beneficial effects of tissue-specific PP5 knock-out.

5 CONCLUDING REMARKS

Obesity, the metabolic syndrome and T2DM are all pathological conditions increasing rapidly world-wide. Therefore it is of great importance to elucidate the mechanisms underlying the progression from normoglycemia to disturbed glucose tolerance and further to hyperglycemia and development of T2DM. Without knowledge on how and why pancreatic β -cells surrender, giving rise to disease, it is very difficult to find a way to prevent the β -cell apoptosis and loss of β -cell mass that is believed to be the hallmark of T2DM progression. A role for GC excess in the metabolic syndrome, and in the long run unfolding of diabetes, has been proposed and there is no doubt that long-term GC exposure can have considerable negative effects on glucose tolerance and insulin sensitivity, in that it can lead to steroid-induced diabetes in susceptible individuals and that T2DM patients risk a worsening of their disease when given GC medications.

This thesis aimed at elucidating diabetogenic effects of GCs with the use of a mouse model that in many aspects recapitulates the metabolic syndrome in humans. The studies show that pancreatic β -cells adapt to the increased demand for insulin secretion and that a number of the negative effects of the GC treatment were counteracted by the GLP-1R agonist liraglutide, giving GLP-1R agonists a possible preventive role in treatment of risk patients on GC medication. The survival of β -cells that are able to adapt is crucial and works protective against T2DM development, maintaining normoglycemia. However in susceptible individuals, when that adaptive ability is compromised and the β -cells succumb to apoptosis, the progression of disease into frank T2DM is a fact.

GCs have indeed been shown to exert direct cytotoxic effects on pancreatic β -cells, a subject that is further investigated in this thesis, examining pathways behind the GC-induced β -cell apoptosis. However, no signs of apoptosis could be found in the β -cells in the *in vivo* studies, acknowledging the difficulty in determining what is what in an *in vivo* setting and that the contradictory signals to the β -cells, coming from increased insulin demand and proliferative effects due to systemic metabolic consequences of GC treatment, as well as direct negative GC effects, are complex. This further emphasizes the need for both *in vivo* and *in vitro* studies, in the quest of unraveling both systemic effects of a treatment and direct effects on certain tissues, to get the whole picture of the implications in a certain context.

The role of PP5 in the setting of adverse GC effects was also studied, due to the fact that a lack of PP5 has shown to be protective in a high fat-feeding context. This thesis shows that loss of PP5 indeed is partly protective against the deleterious effects of GCs *in vivo*, but in the *in vitro* situation with direct effects of GCs on pancreatic β -cells, a lack of PP5 is instead detrimental. The action of PP5 is still in many ways covered in darkness and in the context of β -cell apoptosis and T2DM development, PP5 might be a double-edged sword. The actions of PP5 may very well be contradictory in different tissues, and what is true about the β -cells might not at all be relevant for other cell types important for glucose homeostasis and metabolism. For that reason, a thorough investigation of PP5 action in different tissues might resolve some of the pieces of the T2DM puzzle and potentially lead to a role for PP5 regulation in a clinical setting in the fight against metabolic disease.

6 SAMMANFATTNING PÅ SVENSKA

Andelen personer som lider av övervikt och fetma ökar, både i västvärlden och i utvecklingsländer. Vår västerländska livsstil med högt kaloriintag förenat med att vi rör på oss relativt lite är en farlig kombination. Fetma är i många fall förknippat med andra problem eller metabola störningar som samverkar, och för att beskriva dessa kombinerade faktorer används ofta samlingsnamnet ”metabola syndromet”. Metabola syndromet är ett begrepp som kan ha olika definitioner men generellt ingår riskfaktorerna fetma (framförallt bukfetma), förhöjt blodtryck, förhöjt blodsocker och förhöjda blodfetter samt insulinresistens, d.v.s. en minskad förmåga hos kroppens celler att reagera på det blodsockersänkande hormonet insulin. Metabola syndromet är starkt förknippat med en ökad risk för att drabbas av diabetes typ 2 och hjärt-kärlsjukdom som till exempel hjärtinfarkt och stroke. I samband med att fetman ökar globalt ökar också metabola syndromet och diabetes typ 2. Världshälsoorganisationen, WHO, har beräknat att ungefär 347 miljoner människor har diabetes i världen idag, och att förekomsten av diabetes kommer dubblas mellan åren 2005 och 2030. Detta förväntas innebära enorma kostnader i hälso- och sjukvård för samhället. För att kunna hindra utvecklingen av diabetes krävs en förståelse för hur sjukdomen uppstår och kunskap om vilka mekanismer som ligger bakom att vissa personer blir sjuka och andra inte. Mycket forskning sker världen över för att belysa orsakssamband och molekylära skeenden under utvecklingen av sjukdomen, men ännu är många gåtor gällande diabetes olösta. Forskningsstudier har visat att patienter med metabola syndromet och patienter med störd blodsockerkontroll kan ha förhöjda nivåer av vårt kroppsegna hormon kortisol och det har lett till att en frågeställning utformats där rollen för kortisol i utvecklingen av metabola syndromet har diskuterats.

Kortisol hos människa, och kortikosteron hos möss och råttor, ingår i en grupp steroidhormoner som kallas glukokortikoider. Glukokortikoider är en sorts stresshormon och har en rad viktiga funktioner för kroppen, bland annat gällande kontroll av ämnesomsättning och immunförsvaret. Glukokortikoider har en starkt hämmande effekt på vårt immunförsvaret och därför används en stor mängd mediciner baserade på glukokortikoider för att trycka ner inflammation och immunförvarsreaktioner vid sjukdomstillstånd som till exempel astma, allergier och autoimmuna sjukdomar. Det är sedan länge känt att dessa typer av mediciner kan ha svåra biverkningar, speciellt då de används under längre tid. Biverkningarna har bland annat att göra med glukokortikoidernas påverkan på ämnesomsättningen. Patienter på långtidsbehandling med glukokortikoider kan utveckla övervikt med bukfetma, förhöjt blodsocker och förhöjda blodfetter och insulinresistens, alltså ett tillstånd som mycket liknar det metabola syndromet. En förhöjd egenproduktion av kortisol, som vid hypofystumör vid Cushings sjukdom, ger samma symptom. Patienter som lider av diabetes typ 2 riskerar också att förvärra sin diabetes om de samtidigt behandlas med glukokortikoider. Bieffekterna av förhöjda kortisol-nivåer kan gå tillbaka om patienten får medicin mot sin Cushings sjukdom eller om behandlingen med glukokortikoid-baserade läkemedel upphör. Men hos vissa individer kan biverkningarna övergå till en steroidorsakad bestående diabetes.

Syftet med denna avhandling var att studera effekterna av glukokortikoider avseende komplikationer som har ett samband med diabetes. För att göra detta

användes en musmodell där vanliga möss fick kortikosteron i sitt dricksvatten under fem veckors tid. Dessa möss jämfördes med möss som fått vehikel, d.v.s. samma typ av behandling men utan glukokortikoider. Studie I beskriver vad som hände med mössen under behandlingens gång och resultaten visar på att de utvecklade ett sjukdomstillstånd som i mångt och mycket har samma kännetecken och symptom som det metabola syndromet hos människa. Mössen blev överviktiga med förhöjda blodfetter och ökad mängd fett i specifika fettdepåer men även inlagrat i organ som lever och skelettmuskel. Detta inlagrade, ektopiska, fett är mycket negativt för vävnadens möjligheter att reagera på insulin och ta upp socker från blodet. Följaktligen visade det sig också att mössen som blivit behandlade med kortikosteron hade insulinresistens, förhöjt blodsocker och förhöjda nivåer av insulin. Även blodtrycket var förhöjt hos mössen. Studie I undersökte också vad som hände med de celler som producerar insulin, beta-cellerna i bukspottkörteln. Insulinresistens tvingar dessa celler att producera avsevärt mycket mer insulin för att kunna sänka kroppens blodsocker, i en situation där vävnaden har svårt att reagera på insulinet. De insulinproducerande cellerna har en betydande kapacitet att anpassa sig genom att växa i storlek och antal. Detta visas också i studie I; mängden insulinproducerande beta-celler ökade genom celledelning och cellerna ökade också sin kapacitet att tillverka och utsöndra insulin.

I studie II visas att ett diabetesläkemedel, liraglutid, kan minska de negativa konsekvenserna av glukokortikoidbehandling i den musmodell som beskrivs i studie I. Behandling med liraglutid ledde bland annat till en minskning av mängden ektopiskt fett i levern och till försening av uppkomsten av förhöjda insulinnivåer och förhöjt blodsocker. Liraglutid ingår i en grupp av läkemedel som kallas inkretin-mimetika, eller GLP-1-analoger. Dessa läkemedel fungerar genom att härma ett kroppseget hormon, GLP-1, som normalt hjälper till att bibehålla en lagom blodsockernivå i kroppen. GLP-1 bidrar också till att minska aptiten och till att öka mättnadskänslan efter en måltid. Kroppseget GLP-1 bryts ner väldigt snabbt efter att det utsöndrats till blodet från celler i tarmen, men GLP-1-analoger som liraglutid är svårare för kroppen att bryta ner och de stannar därför längre i blodcirkulationen och kan verka under en förlängd tid. Studie II visar att denna typ av läkemedel kan vara effektiva för att behandla steroidorsakad diabetes.

I studie III och IV undersöktes effekterna av ett speciellt protein (äggviteämne), proteinfosfat 5 (PP5) med avseende på både vad som händer i kroppen, *in vivo*, och vad som händer i odlade celler utanför kroppen, *in vitro*. I studie III användes modellen från studie I men istället för vanliga möss så behandlades möss som saknade PP5 (*Ppp5c*^{-/-}) och jämfördes med möss som hade PP5 (*Ppp5c*^{+/+}). Studie III visade att mössen som saknade PP5 till viss del var skyddade från de negativa konsekvenserna av glukokortikoidbehandlingen. Honor utan PP5 var delvis skyddade från viktuppgång och hanar var delvis skyddade från förhöjt blodsocker och förhöjda insulinnivåer. I studie IV undersöktes istället den direkta effekten som glukokortikoider utövar på insulinproducerande celler. Odlade beta-celler behandlades med en syntetisk glukokortikoid, dexametason, och då cellerna dog genom en programmerad celledöd, så kallad apoptos, undersöktes vilka signaleringsvägar och molekylära mekanismer som var påslagna i cellerna och påverkade cellernas förmåga att överleva. I denna studie undersöktes också effekten av PP5, och det visade sig, i motsats till resultaten i studie III, att en avsaknad av PP5 gjorde cellerna extra känsliga för behandlingen med glukokortikoider.

Sammanfattningsvis visar studierna att musmodellen med glukokortikoider i dricksvattnet kan vara användbar för att studera metabola syndromet eller negativa konsekvenser av förhöjda nivåer av glukokortikoider, då mössen uppvisar flertalet symptom som även är kopplade till metabola syndromet hos människa. Utöver det visar studierna att liraglutid kan vara fördelaktigt för patienter som riskerar att få negativa biverkningar, relaterade till ämnesomsättning och blodsocker, då de behandlas med glukokortikoider. Till sist, en avsaknad av PP5 var fördelaktigt för möss då de behandlades med glukokortikoider, men resultaten visade tvärtom då odlade celler *in vitro* utsattes för behandling. Detta tyder på att PP5 kan ha motsägelsefull verkan i olika vävnader och att det som händer i beta-celler med avseende på PP5 inte behöver gälla andra typer av celler i kroppen. För att få reda på om PP5 skulle kunna ha en roll kliniskt krävs ytterligare studier. Förhoppningsvis kan det leda till att någon av pusselbitarna faller på plats vad gäller gåtan diabetes typ 2.

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