From the Department of Medical Nutrition and the Department of Molecular Medicine, Karolinska Institutet, Stockholm, Sweden

Increased Glucocorticoid Sensitivity in Pancreatic β-cells: Effects on Glucose Metabolism and Insulin Release

Behrous Davani

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Supervisors:
Professor Sam Okret
Department of Medical Nutrition
Karolinska Institutet

Associate Professor Akhtar Khan
Department of Molecular Medicine, Rolf Luft Center for Diabetes Research
Karolinska Institutet

Opponent:
Professor Tommy Olsson
Dep. of Medicine, Umeå University
Umeå University Hospital

Betygsnämnden:

Professor Leif Jansson
Dep. of Medical Cell Biology
Biomedical Center, Uppsala

Associate Professor Abram Katz
Dep. of Physiology and Pharmacology
Karolinska Institutet, Stockholm

Associate Professor Peteris Alberts
Biovitrum AB, Stockholm

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In memory of my father

ABSTRACT

Type 2 diabetes mellitus (T2DM) is characterized by three pathological alterations: (1) insulin resistance in peripheral tissues, (2) increased hepatic glucose production and (3) impaired insulin secretion from the pancreatic β-cells. Glucocorticoids (GCs) exert profound effects on glucose homeostasis. They decrease glucose uptake and increase hepatic glucose production. In addition, they may directly inhibit insulin release. The main aim of this thesis was to investigate the effect of GCs on β-cell function, insulin release and glucose homeostasis. Furthermore, we studied the mechanisms behind this possible effect. For this purpose two different animal models were used. First, transgenic mice (TG mice) with an increased GC sensitivity restricted to their β-cells were generated by overexpressing the glucocorticoid receptor (GR) under the control of insulin promoter I. The second animal model, was ob/ob mice, in which hyperglycemia co-exists with obesity.

At the age of 3-4 months, TG mice had normal fasting blood glucose but decreased glucose tolerance (paper 1). In an intravenous glucose tolerance test, decreased glucose tolerance was observed in TG mice compared to controls. TG mice exhibited significantly higher blood glucose concentrations than control mice at 60 min after intravenous injection of glucose. Measurement of plasma insulin levels 5 min after glucose load demonstrated a decrease in acute insulin response in the TG mice.

Following the natural history of glucose impairment in TG mice, we found that the transgenic mice developed hyperglycemia both in the fed and overnight-fasted states at the age of 12-15 months (paper III). The basal and stimulated insulin secretion was significantly decreased in the old TG mice both under *in vivo* and *in vitro* conditions. Importantly, glucose elimination after i.p. insulin administration did not differ between the groups, suggesting that insulin sensitivity was normal in TG mice. Therefore, it seems that the development of manifest diabetes in these animals is due to a direct inhibitory effect of GCs on insulin release, and is not related to reduction in insulin sensitivity.

The mechanisms behind the progressive deterioration of glucose tolerance in TG mice are not known. However, data presented in paper II and III shed some light on the mechanisms behind the impaired insulin release in TG mice. First, the activity of islet Glucose-6-phosphatase (G6Pase) was increased in transgenic mice. Secondly, we found a significantly higher density of α_2-adrenergic receptor (α_2-AR) in the islets of TG mice compared to controls. Furthermore, expression of α_2-AR mRNA was increased in islets from TG mice.

G6Pase and α_2-AR are two of the genes that have been reported to be regulated by GC and may be related to the regulation of insulin secretion. Previous reports from some animal models of T2 DM have shown an increased G6Pase activity in the islets which can be attenuated by GC treatment. GCs upregulate also α_2-AR expression and signaling in β-cell lines. Thus, reduced glucose-stimulated insulin release and development of hyperglycemia in TG mice, can be due to enhanced expression of G6Pase and α_2-AR.

The inhibitory effect of GC on islets is partly mediated by 11β-hydroxysteroid dehydrogenase type 1 (11β-HSD-1) through local production of active GC. In the last study (IV), we found evidence for activity and expression of 11β-HSD-1 in pancreatic islets from ob/ob mice. Glucose-stimulated insulin release was inhibited with 11-dehydrocorticosterone in a dose dependent manner. Carbenoxolone restored this effect. Thus, 11β-HSD-1 is an amplifier of GC action in the β-cells of ob/ob mice.

Taken together, our results demonstrate that GCs directly inhibit insulin release and identify the pancreatic β-cells as an important target for the diabetogenic action of GC. Antagonism of GR and their signaling pathways are thus promising therapeutic targets for treatment of T2DM.

Key words: Glucocorticoids, glucocorticoid receptor Pancreatic β-cell, Type 2 diabetes, insulin release

TABLE OF CONTENTS

MAIN REFERENCES

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ABBREVIATIONS

ACTH	Adrenocorticotropic hormone
α_2-AR	α_2- Adrenergic receptor
AP	Alkaline phosphatase
AP-1	Activator protein-1
CAT	Chloramphenicol acetyltransferase
CBX	Carbenoxolone
CBG	Corticosteroid binding globulin
CRF	Corticotropin releasing factor
DEX	Dexamethasone
G6Pase	Glucose-6-phosphatase
GC(s)	Glucocorticoid(s)
GLUT-2	Glucose transporter-2
GR	Glucocorticoid receptor
GRE	Glucocorticoid response element
HPA	Hypothalamic-pituitary-adrenal axis
Hsp	Heat shock protein
KRB	Krebs-Ringer bicarbonate buffer
LIR	Low Insulin response
LSC	Liquid scintillation counting
IVGTT	Intravenous glucose tolerance test
IPGTT	Intraperitoneal glucose tolerance test
IPITT	Intraperitoneal insulin tolerance test
NF-κB	Nuclear factor-κB
OGTT	Oral glucose tolerance test
PGC-1	peroxisome proliferator activator receptor-γ coactivator 1
PEPCK	Phosphoenolpyruvate carboxykinase
POMC	pro-opiomelanocortin
PPAR-γ	peroxisome proliferator activator receptor
RIA	Radioimmunoassay
RIP1-GR	Rat insulin promoter I-glucocorticoid receptor
T1DM	Type 1 diabetes mellitus
T2DM	Type 2 diabetes mellitus

TLC	Thin layer chromatography
TUNEL	TdT-Mediated dUTP-Nick Labelling
UK-14304	Bromo-6-(2-imidazoline-2-amino)-quinnoxaline
WHO	World Health Organization
11-HSD-1	11β-hydroxysteroid dehydrogenase type 1
11-HSD-2	11β-hydroxysteroid dehydrogenase type 2
11-DHC	11-dehydrocorticosterone

GENERAL INTRODUCTION

Intracellular Communication by hormones

Tissues and cells in an organism do not function in isolation, but rather are dependent on advanced mechanisms of communication. The nervous system and hormones mediate this communication. The classical definition of a hormone is a biomolecule which is synthesized and secreted by specific glands, transported to target cells or tissues, and acts as a chemical messenger or signal molecule. Target cells respond to hormones with the help of signal-specific receptors or so-called " information receivers". The receptor thus must specifically recognize and interact with its cognate hormone. The hormones are derived from more essential compounds. Thus, peptides are derived from proteins, steroids from cholesterol, and catecholamines and thyroid hormones from amino acids. Functionally, hormones are predominantly regulators of homeostasis in the body. For example, plasma glucose levels are maintained within a narrow range, both in the basal condition and during and after meals by hormones involved in carbohydrate metabolism. Regulation of these hormones allow the body to store energy when food is available, or use energy, for example, during starvation or stress situations. Any disturbance in this control system results in hyper- or hypoglycemia.

Diabetes mellitus

Classification of diabetes mellitus

Diabetes mellitus is a group of metabolic disorders characterized, defined and diagnosed by chronic hyperglycaemia (WHO, 1985). Diabetes mellitus is a major global health problem. The World Health Organization (WHO) classification recognizes two major clinical forms of diabetes, i.e. Type-1 and Type-2 diabetes mellitus (T1DM and T2DM). T2DM is the predominant form of glucose intolerance, with about 80% prevalence among all diabetic patients. It has been predicted that the number of diabetic people, overwhelmingly T2DM patients, will increase from the present 140 million to 280 million over the next thirty years, and the bulk of the increase will occur in developing countries (Zimmet et al., 2001). T2DM is characterized by three pathological alterations: (1) insulin resistance in peripheral tissues, (2) increased hepatic glucose production and (3) impaired insulin secretion from the pancreatic β-cells (DeFronzo et al., 1992; Efendic et al., 1984; Kahn, 1996). While in T1DM (insulin-dependent diabetes mellitus) the function of the pancreatic β-cell is almost

destroyed, in T2DM (non-insulin-dependent diabetes mellitus), β-cells can still secrete insulin while the glucose-induced insulin release is impaired. The relatively late appearance of T2DM and its mild progressive course compared to T1DM also characterize this disorder, although younger individuals can also develop the disease. Type 2 patients, unlike Type 1 patients, do not depend on exogenous insulin treatment for survival.

Although T2DM has been recognized as a disease for a long time, the primary defect that accounts for this disorder is still a matter of debate. Both genetic and environmental factors contribute to the development of T2DM. Environmental factors, e.g. diet, stress, obesity and lack of physical activity, are involved in the development of the disease (Hamman, 1992; Horton, 1983). A number of candidate genes that could affect insulin secretion and/or insulin action have been investigated, but so far none of these have accounted for more than a few percent of the incidence of T2DM.

There are different opinions as to whether the primary cause of diabetes lies within the dysfunction of the β-cell or the insulin resistance. Many groups have suggested that insulin resistance, a condition of reduced insulin sensitivity, is the primary abnormality and β-cell dysfunction is a late event. In contrast, others have suggested that impaired β-cell function, manifest as decreased insulin release, is a prerequisite for the progression from glucose intolerance to hyperglycemia (Beck-Nielsen et al., 1994; Cerasi and Luft, 1967; Efendic et al., 1984; Ostenson et al., 1993b). In Pima Indians (Lillioja et al., 1993) and in Mexican-Americans (Haffner et al., 1989), insulin resistance is the dominant feature, whereas in Caucasian populations impaired β-cell function appears to be more marked at an early stage in the development of diabetes (Jensen et al., 2002; Turner et al., 1988).

In general, it is accepted that increased hepatic glucose production and/or increased insulin resistance alone or together can not maintain hyperglycemia unless the pancreatic islets fail to function (Kahn, 2003; Porte and Kahn, 2001). It must be noted that the pathological alterations seen in T2DM can differ between individuals. The abnormalities may range from a metabolic state of predominant insulin resistance with a relatively light β-cell dysfunction, to a state where the β-cell dysfunction is severe and accompanied by a mild insulin resistance.

Type 2 diabetes and stress

Stress stimulates the release of so-called “stress hormones”, including glucocorticoids (GCs) and catecholamines, which play an important role in glucose metabolism. The actions of these hormones are often opposing insulin action/ release. This effect could be of adaptive importance in a healthy organism, but in a diabetes-prone individual it can be

problematic, as shown both by human and animal studies (Chrousos and Gold, 1992; Surwit et al., 1992) Surwit et.al. have shown that the degree of hyperglycemia in the genetically obese mouse (C57BL/6J, ob/ob) is dependent, in part, on whether the animal is exposed to stressful environmental stimuli (Surwit et al., 1984). Pima Indians are at high risk for developing T2DM. Approximately 60% of Pima Indians develop T2DM in adulthood, compared with 5% of the white population (Knowler et al., 1990). Normal euglycemic Pima Indians have shown a disturbed glucose response to behavioral stress compared with whites (Esposito-Del Puente et al., 1994). From this study it appears that the diabetes-prone Pima Indians have a specific glucoregulatory defect that becomes apparent during stress stimulation.

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Glucocorticoid hormones

Glucocorticoid synthesis and secretion

The primary glucocorticoid in man and most mammals is cortisol, whereas in rodents and lower vertebrates it is corticosterone. Other known steroid hormones in mammalian systems are mineralocorticoids, androgens, estrogens and progestins. The location of steroid hormone synthesis depends on the class of hormone. These production sites are mainly the " classical" endocrine glands, such as adrenal and gonads. However, auto- or paracrine synthesis of steroids in some other organs, e.g. brain, heart or thymus, has been reported (Baulieu and Robel, 1995; Pazirandeh et al., 1999; Silvestre et al., 1998). GCs are produced from cholesterol in adrenal cortex by a series of enzymatic reactions. Cortisol biosynthesis and secretion are regulated by adrenocorticotropic hormone (ACTH) secreted from the anterior pituitary, which in turn is regulated by corticotropin-releasing hormone (CRH) secreted from the hypothalamus (HPA axis). The HPA axis is kept in balance by the negative feedback effects of GCs, which in a classical endocrine feedback mechanism inhibit the synthesis and secretion of CRF in the hypothalamus and the pro-opiomelanocortin (POMC) gene (precursor for ACTH) in the anterior pituitary.

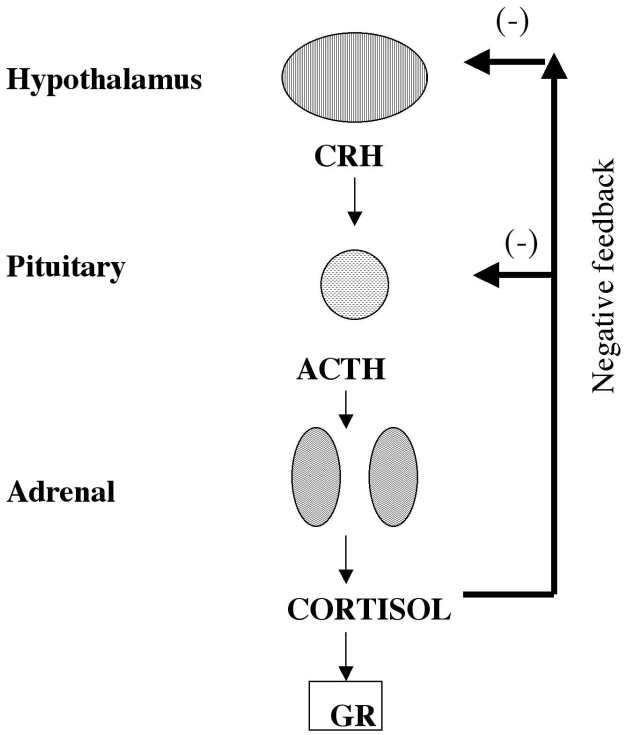

Figure 1. Regulation of the Hypothalamic-Pituitary-Adrenal (HPA) axis by negative feedback.

In the circulation, glucocorticoids are bound to plasma proteins, such as corticosteroid binding globulin (CBG) and albumin (Heyns and Coolens, 1988). The binding to CBG limits the concentration of free biologically active hormone and protects the hormone from degradation in the liver. In fact, the free glucocorticoid levels in blood serum constitute only about 4% of the total hormone concentration.

Glucocorticoid receptor

GCs mediate their effects through a specific intracellular receptor present in almost all cell types, including pancreatic β-cells (Fischer et al., 1990; Matthes et al., 1994). The glucocorticoid receptor (GR) belongs to the superfamily of nuclear hormone receptors, and functions as a ligand activatible transcription factor (Beato et al., 1995; Mangelsdorf et al.,

1995). Receptors for mineralocorticoids, progesterone, androgen and the estrogens also belong to this receptor family. GRs are associated with a heat shock protein (hsp) complex in their non-activated state and located in the cytoplasm. Upon binding of ligand the hsp90 complex dissociates and the receptor is activated and binds as a dimer to bind to specific DNA-sequences, called glucocorticoid responsive elements (GREs), usually located in the promoter region of glucocorticoid responsive genes (Figure 2).

GCs can either upregulate or repress gene activation. One example for positive gene regulation by GR is phosphoenol pyruvate carboxykinase (PEPCK), the rate limiting enzyme in glucocogneogenesis. GC induction of PEPCK is mediated through a complex glucocorticoid response unit consisting of two GREs and three accessory factor elements (Hanson and Reshef, 1997). Some genes regulated by GR, however, seem to be down regulated. One mechanism for repression mediated by GR can occur through binding to negativeGREs (nGREs) (Akerblom et al., 1988; Sakai et al., 1988). An example of this is the osteocalcin gene (Meyer et al., 1997).

Another mechanism of GR action is interaction with other transcription factors, for instance NF-κB or AP-1, in a process called cross talk (Gottlicher et al., 1998; McEwan et al., 1997). In contrast to the GRE-dependent transactivation of GR, the process of cross-talk does not require the DNA binding properties of the GR.

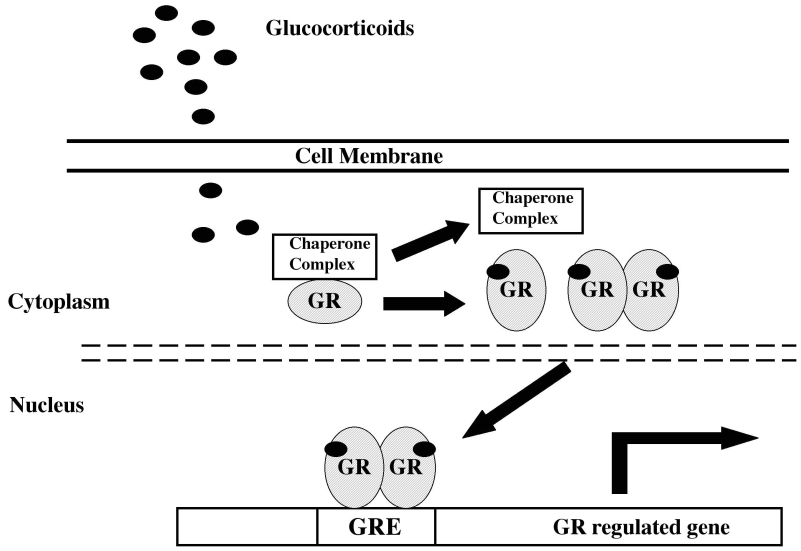

Figure 2. Schematic illustration of the activation process of GR.

There are some other proteins associated with the transcriptional activity of GR. These factors are commonly called cofactors and both coactivators and corepressors have been identified (Jenkins et al., 2001; Xu et al., 1999). An example of such a cofactor is peroxisome proliferator activator receptor-γ coactivator (PGC-1) which was first identified as a coactivator for PPAR-γ (Puigserver et al., 1998) Later reports shown that PGC-1 can enhance transactivation of GR and some other nuclear receptors (Yoon et al., 2001).

The GR contains three functional domains **(**Figure 3). The N-terminal domain is a variable aminoterminal region containing the transactivation domains responsible for gene activation. The ligand-binding domain (LBD) is located in the C-terminal part of the receptor. Apart from its ligand binding property, The LBD contains sequences important for hsp-90 binding, nuclear localization and ligand dependent transactivation.

The DNA-binding domains (DBD) are highly conserved and consist of two so- called zinc finger domains. The DBD of GR recognizes a GRE that consists of two palindromic hexameric half-sites spaced by three nucleotides.

Figure 3: Structural domain of human GR.The transactivation domains τ1, τ2, the DNA-binding domain (DBD) and the ligand- binding domain (LBD) are labelled.

Biological effects of glucocorticoids

Since GR is widely distributed throughout the body, it is reasonable to assume that GCs affect almost all cells and tissues. GCs mediate the stress response, promote production of glucose in the liver and inhibit glucose utilization in the peripheral tissues, regulate fat metabolism and influence the immune system as well as playing a key role during development. In addition, GCs affect growth and maturation of a variety of tissues and

organs, e.g. lung maturation during embryonic development. The importance of the GR in glucocorticoid physiology and during development has been investigated in mice lacking GR (Cole et al., 1995). These mice die within a few days postnatally because of respiratory failure. They also have reduced expression of important gluconeogenic enzymes like PEPCK and glucose-6-phosphatase (G6Pase). Furthermore, elevated levels of ACTH and corticosterone indicate an impaired negative feedback on the HPA- axis.

Glucocorticoids and glucose metabolism

GCs as their name suggests, are among the major hormones concerned with the formation and metabolism of glucose. GCs exert profound effects on glucose homeostasis under both normal and pathological conditions (Andrews and Walker, 1999; Friedman et al., 1996; McMahon et al., 1988). One of the metabolic effects of cortisol and other GCs on metabolism is their ability to stimulate the liver, often increasing the rate of glucoconeogenesis as much as 10- fold (Exton, 1979). This results mainly from two effects of GCs. First, GCs increase activity and expression of all the enzymes required for converting amino acids into glucose in the liver. Second, GCs cause mobilization of amino acids mainly from muscle (Pilkis and Granner, 1992). GCs also induce the expression of PGC-1, which is of major importance for the entire program of key gluconeogenic enzymes in the liver (Yoon et al., 2001)

Various degrees of impairment of glucose tolerance are generally observed in patients with excessive production of endogenous GCs or with long-term glucocorticoid hormone treatment (Andrews et al., 2002; Reynolds et al., 2001). During fasting, GCs increase hepatic glucose production by stimulating gluconeogenesis and decreasing glucose uptake. Patients with a GC deficiency have high insulin sensitivity, whereas GC excess (e.g. Cushing syndrome) is associated with insulin resistance and tendency to hyperglycemia (Chrousos, 1995). In addition, a side effect in long term glucocorticoid treatment, e.g. in inflammatory disease, is precipitation of diabetes (Landy et al., 1988).

In general, GCs are referred to as diabetogenic hormones because they stimulate glucose production and induce insulin resistance. Furthermore, it has been suggested that GCs also inhibit insulin release. In perfused rat pancreas, corticosterone inhibited insulin secretion in response to glucose (Barseghian et al., 1982; Billaudel and Sutter, 1979). A similar effect of glucocorticoids has been reported from cultured islets of Langerhans (Gremlich et al., 1997; Lambillotte et al., 1997; Pierluissi et al., 1986). However, this question has been difficult to address *in vivo* because of the multiple influences of GCs on the whole body, which can have an impact on the endocrine pancreas.

Glucocorticoid sensitivity

Factors that could alter GC sensitivity include intracellular hormone availability, GR expression level or transcriptional activity, structure of response elements within GR target genes, and interaction of GR with other proteins (Bamberger et al., 1996). The GR expression level is a major factor determining cellular sensitivity towards GCs both *in vitro* and *in vivo* (Okret et al., 1991; Pazirandeh et al., 2002; Reichardt et al., 2000; Vanderbilt et al., 1987). The cellular glucocorticoid sensitivity is also determined by intracellular 11β-HSD. This enzyme catalyzes the interconversion of cortisol and cortisone (corticosterone and 11-dehydrycocrticosterone), regulating glucocorticoid access to intracellular receptors (Seckl and Walker, 2001). Tissues may thus be exposed to a relative excess of cortisol without any increase in cortisol secretion or plasma cortisol concentration (Krozowski, 1999; Nobel et al., 2001). Alterations in any of these factors may result in individual differences in GC sensitivity. This is in agreement with clinical findings that GC therapy varies between individuals both with regard to efficacy and to the prevalence and severity of side effects (Ross and Linch, 1982).

Glucocorticoids and the metabolic syndrome

The metabolic syndrome

The metabolic syndrome is characterized by a combination of insulin resistance, hypertension, endothelial dysfunction and dyslipidaemia. The metabolic syndrome or "Syndrome X" shares important symptoms with Cushing's syndrome, *i.e*. dyslipadaemia, hypertension, obesity, glucose intolerance and insulin resistance (Reaven, 1995). All of these features are potentially " steroid sensitive". The combination in patients of the metabolic alterations present in the metabolic syndrome constitutes a high risk for the development of T2DM and cardiovascular disease. The metabolic syndrome and Cushing's syndrome show similar symptoms but one major difference: plasma cortisol is not elevated in the metabolic syndrome (Bahr et al., 2002). However, dysregulation of GR activation has been discussed as a common cause for both syndromes (Friedman et al., 1996; Walker, 2001). Several epidemiological studies have shown, an association between low birth weight and the development in later life of features of the metabolic syndrome (Carlsson et al., 1999; Nyirenda et al., 1998; Phillips et al., 1998). The reasons for these associations between feature of this syndrome remain obscure, but it has been proposed that subtle

abnormalities in cortisol action or signaling are a missing link between these factors in patients with the metabolic syndrome (Seckl et al., 1999).

Glucocorticoids, insulin resistance and the metabolic syndrome

There is an association between the metabolic changes present in metabolic syndrome and metabolic abnormalities due to excessive GC hormone activity, as seen in e.g Cushing's syndrome. It should be noted that GCs are strong antagonists of insulin action. In excess they cause insulin resistance, the key feature in metabolic syndrome (Andrews and Walker, 1999). In the liver, GCs induce gluconeogenesis and antagonize insulin action. This causes increased glucose output and triglyceride synthesis as well as altered lipoprotein export (Brindley, 1995). In the muscle GCs impair insulin signaling, resulting in an insulin resistant state. GCs also stimulate differentiation of fat cells from preadipocytes to adipocytes and promote lipolysis and triglyceride storage, predominantly as visceral fat that is associated with metabolic syndrome and Cushing's syndrome (Bjorntorp and Rosmond, 2000). Increased levels of free fatty acids in the blood, e.g. by GC stimulated lipolysis, is thought to be an important mechanism contributing to insulin resistance (Andrews and Walker, 1999; Brindley, 1995). Furthermore, GCs impair endothelium-dependent vasodilatation, particularly in the muscle. These observations have led to the hypothesis that increased GC activity may constitute a common primary aetiology for the metabolic syndrome (Bahr et al., 2002; Walker, 2001). More recently it has been demonstrated in transgenic mice that an increased GC exposure of the fat tissue results in several metabolic changes that accompany the metabolic syndrome (Masuzaki et al., 2001). However, the precise contribution of GC effects on hepatic lipid and glucose metabolism to the development of metabolic syndrome and T2DM has not been clearly established. It has been suggested that the predisposition to diabetes might arise because of genetic variations which are advantageous in certain environmental situations but can became pathological in different environments (Surwit and Schneider, 1993).

A recent report suggests the elevated plasma cortisol levels may be a link between low birth weight and insulin resistance syndrome (Phillips et al., 1998). Thus, plasma cortisol concentrations were significantly higher in subjects with low birth weight and were significantly related to systolic blood pressure and fasting and 2 h plasma glucose concentration after an oral glucose tolerance test. In this context, it is of interest that environmental exposure in prenatal and early postnatal life may imprint the HPA axis, resulting in permanent modification of the neuroendocrine response to stress throughout life. The administration of dexamethasone to pregnant rats results in low birth weight

offspring that develop hypertension, glucose intolerance, insulin resistance and an overactive HPA axis in later life (Benediktsson et al., 1993). Some authors have sought to explain the finding of low birth weight in terms of early environmental insults, including the actions of glucocorticoids *in utero* to increase predisposition to T2DM (Lindsay and Bennett, 2001). There are studies demonstrating that cortisol levels are increased not only in subjects with low birth but also in other subjects with metabolic syndrome. Thus, in men with high waist- hip ratio 24 h urinary free cortisol output was elevated (Rosmond and Bjorntorp, 1998). They had normal suppression by dexamethasone (1.0 mg) but elevated responses of cortisol to stimulation. In another study it was demonstrated that stress related cortisol secretion is strongly associated with abdominal obesity and with the systolic and diastolic blood pressure (Peppa-Patrikiou et al., 1998). A recent report shows as well that adverse cardiovascular risk is greatest in those with the combination of obesity and high plsama cortisol levels (Walker et al., 2000).

Metabolic programming and glucocorticoids

The term metabolic programming refers to an early adaptation to environmental changes, *e.g*. nutritional or stress stimulus, which permanently change the physiology and metabolism of the organism, the consequences of which are observed much later in life even in the absence of the stimuli that initiated them (Patel and Srinivasan, 2002). As an example of this, it has been suggested that stress exposure of the fetus during pregnancy will result in an increased risk of developing disease in adulthood, particularly impaired glucose tolerance and T2DM, increased blood pressure and vascular diseases (Lindsay et al., 1996). GCs may be important in this process, as fetal exposure to GCs has been proposed to reduce insulin sensitivity in the adult, thus increasing the risk of development of T2DM. Furthermore, it has also been demonstrated that fetal exposure of rats to GCs will change GR levels in the adult tissues (Cleasby et al., 2003; Levitt et al., 1996; Nyirenda et al., 1998). It has also been suggested that an increased GR in skeletal muscle may contribute to the pathogenesis of the metabolic syndrome (Whorwood and Byrne 2002).

Pancreatic islets and Insulin secretion

Pancreatic islets

The human pancreas has 1-2 million islets of Langerhans. Each islet consists of anything from a few hundred to several thousand cells. The islets contain four major types of cells,

*alpha (*α), *beta (*β) *delta (*δ) and PP-cells. β-cells, located at the center of islet, are the most numerous islet-cell type in all species examined. These cells constitute about 65-80 % of the islet population, forming a uniform homocellular medulla and secreting insulin. The α- cells, constitute about 20 % of the population, secrete glucagon and lie in the islet cortex together with the other non-β-cells. The δ-cells account for about 10% of the cell

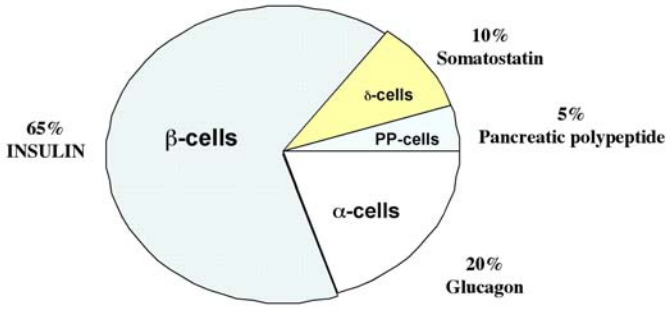

Figure 4. Four major types of cells in islets of Langerhans

Number, are located close to periphery and secrete somatostatin. The PP cells are present in only small numbers and produce pancreatic polypeptide (Figure 4). The islets are highly innervated by sympathetic adrenergic, parasympathethic cholinergic and various peptidergic nerves.

Insulin secretion

The β-cells produce, store and secrete insulin in response to glucose. Glucose is the main regulator of insulin secretion. The β-cells act as glucose sensors to maintain a balance between glucose production by the liver and the rate of insulin dependent- glucose utilization by muscle and adipose tissue. Changes in plasma glucose levels are sensed by the β-cells, leading to an adjustment of insulin secretion to restore normal blood glucose levels. It is generally accepted that glucose is transported into the β-cell via the glucose

transporter GLUT-2 and promotes insulin release by generating ATP, which in turn increases the ATP/ADP ratio, leading to closure of ATP sensitive K^+ channels, depolarization of the plasma membrane, opening of voltage-activated Ca^+ channels, entry of extracellular Ca^+, and, finally exocytosis of insulin secretory granules.(Ashcroft et al., 1994; Efendic et al., 1991) (Figure 5).

Glucose can also stimulate insulin release independently of K^+-ATP channels This mechanism is termed the K^+-ATP independent pathway (Gembal et al., 1992; Sato et al., 1992)

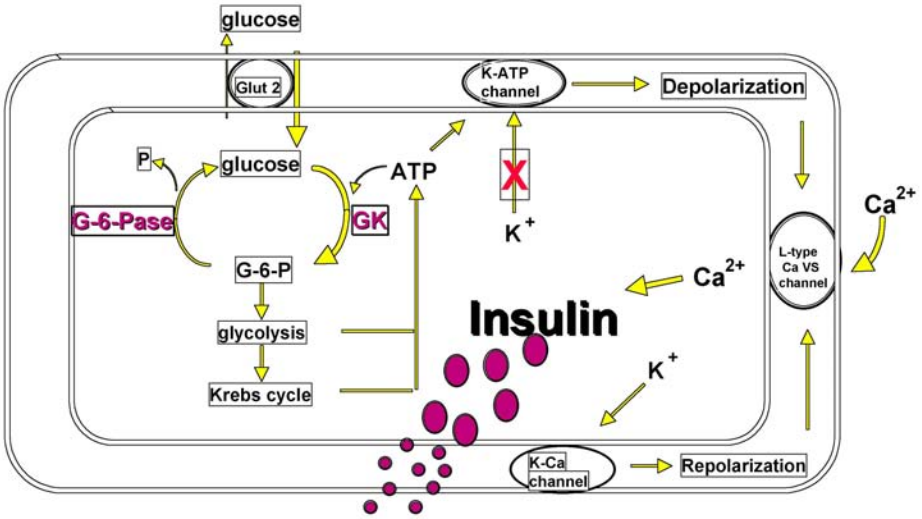

Figure 5. Current model for the mechanism of glucose induced insulin secretion from pancreatic β-cell**.**

This pathway involves direct interaction of signals from glucose metabolism with exocytosis of insulin. In the presence of diazoxide (which opens K^+-ATP channels) and high K^+ (which depolarizes the membrane) glucose can still stimulate insulin release. Such an effect is not coupled to further increase of Ca^+, but to potentiation of the stimulatory effect of Ca^+ on exocytosis.

Insulin secretion also is stimulated by amino acids, fatty acids, and gastrointestinal hormones like GLP-1 and GIP. The secretory pathway of insulin is regulated via a complex intracellular chain of events. Disturbances at any stage of the secretory process, *i.e* from transport of glucose into the cell to insulin exocytosis, can impair glucose-stimulated insulin secretion. Glucocorticoids regulate some factors involved in the secretory process of insulin as discussed below.

Role of glucose-6-phosphatase in insulin release

Glucose-6-phosphatase (G6Pase) catalyses the terminal step in gluconeogenesis, the hydrolysis of glucose-6-phosphate to free glucose in the liver and kidney (Nordlie et al., 1993; van de Werve et al., 2000). In liver, G6Pase plays an important role in regulating glucose metabolism in concert with the opposing actions of the glucose phosphorylating enzyme glucokinase (Mithieux, 1996; Nordlie et al., 1999). Hepatic G6Pase activity is the composite result of the action of at least two membrane proteins: a 36 kDa catalytic subunit and a 46 kDa G6P translocase subunit (Foster and Nordlie, 2002) The genes encoding G6Pase and G6P translocase have been cloned (Shelly et al., 1993). The promoter of the G6Pase gene contains two potential GREs (Lin et al., 1998) Injection of glucocorticoids *in vivo* increases by about 40 % the activity of G6Pase in the livers of control and adrenalectomized rats (Nordlie et al., 1965). Dexamethasone causes up to 10-fold increase in G6Pase activity (Garland, 1988) and in the levels of its mRNA in cultured hepatoma cells (Lange et al., 1994). In addition to liver and kidney, G6Pase is also present in pancreatic β-cells (Ashcroft and Randle, 1968; Taljedal, 1969). The gene encoding a G6Pase related protein, expressed specifically in pancreatic islets, has also been cloned (Arden et al., 1999). In diabetic animals, the activity of islet G6Pase is several fold higher than in normal (Khan et al., 1990a; Khan et al., 1995; Ostenson et al., 1993a). Simultaneous phosphorylation of glucose to glucose-6-phosphate, catalyzed by glucokinase, and dephosphorylation of glucose-6-phosphate to glucose, catalyzed by G6Pase, with the resulting conservation of ATP has been termed glucose cycling (Katz and Rognstad, 1976). It has been shown that islets from ob/ob mice have a markedly enhanced rate of glucose cycling. In islets from ob/ob mice, 30-40% of phosphorylated glucose was dephosphorylated, compared to 3% in islets from normal mice (Khan et al., 1990b). Islet glucose cycling was further increased in ob/ob mice treated with dexamethasone (Khan et al., 1995; Khan et al., 1992). This group has also demonstrated an increase in glucose cycling in islets of neonatally streptozotocin-induced diabetic rats (STZ-D) (Khan et al., 1990b), probably contributing to the decreased insulin secretion found in these animals.

Reduced glucose-stimulated insulin release, a feature of T2DM, can be due to enhanced expression of G6Pase, thereby enhancing glucose cycling and preventing the glycolytic flux.

Role of α_2-adrenergic receptor in insulin release

Insulin secretion is modulated by neurotransmitters which can either increase or decrease the rate of insulin release (Rasmussen et al., 1990) . Thus, α_2-adrenergic receptor (α_2-AR) agonists inhibit insulin release in humans and in experimental animals *in vivo* (Metz et al., 1978; Ostenson et al., 1988). Furthermore, α_2-AR agonists such as UK 14304, clonidine and oxymethazoline inhibit glucose-stimulated insulin release from isolated islets and insulin-producing cell lines. The inhibitory effect of α_2-AR agonists on insulin release is blocked by α_2-AR antagonists, *e.g*. yohimbine or idazoxan (Ito et al., 1995; Ostenson et al., 1988). Although three α_2-AR subtypes ($\alpha_{2A,}$ $\alpha_{2B,}$ α_{2C}) are expressed in pancreatic islets, pharmacological evidence shows that the inhibitory effect of α_2-adrenoceptors on insulin release is mediated by the α_{2A} subtype (Chan et al., 1997; Lacey et al., 1993). An altered signaling of α_2-AR in the pancreas may be related to the pathophysiology of T2DM (Surwit et al., 1992), which is characterized by impairment in insulin secretion.

Overexpression of α_2-AR attenuates basal and stimulated insulin secretion in RIN cell lines (Rodriguez-Pena et al., 1997). Moreover, transgenic mice overexpressing α_2-AR in pancreatic β-cells showed decreased glucose-stimulated insulin release, resulting in glucose intolerance (Devedjian et al., 2000). In pancreatic cell lines, α_2-AR mRNA and signaling are upregulated by GCs (Hamamdzic et al., 1995).To our knowledge no report is available whether a similar mechanism occurs *in vivo*.

11β-hydroxysteroid dehydrogenase (11β-HSD)

Two different isoenzymes of 11β-HSD have been characterized (Stewart and Krozowski, 1999) . The type 1 isoenzyme (11β-HSD-1) is a bi-directional enzyme which mainly acts as a reductase and generates active glucocorticoids from an inert 11-keto form. 11β-HSD-1 encodes predominantly low affinity NADP(H)-dependent reductase. This enzyme is expressed in the liver, lung, adipose tissue, and brain, largely localized to cells expressing glucocorticoid receptors but not mineralocorticoid receptors. The type 2 isoenzyme (11β-HSD-2) converts active glucocortoicoids to its inactive congener, hence protecting the mineralcorticoid receptor against occupancy by GCs. 11β-HSD-2 encodes a high affinity

unidirectional NAD(H)-dependent 11-dehydrogenase. Type 2 enzyme is mostly expressed in kidney and placenta. The important function of 11β-HSD-2 is to protect mineralocorticoid receptor from inappropriate activation of cortisol/corticostrone. Mice with deleted 11β-HSD-2 develop hypertension (Kotelevtsev et al., 1999).

The expression of hepatic 11β-HSD-1 was shown to be increased by dexamethasone and decreased by insulin. (Bahr et al., 2002; Bujalska et al., 1999). The physiological role of hepatic 11β-HSD-1 has been studied using 11β-HSD-1 knockout mice (Kotelevtsev et al., 1997). These mice were unable to convert inert 11-dehydrocorticosterone (11-DHC) to corticosterone *in vivo*. Furthermore, the activity of the key gluconeogenic enzymes, PEPCK and G6Pase, was not increased both after starvation and when fed with fat diet. Furthermore, these animals resist hyperglycemia upon stress. The inhibition of 11β-HSD activity by carbenoxolone and thereby reduction of intrahepatic cortisol concentration causes an increase in hepatic insulin sensitivity in man and decreases glucose production (Lindsay et al., 1996).These results demonstrate that basal 11β-HSD activity plays an important role in maintaining adequate exposure of GR to cortisol in the liver.

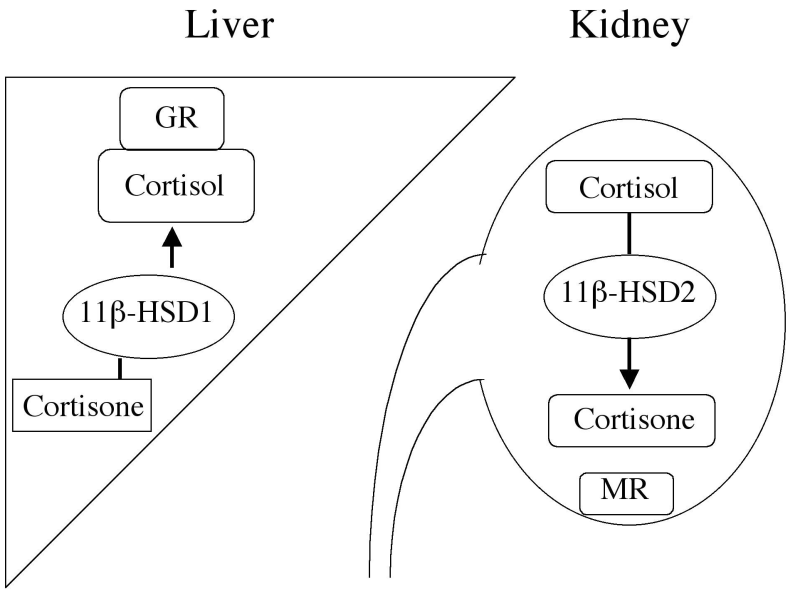

Figure 6: Contrasting effect of 11β-HSD on glucocorticoid sensitivity in liver and kidney (Adapted from Andrews and Walker, 1997)

The pathophysiological consequences of elevated levels of 11β-HSD-1 in visceral adipose tissue were recently demonstrated in mice selectively overexpressing 11β-HSD-1 in adipose tissue (Masuzaki et al., 2001). These mice exhibit increased concentrations of corticosterone in adipose tissue and develop visceral obesity that is exaggerated by a high-fat diet. Concomitantly, these mice develop an insulin resistance syndrome including diabetes and hyperlipidemia, strikingly resembling the metabolic syndrome in humans. Rask and collaborators have found upregulation in adipose tissue activity of 11β-HSD-1 in obese females (Rask et al., 2002). Therefore 11β-HSD-1 appears a key contributor to obesity and metabolic dysfunction, which may explain the clinical similarities between Cushing's syndrome and the metabolic syndrome. The circulating pool of cortisone is therefore physiologically important as a source of active glucocorticoid in sites where 11β-HSD-1 is expressed.

AIMS OF THE PRESENT STUDY

The current studies aimed to investigate:

- The effect of increased glucocorticoid sensitivity in β-cells on glucose homeostasis.
- The effect of glucocorticoids on β-cell function and insulin release.
- The molecular mechanisms behind the inhibitory effect of glucocorticoids on insulin release, particularly the role of glucose-6-phosphatase (G6Pase) and α_2-adrenergic receptors (α_2-AR).
- The possible mediatory effect of 11β-hydroxysteroid dehydrogenase type 1 in pancreatic β-cells on insulin release.

METHODOLOGY

Transgenic mice

Due to ethical and practical reasons, studies on β-cells from Type-2 patients are difficult to perform. Over the past decade, transgenic mice have become one of the most useful tools in the study of the diabetes. By using specific promoters one can drive tissue- specific expression of the transgene, *e.g*. by producing transgenics carrying a transgene linked to the insulin promoter, one can limit expression of the transgene to pancreatic β-cells.

The most commonly used method for making transgenic animals is by direct injection of foreign DNA into a fertilized egg. Embryos are then reimplanted into pseudopregnant female mice and transgenic pups are born after 19 or 20 days of gestation. The transgene integrates randomly into the genome and can be transfected to the next generation via germ cells.

In our studies the full-length coding region of the rat GR cDNA was fused to the (-410/+1) promoter region of the rat insulin gene I and an SV40 polyadenylation signal. A 3.6-k.b PvuII linear fragment of this construct was isolated and introduced into male pronuclei of zygotes obtained from F1 (C57BL/6J X CBA/J) mice using standard microinjection technique.

ob/ob mice

The ob/ob mouse, an animal model of Type-2 diabetes, exhibits hyperglycemia, hyperinsulinemia and obesity (Westman, 1968) . The animals are already obese at 3-4 weeks of age, and a significant difference between lean and obese animals in blood glucose and plasma insulin concentration is observed at 4-5 weeks. The hyperglycemia and hyperinsulinemia increase and the highest mean blood glucose and insulin values are obtained in 3-5 month-old obese mice. Islets of ob/ob mice are often used to study β-cell functions because the pancreatic islets of ob/ob are greatly enlarged and these islets contain about 90% β-cells. Insulin release from isolated islets of ob/ob mice is high at basal glucose levels (5.5 mM) and is stimulated only 2-3 fold by 16.7 mM glucose (Khan and Efendic, 1995), indicating impaired insulin response to glucose.

Glucose Tolerance Test and Intraperitoneal insulin tolerance test (IPITT)

Intravenous Glucose Tolerance Test (IVGTT) and Intraperitoneal Glucose Tolerance Test (IPGTT) were performed in overnight fasted animals. Blood was collected before (0 min) and after the injection of glucose (2 g/kg body weight) at different times either from the orbital plexus (3 months old) and tail (12-15 months old).

IPITT was performed in overnight fasted animals. At -10 min, blood glucose levels were measured prior to injection of insulin (0.25 U/kg body weight) Ten min after administration of insulin, blood glucose was measured (0 min) and a bolus of glucose (1 g/kg body weight i.p.) was given to the animals. Blood was then collected at an interval of 15, 30, 60, 90 and 120 min to determine glucose concentrations.

Experiment with isolated islets

Mice were killed by decapitation, and the pancreata were removed, cleaned of fat tissue, and washed in Hanks' solution. Each pancreas was cut into small pieces and placed in a scintillation glass bottle with 2.5 - 3 ml of Hanks' solution containing 6-8 mg of collagenase. The tissue was digested at 37^{o} C for 10-12 min under continuous shaking (150 strokes/min). The sediment was washed several times with the cold Hanks' solution and the islets from the sediment were collected using a glass pipette under stereomicroscopy. The islets were either used directly or preincubated with different substances before the measurement of enzyme activity, insulin release and ligand binding.

For the measurement of insulin release .in pancreatic islets, static incubations were used. Islets were preincubated for 45 min at 3.3 mM glucose at 37 °C prior to incubation with different glucose concentration and appropriate test substances. Incubation of islets was performed at 37 °C for 1 h under continuous shaking. A part of the incubation medium was removed and stored at – 20 °C for the measurement of insulin release by radioimmunoassay (RIA) with the addition of charcoal to separate free and bound antibody and by using rat insulin as a standard. (Herbert et al., 1965). The incubation was terminated by cooling the incubation tubes on ice .

Measurement of glucose-6-phosphatase activity and glucose cycling

Glucose cycling was measured from the incorporation of ^{3}H at position 2 of glucose after incubating the islets with $^{3}H2O$ and unlabeled glucose. The measurement of G6Pase is

based on the assumptions that before each molecule of glucose-6-phosphate is hydrolyzed to glucose, hydrogen from the medium equilibrates with the hydrogen bound to carbon of glucose-6-phosphate, because of the rapid equilibration between glucose-6-phosphate and fructose-6- phosphate**.**

G6Pase activity was measured in permeabilized and sonicated islets, reflecting translocase and hydrolase activity (Khan et al., 1995). The activity of G6Pase in intact ER membrane estimates the translocase function, whereas complete disruption of the membranes provides a measure of the hydrolase component. Glucose-6-phosphatase activity was equal to the amount of glucose-6-P hydrolyzed to glucose. For the measurements of glucose oxidation and glucose utilization, islets were incubated in an identical manner to that for the experiments using $^{3}H_2O$, except that instead of incubating with $^{3}H_2O$, the islets were incubated either with [U-^{14}C] glucose or [5-^{3}H] glucose. Oxidation of glucose was measured from the yield of $^{14}CO_2$ from [U-^{14}C] glucose and glucose utilization from the yield of $^{3}H_2O$ from [5-^{3}H] glucose.

Transient cell transfection of HIT-cells and pancreatic islets

The expression and regulation of a gene can be studied by introducing it into cultured cells. Genetic reporter systems have contributed greatly to the study of eukaryotic gene expression and regulation. Typically, a reporter gene is joined to a promoter sequence in an expression vector that is transfected into cells. Following transfer, the cells are assayed for the presence of the reporter by the enzymatic activity of the reporter gene. Several reporter genes have been described for many molecular biologic applications. Commonly used systems for monitoring genetic activity in eukaryotic cells include chloramphenicol acetyltransferase (CAT), β-galactosidase, firefly luciferase, alkaline phosphatase (AP) and green fluorescent protein (GFP). We used luciferase and AP in our transfection assays.

Hamster HIT-β cells were grown in RPMI 1640 medium supplemented with 10% FBS, 2 mM L-glutamine, 100 μU/ml penicillin, and 100 μg/ml streptomycin at 37 °C 5% CO_2. Cells were transiently transfected with 6 μg GR expression plasmid DNA by the calcium phosphate coprecipitation technique. The mixture contained 1 μg mouse mammary tumor-virus-alkaline-phosphatase (pMMTV-AP) reporter plasmid, 0-2.5 μg GR expression plasmid, and 2.5 μg pGEM plasmid as carrier DNA. After transfection, cells were grown in the absence or presence of 1 μM dexamethasone for 48 h, and alkaline phosphatase was determined.

In experiments with ob/ob islets, pancreatic islets were isolated and were resuspended in the same medium as above. Cells were incubated at 37 °C in 5% CO_2. and transfected by LipofectAMINE plus with 400 ng of a GRE-tk-luk reporter plasmid. The following day, fresh medium was added containing either 100 nM 11-DHC or 100 nM corticosterone with or without 5 µM CBX. After 16 h, cells were lysed and analyzed for luciferase activity.

Ligand binding Assay

Islets were preincubated for 60 min in KRB at 3.3 mM glucose, after which triplicate batches of 25 islets in 100 µl KRB (without glucose) were placed into microfuge tubes containing 150µl of a mixture of dibutyl- and dinonylphthalate (10:3), layered above 40 µl 6mol/l urea. Then 50µl of KRB containing 50 nCi [^{3}H]UK-14304 (specific activity 75 Ci/mmol; NEN) and 50 nCi [^{14}C] sucrose (specific activity, 0.67 Ci/mmol; NEN) were added to the tubes. The final concentration of [^{3}H]UK14304 in the medium was 4.5 x 10^{-9} M except in experiments used for Scatchard analysis, in which serial dilutions (1:2) from 9x10^{-9} M to 2.8x 10^{-10} was used. The tubes were incubated for 40 min (unless indicated otherwise) at 37^{o}C and then centrifuged for 15 sec. The lower section of each tube containing the islets was cut off and placed in a liquid scintillation vial, 5 ml of scintillation fluid was added, and radioactivity in the ^{3}H and ^{14}C peaks was determined by liquid spectrometry. Total binding was calculated as the radioactivity remaining in the islets after subtraction of radioactivity in the sucrose space. Specific binding was defined as the difference between total binding and non-specific binding obtained in the presence of 3.3 x 10^{-6} M unlabelled UK14304.

TdT- mediated dUTP nick-end labelling assay for apoptosis

Extensive DNA degradation is a characteristic event which occurs in the early stages of apoptosis. Cleavage of DNA may yield double-stranded, single strand breaks or low molecular weight DNA fragments. Those DNA strand breaks can be detected by enzymatic labelling of the free 3' –OH termini with modified nucleotide biotin-dUTP and labelling enzyme terminal deoxynucleotidyl transferase (TdT). The end labeling method has been termed TUNEL (Terminal Transferase-mediated dUTP Nick End Labeling) and is a common method for study of apotosis.

TUNEL was performed on 4 µm thick paraffin sections of formaldehyde-fixed pancreatic tissue, using an *in situ* cell death detection kit, according to the manufacturer's instructions as described earlier (Heiden et al., 2000) The sections were counterstained for 10 min with

4'-6-diamino-2-phenylindole, (DAPI) solution (10 µmol DAPI in 800 mmol disodiumhydrogenphosphate) and embedded with Vectashield. The stainings were analyzed with a fluorescence microscope.

Tissue processing for immunocytochemistry

Pancreatic specimens were fixed by immersion overnight in Stefanini's fixative (2% formaldehyde and 0.2% picric acid in phosphate buffer, pH 7.2), rinsed repeatedly in Tyrode solution enriched with sucrose (10% w/v), and frozen on dry ice. Sections (10 µm thickness) were cut in a cryostat, collected on chrome alum-coated slides and then processed for immunocytochemistry. The primary antibodies were: for insulin detection, an anti-unconjugated human proinsulin antibody raised in guinea pig (code 9003, dilution 1:1280; Euro-Diagnostica, Malmö, Sweden), for glucocorticoid receptor detection, a mAb against rat GR raised in mouse (Okret et al., 1984), for glucagon detection, an anti-protein-conjugated glucagon antibody raised in rabbit (code 7811, dilution 1:5120, Euro-Diagnostica), and for detection of GLUT-2, a rabbit anti-rat GLUT-2 antiserum (Chemicon International Inc., Temecula, Ca, dilution 1:200). The sections were then incubated for 1 h at room temperature with a secondary antibody, coupled to fluorescein isothiocyanate (FITC) with specificity for immunoglobulin G (IgG) (Euro-Diagnostica, dilution 1:80) of the primary antibody and examined in a fluorescence microscope.

PCR and RT-PCR

Transgenic animals were identified by PCR on DNA from tail biopsies. Total RNA from pancreatic islets was isolated by Trizol reagent (Invitrogen-Life Technologies) according to the manufacturer's instructions. Expression of the α_{2A}-AR mRNA in pancreatic islets isolated from control and transgenic mice was examined by semi-quantitative RT-PCR. 11β-HSD-1 expression in ob/ob pancreatic islets and human islets was detected by RT-PCR. Primers for performing PCR and RT-PCR are presented in Table 1.

Table 1. Primer sequences for PCR and RT-PCR. TG= transgenic mice, INS-GR = transgenic mice with overexpression of GR in β-cells, α_2-AR= α_2-Adrenergic receptor, R.L30= Ribosomal protein L30, 11β-HSD-1= 11β-Hydroxysteroid dehydrogenase type 1, GAPDH= glyceraldehyde-3-phosphate dehydrogenase

Gene	sense primer	antisense primer
INS-GR (TG mice)	TGATTGTGCCTGTGAACTGCTT	CTCCTCCCCTCAGGCTTTTAT
α_2-AR (TG mice)	AGCTCCCCAAAACCTCTTCCT	CCAGCGCCCTTCTTCTCTATG
R. L30 (TG mice)	AAGTGGGAAGTACGTGCTGG	CACCAGTCTGTTCTGGCATG
11β-HSD1 (ob/ob mice)	TTATGAAAAAATACCTCCTCCC	CTTTGATCTCCAGGGCGCATTC
11β-HSD1 (human)	ATGCTCCAAGGAAAGTCATTGT CAGGGGCC	CTACTTGTTTATGAATCTGTC CATATTCA
GAPDH (ob/ob mice and human)	TGAAGGTCGGGTGTCAAC	CATGTAGGCCATGAGGTC

11β-hydroxysteroid dehydrogenase activity

Oxidative and reductive conversions of corticosterone and 11-dehydrocorticosterone (11-DHC) respectively, were analyzed by incubating intact islets in the presence of (^{3}H) corticosterone or (^{3}H) dehydrocorticosterone. In kinetic experiments, islets were incubated at different concentrations of unlabeled steroid, ranging from 10 to 500 nM and supplemented with tracer steroid. After incubation reaction mixtures were extracted with a 5-fold volume of ethyl acetate after the addition of excess unlabeled corticosterone and 11-DHC. The organic phase was dried under nitrogen, redissolved in methanol, transfected to silica TCL plates, and steroids were seperated using a mobile phase of dichloromethance/acetone (4:1, v/v). Substrates and products were cut out and eluted into scintillation fluid, and the fractional conversion into product was determined by LSC.

RESULTS AND DISCUSSION

GCs directly inhibit insulin release both in vivo and in vitro (Papers I, II, III)

GSs exert a marked diabetogenic effect in man and animals. It is well characterized that this effect is due to enhanced gluconeogenesis in the liver and decreased glucose uptake in muscle and adipose tissue. Since GCs decrease glucose-stimulated insulin release in isolated perfused pancreas and in isolated islets, these inhibitory effects may also contribute to the diabetogenic action of the hormone. To investigate this hypothesis *in vivo*, we have generated transgenic mice with an increased β-cell GC sensitivity by overexpressing the rat GR in β-cells under the control of the rat insulin promoter I (papers I, II, III).

Transgenic and control mice had similar body weight and blood glucose levels. Expression of transgene was restricted exclusively to the β-cells of transgenic mice. No sex differences were observed in blood glucose and plasma insulin concentrations. Plasma corticosterone levels did not differ significantly between transgenic and control mice. Islet DNA, protein and insulin content were similar in transgenic and control mice. At the age of 3-4 months, transgenic animals had normal fasting blood glucose but decreased glucose tolerance (paper 1). In an intravenous glucose tolerance test (IVGTT), using a glucose dose of 0.5 g/kg body weight a decreased glucose tolerance was observed in transgenic mice compared to controls. Similar results were found with an intravenous dose of 2 g/kg-body weight. Transgenic mice exhibited significantly higher blood glucose concentration than control mice at 60 min after intravenous injection of glucose. Measurement of plasma insulin levels at 5 min after intravenous glucose load demonstrated a markedly decrease in acute insulin response in the transgenic mice. After 60 min, plasma insulin concentrations were not different.

The acute insulin responses to intravenous glucose load were abolished in the transgenic animals *in vivo*, while glucose tolerance was decreased only moderately. This mild glucose intolerance could be the result of increased insulin sensitivity in the peripheral tissues, as suggested by the lower fasting plasma insulin levels in some transgenic mice. Interestingly, about 20% of healthy humans also exhibit a markedly decreased insulin response to glucose, but maintain almost normal glucose tolerance due to enhanced insulin sensitivity (Luft and Efendic, 1979). It has been demonstrated that many individuals with low insulin

response (LIR) develop impaired glucose tolerance after a short treatment with GCs due to their inability to increase insulin release in response to hyperglycemia (Rull et al., 1970; Wajngot et al., 1992). Extending our results with the transgenic mice, it may be suggested that impaired cortisol OGTT in LIR humans reflects increased GC sensitivity in the β-cells. Following the natural history of glucose impairment in transgenic mice, we found that at 12-15 months the transgenic mice had significantly higher fasting blood glucose levels and further deteriorated glucose tolerance (paper III). They developed hyperglycemia both in the fed and overnight-fasted states. The basal and stimulated insulin secretion was significantly decreased in the old transgenic mice both under *in vivo* and *in vitro* conditions. Importantly, glucose elimination after i.p. insulin administration did not differ between the groups, suggesting that insulin sensitivity was normal in transgenic mice. Therefore, it seems that the development of manifest diabetes in these animals is due to a direct inhibitory effect of GCs on insulin release, and is not related to reduction in insulin sensitivity. Similarly the acute administration of dexamethasone in healthy humans impairs oral glucose tolerance without significantly increasing insulin resistance (Scheneiter, Tappy, 1998).

Mechanisms behind the inhibitory action of GCs on β-cells (Paper II, III)

The mechanisms behind the progressive deterioration of glucose tolerance in transgenic mice are not known. However, data presented in paper II and III shed some light on the mechanisms behind the impaired insulin release in transgenic mice. First, the activity of islet G6Pase was increased in transgenic mice. Secondly, we found a significantly higher density of α_2-AR in the islets of transgenic mice compared to controls. Furthermore, expression of α_2-AR mRNA was increased in islets from transgenic mice. The mechanism by which GCs directly inhibit insulin release in β-cells is likely to involve up- and down-regulation of genes important for the glucose-stimulated insulin secretion. G6Pase is one of the genes that has been reported to be regulated by GC in pancreatic islets and that may be related to insulin secretion. Experiments with islets from transgenic mice showed that islet G6Pase activity was 70% higher, glucose cycling was increased threefold and insulin release was 30% lower in these animals. These results are in agreement with the previous reports from some animal models of T2DM, namely ob/ob mice and GK rats (Khan et al., 1995; Ostenson et al., 1993a). Islet glucose utilization and oxidation in TG mice were comparable to those in control animals, demonstrating that the enhanced glucose flux through G6Pase did not alter glucose utilization.

Results from study II indicate that GCs enhance islet G6Pase activity by acting directly on β-cells. Since an increased G6pase activity results in increased ATP consumption, a linkage between the increase in G6Pase activity and decreased insulin release in TG animals is possible.

Previously, increased G6Pase activity has been reported in islets of ob/ob mice after dexamethasone treatment (Khan et al., 1995). A glucocorticoid responsive element has been identified in the human G6Pase gene promoter, suggesting that the effects of GCs on G6Pase are directly mediated by GCs (Schmoll et al., 1996). According to Iizuka *et al.,* stable overexpression of the G6Pase catalytic subunit attenuated glucose sensitivity of insulin release from a mouse pancreatic β-cell line, MIN6 (Iizuka et al., 2000). The degree of impairment in insulin release correlated positively with the level of overexpression. In clones with a 24-fold G6Pase increased activity, glucose-stimulated insulin release was completely abolished, while the basal insulin secretion was not altered. In another experiment in mouse pancreatic INS-1 cell lines, a fourfold increase in G6Pase activity resulted in a proportional 30% decrease in glucose-stimulated insulin release (Trinh et al., 1997), indicating that overexpression of G6Pase catalytic subunit significantly affects insulin secretion in islet β-cells.

Since β-cells express α_2-ARs and GCs upregulate α_2-AR expression and signaling in β-cell lines (Hamamdzic et al., 1995) we investigated the possibility whether inhibition of insulin secretion in TG mice involves a signaling pathway via α_2-AR. In paper III, we found a significantly higher density of α_2-ARs in islets from transgenic mice, as revealed by binding studies using the α_2-AR agonist [^{3}H]UK-14304 as a ligand. Furthermore, analysis by RT-PCR also showed increased expression of α_2-ARs mRNA in transgenic mouse islets.The incubation of islets with benextramine, a selective antagonist of α_2-AR, completely restored the insulin response to glucose in isolated islets from transgenic mice, while it had no effect on control islets. These findings suggest that the increased expression and binding by α_2-ARs are involved in the inhibition of insulin secretion in transgenic mice. In the present study, not only glucose-, but also arginine-induced insulin release was reduced in islets of transgenic mice. Since catecholamines also inhibit arginine-induced insulin release (Efendic et al., 1978), this finding is compatible with the hypothesis that the increased signaling by α_2-AR is involved in the regulation of insulin secretion in the β-cells

of transgenic mice. These results are supported by other studies showing that GGs increase the expression α_2-AR mRNA and upregulate its signaling in β-cell lines (Hamamdzic et al., 1995). The inhibition of insulin release by dexamethasone was prevented when cultured islets were preincubated with pertussis toxin (Lambillotte et al., 1997), which inhibits G proteins involved in the transduction of α_2-AR signaling in the β-cell. Further evidence for the involvement of G-proteins in α_2-AR signaling in the β-cell was provided by Lang *et al*. Using HIT-T15 cells, they demonstrated that the activation of α_2-ARs caused pertussis toxin-sensitive inhibition of insulin secretion (Lang et al., 1995).

We had reported previously that the concentration of UK 14304 required to cause a 50% reduction of insulin release from transgenic mice islets was 10 fold lower than the concentration of agents required for a similar response in control mice (Ling et al., 1998). Benextramine, an inhibitor of α2-AR, normalized both insulin release and the activity of G6Pase in transgenic mice. Moreover, the α_2-AR agonist, UK-14304 increased G6Pase activity in transgenic but not in wild type animals (Ling et al., 1998). This suggests that GCs modulate G6Pase through α_2-AR signaling.

Since GC induces apoptosis of β-cells (Pick et al., 1998), we speculated that apoptotic cell death is also involved in the progressive inhibition of insulin release and development of diabetes in transgenic mice (paper III). However, the development of the diabetic state in transgenic mice did not involve apoptosis of β-cells, as a TUNEL assay did not show any evidence of apoptotic nuclei in islets from these mice. In accordance with our results, dexamethasone treatment of partially pancreatectomised hyperglycemic rats did not cause any apoptotic changes in the β-cells (Laybutt et al., 2002).

Progression from impaired glucose tolerance to manifest diabetes was not associated with insulin resistance or morphological change in pancreatic islets. Glucose elimination after i.p. insulin administration did not differ between the groups, suggesting that insulin sensitivity was not reduced in transgenic mice. Morphological studies of pancreatic islets revealed that the islet cytoarchitecture was intact in the transgenic animals, as judged by the normal distributions of insulin- and glucagon- producing cells. Impaired insulin secretion from transgenic islets was not due to decreased insulin content, which was similar to controls in all experiments (paper I-III).

The novel finding in paper III is that transgenic mice become diabetic over a period of 12-15 months. Hence, increased GR sensitivity in the pancreatic β-cells is sufficient for development of the diabetic state and our results suggest that this is due to overexpression of α_2-ARs.

Overview of results (papers I-III)

Table 2: Summary of results from the studies I-III as compared to control animals. The symbols used in the table are as follows; ↑ = increase, ↓ = decrease, ↔ = no change, N.D. = not determined. GSIR= glucose stimulated insulin release

	3-4 months age	**12-15 months age**
Body weight	↔	↔
Corticosterone levels	↔	↔
Islet DNA	↔	↔
Insulin content	↔	↔
Islet protein content	↔	↔
Non-fasting glucose	↔	↑
Fasting glucose	↔	↑
Glucose after GTT:		
(5 min)	↔	↔
(60 min)	↑	↑
(120 min)	↑	↑
Fasting plasma insulin	↔	↓
Plasma insulin after GTT:		
(5min)	↓	↓
(60 min)	↓	↓
GSIR in islets (16.7 mM glucose)	↓	↓
Glucose utilization	↔	N.D.
Glucose oxidation	↔	N.D.
G6Pase activity	↑	N.D.
Glucose cycling	↑	N.D.
GSIR in islets + benextramine	↑	↑
GSIR in islets + UK 14304	↓	↓
α_2-AR density and expression	N.D.	↑
Apoptotic changes	N.D.	↔
Morphological changes	↔	↔

11β-HSD-1 is an amplifier of GC action in the β-cells of ob/ob mice (Paper IV)

The presence and functional activity of this enzyme in pancreatic islets has not yet been studied. In the present study (IV) we have found evidence for activity and expression of 11β-HSD-1 in pancreatic islets.

Increasing amounts of substrate for 11β-HSD-1 resulted in a dose-dependent production of corticosterone. Expression of 11β-HSD-1 mRNA was detected by RT-PCR in islets isolated from ob/ob mice and also from human tissues. Glucose stimulated insulin secretion from pancreatic islets in a concentration dependent manner. This secretion was inhibited with 11-dehydrocorticosterone**.** We evaluated the inhibitory effect of 11-DHC on insulin release in the presence and absence of the 11β-HSD inhibitor carbenoxolone (CBX). While in the absence of CBX, 11-DHC markedly inhibited insulin release, a reversal of this effect was noted in the presence of CBX, indicating an important role of 11β-HSD-1 in the regulation of insulin release. These novel findings suggest that 11β-HSD-1 is present in pancreatic islets and plays a role in insulin release. The relevance of this finding to disease processes, such as GC-induced T2DM, remains to be explored. Furthermore, enzymatic properties of 11β-HSD-1 support the concept and importance of cell-specific activation of GR ligands from inactive glucocorticoid hormone precursors.

The results of this study have several important consequences. First, the principle that 11β-HSD-1 is an amplifier of glucocorticoid action has now been extended in all tissues critically involved in glucose and carbohydrate metabolism and homeostasis (i.e. liver, adipose tissue, pancreatic islets, and muscle). In all cases, the tissue specific effects are related to intracellular reductive activation of 11-oxo GCs via 11β-HSD-1 to active GR ligands, followed by tissue specific induction or repression of GC controlled genes. This concept implies that circulating cortisone or 11-dehydrocorticosterone, which is not bound to plasma CBG, displays favorable pharmokinetics and can freely enter the cell, in contrast to cortisol and corticosterone. These are tightly bound to CBG, thus indicating that the level of free 11-OH-GC is low. This clearly points to a novel role of 11-oxo glucocorticoids as pre-hormones, which can be activated in a tissue-specific manner via 11β-HSD-1 to its receptor ligand. Finally, the present study suggests that the resistance to hyperglycemia in 11β-HSD-1 knockout mice provoked by obesity or stress was probably partially mediated by improved insulin release, in parallel with attenuation of gluconeogenesis.

Summary

A schematic pathway by which GCs can influence insulin secretion in β-cells is shown in figure 7.

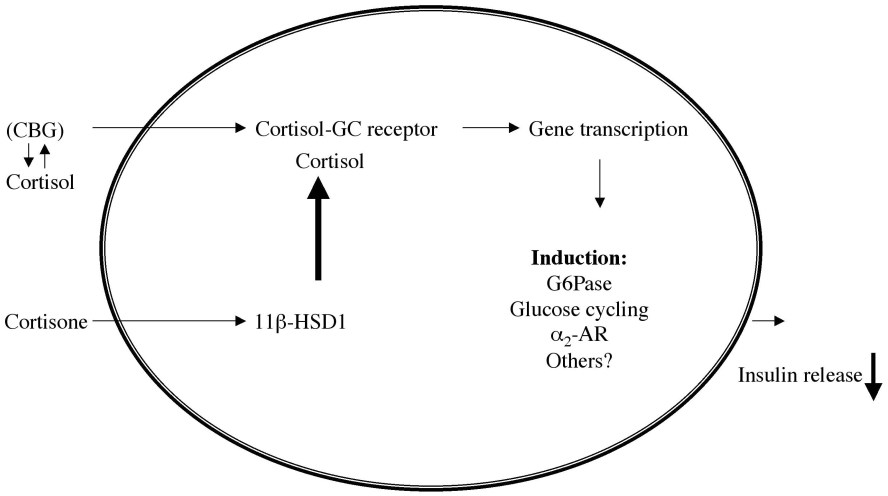

Figure 7: Schematic pathways by which glucocorticoids can influence insulin secretion

GENERAL DISCUSSION

Physiological and pathophysiological implications of GC mediated suppression of insulin release

The results presented in this thesis show that GCs, in addition to their well-known diabetogenic effects, also exert a direct suppressive effect on pancreatic β-cells, which results in a marked inhibition of insulin release. The physiological role of the direct inhibition of glucose-stimulated insulin release by GC is not clear. However, it could be an important regulatory mechanism during stress, allowing a transient attenuation of the insulin response to hyperglycemia in order to ensure sufficient glucose for the needs of the brain. This β-cell-directed action of GCs thus operates in concert with other well-established effects of these steroid hormones, such as decreased glucose uptake and increased hepatic glucose production. Importantly, we demonstrated that the prolonged increased GR sensitivity in the pancreatic β-cells is sufficient for development of the diabetic state. This would support the notion of a role for stress in the development of T2DM, which thus may be mediated by increased GC on islet function via increased G6Pase activity and expression of β-cell α_2-ARs. The involvement of other factors can not be excluded. Another important aspect of the results presented here is the novel finding suggesting that 11β-HSD-1 is present in pancreatic islets and participate in inhibition of insulin secretion by GC. Enzymatic properties of 11β-HSD-1 support the concept and importance of cell-specific activation of GR ligands from inactive GCs precursors.

Clinical and Pharmacological implications

These results could have important clinical implications. We believe that these direct effects of GC on the β-cell may diminish the survival of transplanted islets. Thus, the harmful effect of GCs, used for immunosuppression on β-cells, may be a very important factor, as recently noted by Shapiro *et.al*, who reported 100% success for an average of 1 year in 7 patients, when immunosuppressive therapy did not include glucocorticoids (Shapiro et al., 2000). For future use of GCs in the clinic it would be a great improvement if one could develop novel synthetic GCs with less side effects. It is known that persons who have diminished β-cell functions or are low-insulin responders are predisposed to develop overt diabetes during GC therapy (McConnell et al., 2001; Wajngot et al., 1992). The results presented in this thesis have important pharmacological implications as well. In recent years several investigators have searched for selective inhibitors of GCs and glucocorticoid signaling in order to reduce diabetogenic effects of GCs (Kurukulasuriya et

al., 2003). A number of promising therapeutic targets for inhibition of glucocorticoid signaling have been reported. Barf *et. al* have reported the compound BVT.2733 as a selective inhibitor of murine 11β-HSD 1(Barf et al., 2002). Treatment of KKA^y mice, a hyperglycemic mice model, with BVT.2733 results in lowering of blood glucose and serum insulin concentrations (Alberts et al., 2002). Administration of BVT.2733 also reduced hepatic concentrations of mRNA encoding PEPCK and G6Pase. However, these investigators have not reported on the effect of this selective inhibitor in tissues other than liver. However, this data suggests the idea of inhibition of 11β-HSD-1 as an interesting approach for T2DM treatment. It is important to mention that it has been reported that down regulation of 11β-HSD 1 in the liver is one of the mechanisms of antidiabetogenic effects of thiazolidinediones (Berger et al., 2001) .

More recently Abbott Laboratories and Karo Bio AB announced that they have identified a novel, first-in-class compound, A-348441, for the treatment of T2DM. Currently in preclinical study, A-348441 targets the GR in the liver.

In preclinical animal studies, A-348441 normalizes blood glucose levels and has beneficial effects on elevated lipids in diabetic, dyslipidemic animals. In multiple species, A-348441 significantly reduces hepatic glucose output with secondary improvements in insulin sensitivity. Although GRs are present in a variety of tissues, A-348441 is pharmacologically selective for GR in the liver, thereby minimizing potential systemic side effects associated with this drug target (*www.karobio.se/presslease*).

CONCLUSION

1) Glucocorticoids directly inhibit insulin release *in vivo* and pancreatic β-cells are important targets for the diabetogenic effects of glucocorticoids.

2) Glucocorticoids stimulate islet G6Pase activity and glucose cycling by acting directly on the β-cells.

3) Increased GR sensitivity in pancreatic β-cells is sufficient for the development of the diabetic state and our results suggest that overexpression of α_2 –ARs play an important role in this context.

4) The inhibitory effect of glucocorticoids on β-cells is partly mediated by 11β-HSD-1 through local production of active glucocorticoids.

5) Antagonism of glucocorticoids and their signaling pathways are promising therapeutic targets for treatment of Type 2 diabetes mellitus.

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