LUND UNIVERSITY

Mechanisms underlying lipotoxicity and glucotoxicity in pancreatic islets

Meidute, Sandra

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Sandra Meidutė-Abaravičienė

Doctoral Thesis

LUND UNIVERSITY

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Abstract:

Although it has been known for many years that long-term exposure of pancreatic islets to a high level of free fatty acids (FFA) or glucose negatively modulates islet hormone release, the identities of signals mediating these effects are not yet fully clarified. The overall objective of this study was to investigate signal transduction pathways involved in FFA- and glucose-exerted effects on β-cell function during both short-term as well as long-term exposure. The data presented here confirmed that the G-protein coupled receptor 40 (GPR40) is a functional FFA receptor expressed in pancreatic islets. We now show that both the acute stimulatory action of palmitate on IP_3-generation, increased $[Ca^{2+}]_i$ and insulin release as well as the long-term effects of palmitate on the activation of MAPKs (SAPK/JNK and p38), iNOS expression and caspase-3 activity is mediated *via* activation of GPR40. A modulated expression of GPR40 in the islets *i.e.* overexpression (fa/fa rats) was accompanied by an exaggerated secretion of insulin, glucagon and marked suppression of somatostatin release in response to palmitate. In contrast, a low expression pattern of islet GPR40 (GK rats) was associated with a negligible hormone response to palmitate. The islet expression of iNOS seems to be, at least in part, a common signalling pathway in both lipotoxicity and glucotoxicity. Rosiglitazone (ROZ) effectively counteracted the deleterious effects of palmitate but not that of high glucose on the islet function. Glucose-induced expression of iNOS with subsequent β-cell dysfunction was markedly counteracted by the imidazoline derivative RX871024.

We conclude that, in addition to mediating acute stimulatory effects of FFA on insulin release, GPR40 is also an important mediator of dysfunctional effects of FFA overtime. The adverse effects of FFA and high glucose on the islet function involve different or at least partially different signal systems and the FFA signalling is counteracted by ROZ at GPR40, suggesting the thiazolidinediones are protective against β-cell lipotoxicity.

Key words Pancreatic β-cell, GPR40, cellular signalling, hormone secretion, cell stress, diabetes		
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If I began to think that I know, then I'd stop immediately.
If I say I know nothing, then that means I am on my way…

Vilius Orvidas

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ORIGINAL PAPERS

The thesis is based upon studies reported in the following papers, referred to in the text by their respective Roman numerals:

I. Palmitate-induced β-cell dysfunction is associated with excessive NO production and is reversed by thiazolidinedione-mediated inhibition of GPR40 transduction mechanisms Sandra Meidute Abaraviciene, Ingmar Lundquist, Juris Galvanovskis, Erik Flodgren, Björn Olde and Albert Salehi. *PLoS ONE, Volume 3, Issue 5, e2182, May14, 2008*

II. Rosiglitazone counteracts palmitate-induced β-cell dysfunction by suppression of MAP kinase, iNOS and caspase 3 activities Sandra Meidute Abaraviciene, Ingmar Lundquist and Albert Salehi *Cell.Mol.Life Sci. Birkhäuser Verlag, Basel, June14, 2008*

III. Palmitate-stimulated hormone secretion in relation to GPR40 expression in pancreatic islet of spontaneous obesity and type 2 diabetes in rats
Sandra Meidute Abaraviciene, Ingmar Lundquist, Stefan Amisten, Björn Olde and Albert Salehi *Manuscript, 2008*

IV. Excessive islet NO generation in type 2 diabetic GK rats coincides with abnormal hormone secretion and is counteracted by GLP-1 Albert Salehi, Sandra Meidute Abaraviciene, Javier Jimenez-Feltström, Claes-Göran Östenson, Suad Efendic and Ingmar Lundquist *PLoS ONE, Volume 3, Issue 5, e2165, May14, 2008*

V. Insulinotropic effects of an imidazoline derivative include a marked suppression of mouse islet NO generation Sandra Meidute Abaraviciene, Henrik Mosen, Ingmar Lundquist and Albert Salehi *InPress, Acta Physiologica(Oxf). September 1, 2008*

List of publications not included in this thesis

I. Abnormally decreased NO and excessive CO production in islets of the leptin-deficient ob/ob mouse might explain hyperinsulinemia and islet survival in leptin-resistant type 2 obese diabetes Javier Jimenez-Feltstrom, Sandra Meidute Abaraviciene, Ragnar Henningsson, Albert Salehi and Ingmar Lundquist. *Submitted*

II. Uridine diphospate (UDP) stimulates insulin secretion by activation of $P2Y_6$ receptors Fariborz Parandeh, Sandra Meidute Abaraviciene, Stefan Amisten, David Erlinge and Albert Salehi *BBRC 6;370(3):499-503, Jun 2008*

III. GPR40 is expressed in glucagon producing cells and affects glucagon secretion Erik Flodgren, Björn Olde, Sandra Meidute Abaraviciene, Maria Sörhede-Winzell, Bo Ahren and Albert Salehi *BBRC 2; 354(1):240-5, Mar 2007*

ABBREVIATIONS

AC	Adenylate Cyclase
ACh	Acetylcholine
ADP	Adenosine diphosphate
ATP	Adenosine Tri-Phosphate
BSA	Bovine serum albumin
$[Ca^{2+}]_I$	cytoplasmic free Ca^{2+} concentration
cAMP	cyclic Adenosine Monophospate
CCK	Cholecystokinin
DAG	Diacylglycerol
DMEM	Dulbecco's modified Eagle's medium
DTT	Dithiothreitol
GIP	Glucose-dependent insulinotropic peptide
GPR40	G protein-coupled receptor 40
GRP	Gastrin-releasing peptide
GSIS	Glucose Stimulated Insulin Secretion
GK rat	Goto-Kakizaki rat
GLP-1	Glucagon-Like Peptide-1
GTP	Guanosine triphosphate
HPLC	High Performance Liquid Chromatography
HEPES	Hydroxyethyl-piperazineethanesulfonic acid
FFA	Free Fatty Acid
IDF	International Diabetes Federation
IFN-γ	Interferon-gamma
IL-1β	Interleukin-1 beta
IP_3	Inositol Triphophate
K_{ATP} channel	ATP-dependent potassium channel
KRB	Krebs-Ringer bicarbonate buffer
L-NAME	N^G-Nitro-L-Arginine-Methyl-L-ester
T2D	Type 2 Diabetes
T1D	Type 1 Diabete
MAPKs	Mitogen-activated protein kinases
NADPH	Nicotinamide adenine dinucleotide phosphate
NO	Nitric Oxide
NOS	Nitric Oxide Synthase
ncNOS	neuronal constitutive NOS
iNOS	inducible NOS
eNOS	endothelial NOS
PBS	Phosphate-buffered saline
PACAP	Pituitary Adenylate Cyclase-Activating Polypeptide
PKA	Protein Kinase A
PKC	Protein Kinase C
PLC	Phospholipase C
PIP_2	Phosphatidyl inositol-bisphosphate
ROZ	Rosiglitazone (Thiazolidinedione derivative)
RX	RX781024 (Imidazoline derivative)
SAPK/JNK	Stress-activated protein kinase /c-Jun N-terminal kinase
VIP	Vasoactive intestinal peptide
ZDF rat	Zucker Diabetic Fatty Rat

INTRODUCTION

The prevalence of type 2 diabetes (referred to from here on as T2D), which is a metabolic syndrome with a multifactorial etiology, is high and increasing. The complex nature the disease is characterized by a reduced glucose utility by peripheral tissues accompanied by an increased plasma glucose level, which is a further consequence of reduced insulin secretion by pancreatic islets (1). A sustained high level of blood glucose in turn would adversely affect the function of not only different blood proteins and cells but also all other cells within the body including the β-cells themselves, a phenomenon regarded as *glucotoxicity* (2). Both long-term elevation of plasma glucose levels or culturing of pancreatic islets at high glucose concentrations results in reduced insulin response upon a subsequent challenge with a variety of stimuli (3). Thus an important etiology of T2D seems to be a β-cell failure to properly respond to the carbohydrate challenge by secreting the demanded levels of insulin although the insulin resistance might appear under a longer period prior to this (4). In association with the reduced β-cell function and increased blood glucose levels, during the process of glucotoxicity, the apoptotic signals would appear within pancreatic islets. In addition to disrupted glucose balance diabetic patients also display increased levels of plasma free fatty acids (FFA). An increased level of free fatty acids (FFA) during a long time would have a beneficial effect on the function of almost all kind of cells within the not body, a process known as *lipotoxicity*. It has been argued that the lipotoxicity by itself might not have any harmful effect on the β-cell function and could only be of significance in combination with glucotoxicity (2). The present thesis presents some new aspects on the mechanisms underlying lipotoxicity and glucotoxicity in the endocrine pancreas.

PANCREAS

In 1869, Paul Langerhans was the first to describe the endocrine pancreas, which consists of islet-like structure within the surrounding tissue. Also, he found that the islets were richly innervated but no functional data were presented (5).

On 1889, Oscar Minkowski and Josef von Mering were establishing for the first time a relationship between the pancreas and diabetes, after the observation of hyperglycemia following pancreatectomy in dog. Only a few years later, in 1893, Gustave Laguesse gave birth to the term "Islets of Langerhans". Their work led other scientists to pursue further research on the relation of the pancreas to diabetes. It did not wait too long when the term "insulin" (from Latin "insula" – *i.e.* island) was introduced by Jean de Meyer. In 1921, Frederick Banting, Charles Best, Jonh Macleod and James Collip provided evidence that, indeed, the pancreatic islets produced a hormone that was responsible for the control of blood glucose levels (Banting, F. and Best, C. J.Lab.Clin.Med. 7:465-80, 1922). The successive results were soon tested on patients with T1D and the first patient who received an insulin injection was a 14-year-old diabetic. The discovery of insulin was one of the most revolutionary moments in the treatment of diabetes. The primary structure-crystalline form of insulin was determined by Fredrick Sanger in 1955 and it was the first protein to have its sequence to be determinated.

Pancreas and Islets of Langerhans

The pancreas is a complex organ since it contains both an exocrine and an endocrine part. It is located across the back of the abdomen, behind the stomach, opposite to the liver along the gastrointestinal tract. The right side of the organ (called the head) is the widest part of the organ and lies in the curve of the duodenum. The tapered left side extends slightly upward (called the body of the pancreas) and ends near the spleen (called the tail).

The Exocrine Pancreas

The exocrine pancreas constitutes the bulk of pancreatic tissue and is comprised of primarily acinar cells. These cells are arranged in clusters which are called *acini***.** Acinar cells are pyramidal in shape and contain en extensive secretory apparatus. The primary function of exocrine pancreas consists of the release of digestive juice into the small intestine. This juice is collected into small ducts, which merge to form one main pancreatic duct. This is joined by the common bile duct just before it enters the duodenum. Pancreatic juice is a clear alkaline fluid, which contains the precursors of enzymes of all classes necessary to break down the main components of the diet.
Proteolytic enzymes are secreted as *zymogens* - inactive precursors of the enzymes. They are activated in the lumen of the digestive canal. It's important, that enzymes are secreted as inactive proenzymes, which are then activated once they enter the digestive tract to avoid autodegradation, which can lead to pancreatitis. This part of the pancreas is the main source of enzymes for digesting fat (lipids), proteins and carbohydrates (6; 7)**.** There are also reports about the interplay between exocrine and endocrine pancreas in the development of T2D (8).

The Endocrine Pancreas

Scatted amongst the exocrine tissue are little groups of cells, appearing like islands under the microscope. They are known as Islets of Langerhans with the main function of producing and secreting hormones into the bloodstream. Each islet is normally surrounded by a capsule made of fibroblasts and collagen fibers. The size of an islet can range from only 40 μm to proximately 400 μm in diameter and from few cells to 10 000. The pancreas of adult human contains 300 000 - 1.5 million islets. Adult mice have approximately 200-300 islets. So islet forms 2-3 % of the whole pancreas mass. Their combined weight is 1 to 1.5 grams in adult human pancreas. Islets are not evenly distributed throughout the organ. The major part of islets spreads toward the pancreatic tail (9).
Pancreatic islets contain at least 5 different types of cells in both human and rodent pancreas. The distribution of cells is nonrandom, with a core of β-cells surrounded by a

discontinuous mantle of α, δ and PP cells. The islet organ a complex structure of cells and might function both separately and in concert as the endocrine pancreas (10; 11).

Insulin-producing β-cells

Insulin producing β-cells in the islets of Langerhans comprise about 65-80% of total the islet cell mass. Insulin is a polypeptide consisting of A and B chains of 21-30 amino acid, which are linked through a pair of disulfide bonds. Insulin is synthesized from a preproinsulin of 110 amino-acid residues, which by proteolytic cleavage in endoplasmic reticulum to proinsulin, is transported to the Golgi apparatus and then to secretory vesicles. Proteolytic removal of the connecting C-peptide yields the resulting insulin molecule. Insulin is stored in the secretory vesicles and is released upon stimuli e.g. glucose, amino acids, FFA and different incretin hormones. Moreover, it has been shown that insulin is released in a pulsatile manner (12; 13). Physiologically insulin causes a rise in glucose uptake from the blood in liver, muscle and fat tissue. Insulin has a marked antilipolytic effect on the adipose tissue (14; 15). The pancreas of the adult human contains 8 mg of insulin and that of the adult rat approximately 100 μg. Insulin circulates free in the blood stream; it is not bound to a carrier protein. It affects different tissues by binding to specific insulin receptors (16).

Apart from insulin, β-cells release C-peptide into the blood in equimolar amounts with insulin. Measuring of C-peptide helps to estimate the amounts of insulin released and also is a good parameter for the determination of the viable β-cell mass (17). The β-cells also release amylin (IAPP – islet amyloid polypeptide) and since amylin reduces food intake upon administration, an analog of this polypeptide has been considered as therapeutic agent in the treatment of T2D (18).

Glucagon-producing α-cells

Glucagon producing α-cells takes up around 10-15% of the total cell population in the islet. Glucagon cells are mainly localized in the peripheral part of the islet around the core of the islet (10; 11). Glucagon is a single polypeptide chain of 29 amino acids. It is synthesized as a larger protein called proglucagon. Proteolytic cleavage gives rise to

glucagon (19). Glucagon produces its effect on intracellular metabolic pathways by binding to specific receptors in the cell membrane. These receptors are coupled to a G-protein exerting effects on the cAMP levels (20). Glucagon's major action is to elevate the blood glucose concentration, when the glucose level in the blood is low and hypoglycemic (< 3.5 mM). This is accomplished by stimulation glycogenolysis (breakdown of glycogen) and gluconeogenesis (formation of glucose from pyruvate, lactate and glycerol). Gluconeogenesis is also a target of therapy for the treatment of T2D. Some anti-diabetic drugs *i.e.* metformin, which inhibits glucose formation and stimulates glucose uptake in the peripheral tissues seems to inhibit gluconeogenesis (21). Moreover, glucagon is also a potent lipolytic hormone. It has a great effect on adipose tissue and stimulates release of free fatty acids and glycerol, and these substances circulate to the liver where they play a role in the gluconeogenic processes. Insulin and somatostatin inhibits glucagon secretion and action (22).

Somatostatin-producing δ-cells

Somatostatin secreting δ-cells fill almost up to 5 % of the total cell population of the islets. They are localized in the mantle of the islet. Somatostatin has two active forms produced by alternative cleavage of a single preproprotein; one with 14 amino acids and one with 28 amino acids (23). It is worth mentioning that the secretion of somatostatin follows almost the same pattern as that of insulin (24). Somatostatin is classified as an inhibitory hormone, whose actions are spread to different parts of the body. Regarding pancreas and pancreatic islets, somatostatin inhibits the release of insulin and glucagon and also suppresses the exocrine secretory action of pancreas (25).

Pancreatic polypeptide-producing-cells (PP-cells)

PP consists of 36 amino acids. It is found predominantly in the head of the pancreas (11). The physiological functions of PP are not fully clear, but it might exert some effects on the hepatic glycogen levels and gastrointestinal secretions. In humans PP is increased after a protein meal, fasting, exercise, and acute hypoglycemia (26). It has also been

proposed that the somatostatin and PP cells control the secretory activity of each other in a paracrine manner (11).

Innervation and microvascularisation of pancreatic islets

The endocrine part of the pancreas is highly vascularised and has a direct arteriolar blood supply. Arterioles enter the islet β-cell core directly, where they break into a number of capillaries. The efferent capillaries coalesce into colleting venules and drain into the venous system (27). The islets thus have a very rich blood supply and it accountes for about 10% of the total pancreatic blood flow. This indicates how important the islet might be in regulation of whole body metabolism (28). The vascularisation of the exocrine part of the pancreas, on the other hand, greatly differs from the islets (29).

Regarding the innervation of the pancreatic islets, it has been reported that they receive an extensive and complex innervation that includes sympathetic, noradrenergic nerve fibers also storing neuropeptide Y. The nerve fibers enter the islets along the blood vessels and then form a network or pass directly to an endocrine cell (30). Parasympathetic *i.e.* the cholinergic (vagal) nerves also enter the islets and stimulate the release of 4 different neurotransmitters (acetylcholine, VIP, PACAP and GRP). All these neurotransmitters stimulate insulin and glucagon secretion, but factors promoting parasympathetic islet innervation are unknown (31). The sympathetic nerves have an opposite effect and inhibit basal and glucose-stimulated insulin secretion, although, activation of β-adrenoreceptors stimulates insulin release (32; 33).

PANCREATIC ISLET PATHOPHYSIOLOGY

Historic approach to diabetes

The earliest known record of diabetes, back to 1500 B.C., as it was mentioned by Egyptians. It was then set in Roman books by Gallinious. The name "diabetes" comes from the Greek word for siphon. The sweet taste of the diabetic urine was first mentioned by Araeteus of Cappodocia in 130 A.D. Only much later in 17th and 18th century, the British physician Thomas Willis added the word "mellitus" (honey sweet) and Dobson confirmed the presence of excess sugar in urine and blood of diabetic patients as a cause of their sweetness (34).

Definition of diabetes

Diabetes has a broad definition, because the disease itself is heterogeneous and divided into many subtypes. Diabetes mellitus is a chronic state of a pathological condition associated with hyperglycemia in the blood. Diabetes could be briefly divided into two subgroups depending on: a) an absolute insulin deficiency or b) inadequate secretion of insulin in combination with reduced sensitivity of peripheral tissues to insulin. The two main types of diabetes correspond to these conditions are called insulin dependent diabetes mellitus (IDDM) or "type 1 diabetes mellitus" and non-insulin dependent diabetes mellitus (NIDDM) or "type 2 diabetes mellitus" respectively.
In *type 1 diabetes mellitus* (referred to from here on as T1D) is also called "juvenile diabetes mellitus" as it mainly affects children in the age group from 7-14 years. However, it is not exceptional that adults could be diagnosed with T1D (35). The cause of T1D is still not fully uncovered. However, it is generally referred to as an autoimmune disease. A well described feature of this autoimmune disease is a humoral autoimmunoresponse against multiple agents of a given tissue. Regarding, T1D, the immunological events leads to β-cell destruction, which appears to involve an increased activity of T-cells. Evidence has been presented that T1D develops in genetically susceptible subjects, and that the autoimmune process results in selective lymphocyte-

mediated β-cell destruction (36; 37). The disease is associated with the development of islet cell antibodies, insulin and GAD-antibodies, in the blood of patients often many years before the clinical onset of the disease (38). The efficient treatment for patients with T1D so far is only one, daily injections of insulin throughout life time, along with attention to dietary management, typically including carbohydrate tracking, and careful monitoring of blood glucose levels (39; 40). Currently, the only possible treatment for T1D is insulin injections. Pancreatic transplantation is generally recommended if a kidney transplant is also necessary. However, immunosuppression is required and many physicians think that it's more dangerous than continued insulin replacement therapy. Islet transplantation is less invasive than pancreas transplant, but it still remains controversial.

In *type 2 diabetes mellitus* or "adult onset diabetes mellitus" is the most common form of diabetes and it constitutes about 90% of all individuals with diabetes mellitus in developed countries. Epidemiological studies have suggested that the number of diabetes patients world wide will be about 380 millions in 2025 (*www.who.int*). T2D is a metabolic disorder that is primarily characterized by insulin resistance (a reduced response to insulin), relative insulin deficiency (β-cell does not produce enough insulin) and hyperglycemia. It is well established that both genetic (inherited) and environmental (lifestyle) factors are involved in the pathogenesis of T2D (41).

Maturity-onset diabetes of the young (MODY) is a genetically and clinically heterogeneous subtype of T2D characterized by early onset, autosomal dominant inheritance, and primary defects in insulin secretion. Most commonly MODY acts like a very mild version of T2D, with continued partial insulin production and normal insulin sensitivity (42).

Gestational diabetes mellitus (GDM) is recognized in women during pregnancy. Gestational diabetes affects about 3-9 % of pregnancies, depending on the population studied. No specific cause has been identified, but it is believed that the hormones produced during pregnancy contribute to the reduced peripheral sensitivity to insulin, resulting in high blood sugar levels (43).

Insulin resistance

Insulin resistance has classically been defined as a greater requirement for insulin to induce a normal rate of glucose uptake (3). Insulin resistance has been considered as a key factor in the pathogenesis of T2D and as a co-factor in the development of dyslipidemia, hypertension and atherosclerosis. The mechanisms behind insulin resistance are of multifactorial nature including obesity, elevated plasma FFAs, low physical activity and overproduction of stress hormones as well (44). This however, does not exclude factors related to genetic disorders. It should be noted, that the mentioned environmental factors are always more susceptible in genetically predisposed subjects (45; 46). The common basis for insulin resistance to the action of insulin in a particular cell seems to be related either to a post-receptor defect in the signalling cascade involved in the translocation of glucose transporter 4 (GLUT-4) or to a more sever condition *i.e.* a deficiency in insulin binding to its receptor (47). Briefly, obesity-related insulin resistance involves the release of factors from adipocytes which exert a negative effect on glucose metabolism: free fatty acids and different cytokines *i.e.* tumor necrosis factor-alpha (48).

Among of others, at the onset of T2D, the responses to insulin in skeletal muscle, adipose tissue and liver are significantly lowered, leading to a decreased disposal of excessive glucose and fatty acids. In addition to the inability to exert a glucose lowering effect, insulin also fails to inhibit catabolic pathways, such as lipolysis, proteolysis and glycogenolysis in these tissues. Excessive glucose production through gluconeogenesis during the state of insulin resistance even worsen this condition (49). Moreover, insulin resistance at the level of the β-cell itself has also been demonstrated (3). Hence, overt diabetes develops when insulin secretion cannot compensate for the defective insulin action in peripheral tissues. If the disease remains untreated, this decompensation may lead to severe complications. The consequence of insulin resistance in the absence of an adequate insulin level on the function of insulin sensitive tissues is briefly illustrated in Figure 1.

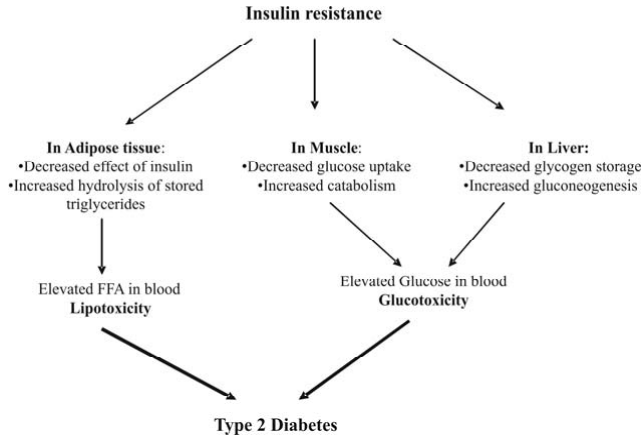

Figure 1. Illustrates the tissues involved during development of insulin resistance

INSULIN SECRETION

Characterization of insulin secretion

Secretion of insulin is stimulated by a wide variety of nutrients such as glucose, FFA and amino acids. Among these nutrients glucose is the most powerful initiator of insulin release. This makes glucose as the primary physiological stimulus of insulin exocytosis (3). The insulin secretory response to glucose is of biphasic character. An increased glucose level is accompanied by a rapid and transient increase in insulin release (first phase) which rapidly declines to basal levels and thereafter increases in an oscillatory manner and lasts for several hours when the glucose is kept high (second phase) (50-52). It has been demonstrated that patients with NIDDM have impaired or lost first phase of insulin secretion and that the second phase is much more enhanced (53). In addition to nutrients, there are also other secretagogues such as hormones (incretins) or neurotransmitters, which exert a stimulatory effect on the insulin secretion in the presence of an adequate glucose concentration. The incretins *e.g.* GLP-1, GIP and CCK or neurotransmitters *e.g.* PACAP and Acetylcholine (ACh) act through a receptor-mediated pathway mainly coupled to G-proteins (Gs or Gq) with subsequent increase in intracellular messenger molecules upon stimuli (51; 54-57). A disturbed secretory pattern of these incretins in T2D patients has been reported (58).

Glucose-stimulated insulin secretion

Glucose-stimulated insulin secretion (GSIS) is a very complex process involving a number of signaling pathways. The detailed nature of GSIS is not fully clarified. As glucose enters the β-cells through a specific glucose transporter (GLUT-2) in rodents (in humans GLUT-1) it is rapidly phosphorylated by glucokinase (59; 60). The metabolism of glucose stimulates insulin secretion through at least 2 distinct pathways *i.e.* 1) a K_{ATP} - dependent and 2) an amplifying pathway which operates independent of K_{ATP} (61).

1. The K_{ATP} channel-*dependent* pathway is also called the *triggering pathway.* This pathway could be shortly described as follows: after glucose entering the cell, glycolysis occurs in the cytoplasm and the mitochondria, which leads to an increase in the ATP/ADP ratio and closure of the K^+_{ATP} channels. The plasma membrane of the β-cell is then depolarized upon closure of the K^+_{ATP} channels. This leads to activation of voltage-dependent L-type Ca^{2+} channels (VDCC) and influx of extracellular Ca^{2+}. An almost 10-20 times increase in intracellular Ca^{2+} triggers the exocytotic machinery and the readily releasable insulin granules are released (62). This pathway is mainly operative in the first phase of insulin release but evidence has been presented that it is also operating, to some degree, during the second phase (63; 64).
2. The K_{ATP} channel-*independent* pathway is also called the *amplifying pathway*. The detailed mechanisms involved in this pathway are not well established that might lead to some controversy. On the other hand it has been clearly demonstrated that glucose is still able to stimulate insulin secretion even when the K^+_{ATP} channels and membrane depolarization events is bypassed pharmacologically (65). The amplifying pathway seems to be operative during the second phase of insulin secretion (51; 66). It should be kept in mind that the amplifying pathway is at least to some extent dependent on the presence an increased $[Ca^{2+}]_{i.}$ (51; 62; 67; 68). Interestingly, several factors have been suggested to amplify insulin secretion through the K_{ATP} independent pathway, *e.g.* cAMP, glutamate, FFA, NADPH and GTP (61; 69-71). However, an integrated symphony of these two pathways in the releasing process, during both phases of insulin secretion could not be excluded (61).

Under normal physiological conditions blood glucose levels are kept within a narrow range between 4-8 mmol/l. A prolonged period of elevated blood glucose levels would adversely influence the function of different tissues, a condition known as glucotoxicity. Hyperglycemia reportedly is the driving force of the late diabetic complications (72). Previous studies has suggested that glucotoxicity in addition to induction of peripheral insulin resistance also might be an important player in the pathogenesis of β-cell

dysfunction (73). Evidence for the support of this hypothesis is derived from the observations that in T2D patients tightly achieved metabolic control leads to the improvement of β-cell response to a glucose-challenge (74; 75). In accordance with these studies, observations in our laboratory have shown that islets isolated from a chronically hyperglycemic rat *i.e.* the Goto-Kakizaki (GK) rat showed a markedly impaired insulin secretory response to glucose both *in vitro* and *in vivo* (76-80). Hence in pancreatic β-cells multiple metabolic, hormonal and neuronal events contribute to the stimulus-secretion coupled cascade and they are also potential sites for the toxic action of long-lasting high glucose concentrations (81). To study the effects of glucotoxicity on islet function *in vitro* we used isolated islets from hyperglycemic GK rats that represent a state of glucotoxicity and also are regarded as a spontaneous model of T2D (77-79). In contrast to Zucker Diabetic Fatty (ZDF) rats, GK rats remain lean during their life time (79; 82) and further allows for the avoidance of the complicating consequences of obesity-related increase in the blood levels of FFA, followed by a negative impact of FFA on the pancreatic islets.

Effects of free fatty acids (FFA) on β-cell function

FFAs serve as an important fuel in the physiological processes in different tissues such as heart, liver, muscle and pancreatic islets. An elevated level of FFAs is considered as risk factor for several pathophysiological conditions and exerts harmful effects on cellular functions such as an increased tissue production of apoptotic signals *i.e.* NO, O_2^- and H_2O_2. In obese subjects the FFA levels are usually increased and lead to deregulation of both FFA-mediated signaling pathways and metabolism *i.e.* lipotoxicity that is linked to insulin resistance, T2D as well as liver steatosis and cardiovascular diseases (83-85).
Regarding FFA involvement in the insulin secretory processes there are two hypotheses. An earlier theory, which is based on the entrance of FFA into the β-cell and conjugation with coenzyme A with subsequent formation of long-chain acyl-CoA (LC-CoA), has suggested that LC-CoA serves as major effector molecule in the insulin secretory mechanisms (86; 87). Despite several years of research targeting the transport of FFA across the plasma membrane and entrance into the β-oxidation with subsequent

generation of signals, there are still many questions and speculations on this hypothesis! A later and more prominent hypothesis is based on the identification of GPR40 as FFA receptor with a ligand potency in the μmolar range and dependent on the chain length for saturated FFA, with increasing number from hexanoic acid (C6:0) to lauric acid (C12:0) (88; 89). Several groups as well as ours have shown that glucose-stimulated insulin secretion is potentiated by FFA through the GPR40 (88; 90; 91). Activation of GPR40 by FFA is due to FFA being potent agonists of the receptor (92). The intracellular coupling-signaling pathway of GPR40 proceeds through the activation of the Gq protein leading to mobilization and release of intracellular calcium as well as activation of PKC (93). Although GPR40 is preferentially expressed in the pancreatic β-cell evidence has been presented for the expression of GPR40 in the glucagon cells too (94). Expression of GPR40 has been detected also in brain and gut (95-98).

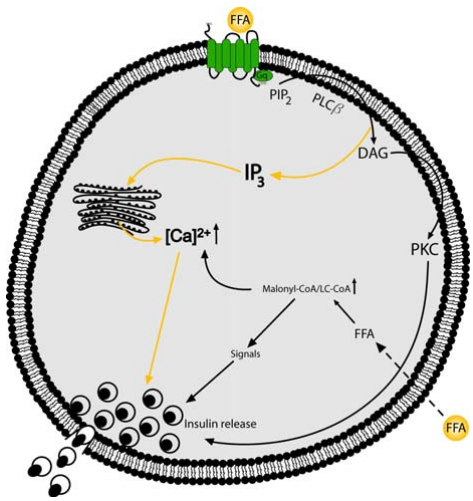

Figure 2. Schematic illustration of biochemical events involved in FFA-stimulated insulin release

Effect of elevated plasma free fatty acids (FFA) on liver and muscle tissue

Energy supply in liver and muscle is stored as glycogen and in adipose tissue as triglyceride (TG). When these tissues cannot store any more and fat is still available, TG is ectopically stored in these non-adipose tissues. Excess fat acts on these tissues to counteract insulin-mediated glucose uptake, hepatic regulation of glucose output, and insulin secretion. These lipids when accumulated in the cytoplasm lead to cell dysfunction or cell death, a phenomenon known as lipotoxicity (99).

It has been showed that elevated FFA interacts with muscle tissue function and results in insulin resistance. Mechanisms underlying this pathophysiological condition involve, at least in part, interaction with the insulin-signaling cascade that culminates in the inhibition of cellular glucose uptake at different steps. It has been reported that long-term elevated plasma FFA is associated with impaired GLUT-4 gene expression, followed by impaired translocation of GLUT-4 to the cell membrane as well as reduced glucose utility, thus contributing to hyperglycemia (100). There is also evidence that FFA decrease glucose conversion into glycogen for storage (101; 102).

Elevated plasma FFA negatively modulates the hepatocyte function that leads to hyperglycemia through the interaction with the enzymes involved in the glycolytic/glycogenolytic pathways which in turn might be followed by more hepatic glucose output via an increased gluconeogenesis and glycogenolysis. It should be mentioned that in normal conditions, insulin inhibits these two processes, but sustained and elevated FFA interferes with the signalling downstream of the hepatic insulin receptor that results in enhanced demand of insulin to properly bring about its work (103; 104).

cAMP/Protein Kinase A (PKA) pathway

Several hormones such as GLP-1 and GIP that are released from the gut upon food intake profoundly potentiates GSIS through the interaction with a membrane bound receptor coupled to adenylate cyclase (AC) via a stimulatory G-protein (G_s). Upon activation, AC

will catalyze the formation of cAMP from ATP. An increase in cyclic AMP levels in the β-cell alters the function of several cellular target proteins. The immediate and rapid insulin secretory response of β-cells due to elevation of cAMP seems to involve a direct interaction with the exocytotic machinery (105) and PKA is known to exert direct effects on the distal events of exocytosis, which causes mobilization of insulin containing granules from the reserve pool to the readily releasable pool. Moreover, previous studies have also indicated that cAMP; in addition to these effects also improves β-cell survival through a PKA-dependent pathway (106). Furthermore, cAMP/PKA induces an inward current in the β-cell, which is mainly caused by an influx of Na^+ into the cell, causing depolarization followed by opening of L-type Ca^{2+} channels and raising the cytosolic concentration of Ca^{2+} and thus potentiation of insulin secretion (107). Besides, activation of PKA has several other effects on β-cell function such as inhibition of cell apoptosis and inhibition of iNOS expression (55).

Phospholipase C (PLC) pathway

Receptor mediated activation of phospholipase C (PLC) results in the hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP_2) into the lipid-soluble messenger molecule 1,2-diacylglycerol (DAG) and the water-soluble messenger molecule inositol 1,4,5-trisphosphate (IP_3). The generated DAG subsequently activates the protein kinase C (PKC) (108). The activated PKC will then be translocated to the cell membrane in a Ca^{2+}-depend manner (109). It should be noted that several isoforms of PKC are expressed in pancreatic islets (110). PKC activation by pharmacological agents such as phorbol esters (TPA) results in the potentiation of glucose-stimulated insulin release through largely unknown mechanisms although an increased $[Ca^{2+}]_i$ through voltage gated Ca^{2+} channels (L-type Ca^{2+} channel) has been reported (111; 112).

IP_3, on the other hand, causes the release of Ca^{2+} from endoplasmic reticulum (ER) by binding to specific receptors *i.e.* ligand-activated Ca^{2+}-channels (113; 114). Apparently Ca^{2+} released through activation of IP_3 receptors do not potentiate glucose-stimulated insulin secretion appreciably (115). However, the IP_3-stimulated increase in $[Ca^{2+}]_i$ might serve as a regulatory signal in the distal exocytotic machinery.

Phospholipase D (PLD) and Phospolipase A_2 (PLA_2) pathways

Stimulation of insulin secretion through PLD pathway starts when PLD is activated; it hydrolyses phosphatidyl choline to produce phosphatidic acid, choline and DAG (116). When PLA_2 is activated *e.g.* in response to acetylcholine (ACh) and cholecystokinin (CCK-8) it causes formation of arachidonic acid (AA) and its metabolites, which stimulates insulin secretion by enhancing Ca^{2+} influx into the cell, and release of Ca^{2+} from intracellular stores (117).

NITRIC OXIDE AND ENDOCRINE PANCREAS

Nitric oxide is a small gaseous molecule with a divergent range of physiological (neurotransmission and vascular tonus regulation) and pathophysiological (defense against invading microorganisms) action. This gaseous molecule diffuses freely across cell membranes and it also easily acts in intracellular environment regulating the function of a numerous enzymes and messenger molecules. Nitric oxide is produced by a family of enzymes called Nitric Oxide Synthases (NOS). The NOS enzyme appears in three major isoforms, two constitutively expressed isoforms *i.e*. neuronal constitutive NOS (ncNOS) and endothelial NOS (eNOS) and also an inducible NOS (iNOS). NOS isoforms could be divided in to 2 functional classes and differ in their dependence on calcium, expression and activity.

1. Constitutively expressed ncNOS and eNOS – are neuronal cNOS and endothelial cNOS. These isoforms are Ca^{2+}-calmodulin dependent and produce low (in nano and pico moles) amounts of NO in a pulsatile manner. Thus, increased intracellular Ca^{2+} levels activate these enzymes and they are involved in different physiological processes. ncNOS isoform is found in neurons of the brain and peripheral neural system. The function of this form is negative modulation of insulin secretion and being neurotransmitter. ecNOS enzyme is normally present in the endothelium lining. This isoform is involved in maintaining blood pressure, inhibition of leucocyte adhesion and inhibition of platelet aggregation.
2. Inducible Nitric Oxide Synthase (iNOS) – is expressed in many different cells, like hepatocytes, respiratory epithelia, chondrocytes, keratinocytes, macrophages, endothelium and inflammatory cells (118). iNOS is Ca^{2+}- calmodulin independent, since it has calmodulin tightly bound to the enzyme, which makes this enzyme completely insensitive to calcium oscillations. iNOS produces NO in vast amounts (in μmoles) and constantly. This NOS isoform is also involved in physiological processes and can play a protective role, for example, macrophage defence against invading microorganisms and tumor cells. It has been shown that iNOS can induce healing of the skin and the intestinal mucosa, regulating T cell proliferation and differentiation. In the wrong place and in big amounts NO brings

about a cytotoxic action and leads to pathophysiological conditions, tissue distraction, inflammation and induce apoptosis of the cells. The iNOS isoform is transcriptionally regulated by cytokines and inflammatory mediators (119; 120).

NO is produced from the amino acid L-arginine in equimolar concentrations to the amino acid L-citrulline (121). As NO has a short half life (lifetime of a few seconds) and is highly reactive, it interacts with oxygen and results in oxidation to nitrite (NO^{2-}) and nitrate (NO^{3-}), or interacting with radicals such as superoxide anion (O_2^-) where it forms peroxynitrite $ONOO^-$, and also forms other radicals that are even more reactive then NO itself and are capable to damage targeting cells (122).
There are several mechanisms by which NO have been demonstrated to affect the biology of living cells. These include oxidation of iron containing proteins such as ribonucleotide reductase and aconitase, ADP ribosylation of proteins, protein sulphuryl group nitrosylation, and iron regulatory factor activation. A biologically important reaction of nitric oxide is S-nitrosylation, the conversion of thiol groups, including cysteine residues in proteins, to form S-nitrosothiols (RSNOs). S-nitrosylation is a mechanism for dynamic, post-translational regulation of many important classes of protein. S-nitrosylation inactivates many proteins (123).

Immunohistochemical and Western blot analysis using specific antibodies against different isoforms of NOS as well as RT-PCR technique has shown expression of ncNOS and eNOS normally and iNOS under pathophysiological conditions in the pancreatic islets (124-127). An excessive NO production from iNOS in cytokine-activated β cells has been implicated in β cell disruption in T1D (127). β-cells are very sensitive to NO-induced apoptosis. On the other hand, ncNOS which is constitutively expressed in islet cells (125), produces NO in pulsatile manner in small amounts. The role of ncNOS in insulin release is not fully understood, but most data from mouse and rat pancreatic islets suggest that ncNOS activity serves as negative modulator of glucose-stimulated insulin release (125; 126).

ANIMAL MODELS REPRESENTING OBESITY OR DIABETES

In order to investigate the molecular mechanisms behind FFA-stimulated insulin release in relation to GPR40 in obesity and T2D, two different animal models were used. The Zucker Diabetic Fatty Rats (ZDF) represents a good model of obesity and diabetes and the Goto-Kakizaki (GK) rats represent a state of hyperglycemia and glucotoxicity without weight gain.

The *Zucker diabetic fatty rat* has impaired leptin receptors (*fa/fa*) that results the obesity, impaired insulin secretion and hypertension with associated renal and cardiovascular disease. The *fa/fa* mutation in the gene coding the leptin receptor results in insulin resistance with reduced glucose tolerance, but rats that express the *fa/–* genotype are lean and do not develop hyperglycemia (128; 129). Leptin release is stimulated by insulin and acts to suppress insulin secretion while inducing satiation. ZDF rats are hyperphagic due to the reduction in leptin signal that results in obesity (130). It has been also observed marked accumulation of lipids in ZDF rats islets (131). The ZDF rat presents with increasing insulin resistance with compensatory hyperinsulinemia that quickly progresses to severe glucose intolerance. Plasma free fatty acids rise rapidly at about 5–8 weeks and hyperglycemia occurs between 7 and 10 weeks (132). The animals become severely dyslipidemic, with raised plasma free fatty acids, cholesterol, and triglyceride. Adult ZDF rats also develop complications to the diabetic state, including cardiovascular disease, peripheral neuropathy (133). The ZFD rat was derived from Zucker fat (ZF) rat and ZF from Wistar rats, which were inbred to conserve the phenotypic features of the pre-diabetic state, such as glucose intolerance.

In contrast to ZDF, the GK rat is a nonobese, diabetic strain that presents with primary β-cell defects and peripheral insulin resistance in both males and females. The GK rats remain lean during their life time that further allows for the avoidance of the complicating consequences of obesity on experimental parameters (134). Historically the GK rat colony was established by the repeated inbreeding from Wistar rats displaying

both a high fasting blood glucose levels and a reduced glucose tolerance *i.e.* a glucose tolerance at the upper limit of GK rats were normoglycemic and showed normal islet morphology at birth and changes in islets do not develop simultaneously with hyperglycemia. At 4 weeks of age, GK rats displayed a mild fasting hyperglycemia of 9 mM, raised basal insulin secretion, β-cell insensitivity to glucose, and reduced glucose tolerance (79; 135). The colony originated in Japan in 1975 and other colonies have since been established elsewhere, most notably in Stockholm and Paris.

AIMS

The overall aim of the present thesis was to study underlying mechanisms of lipotoxicity and glucotoxicity for hormone secretion in the islets of Langerhans with special regard to the FFA receptor GPR40 and the NO system.

1. To study both short-time and long-term effects of FFA and its interaction with the PPARγ agonist rosiglitazone on the activities of islet NOS isoenzymes in relation to GPR40 and insulin secretion.

2. To study the effects of FFA on different MAPKs and their role in FFA-induced β-cell dysfunction and whether this effect is modulated by rosiglitazone.

3. To study in more detail the relation between FFA-stimulated hormone secretion and the expression pattern of GPR40 in pancreatic islet of obese and lean diabetic animal models.

4. To investigate the islet NOS activities and the effect of GLP-1 on mildly hyperglycemic, lean type 2 diabetic animal model.

5. To explore whether the imidazolin derivative RX has any impact on the islet NO generation and insulin release after short- and long-term incubations at high glucose levels.

METHODOLOGY

Animals

Female mice of the NMRI (Naval Medical Research Institute) strain, weighing 25-30g, were used**.** Age- and sex-matched GK rats of the Stockholm colony and Wistar controls (B&K Universal, Sollentuna, Sweden) were used. They were given a standard pellet diet (B&K, Sollentuna, Sweden) and tap water *ad libitum* throughout the experiments. ZDF rats were bought from Charles River Laboratories. The experimental procedures were approved by the Ethical Committee for Animal Research at University of Lund; Sweden and were in accordance with the international standard recommended by NIH.

ISOLATION OF ISLETS OF LANGERHANS

Preparation of pancreas

The distal end of the pancreatic duct was clamped and was performed by retrograde injection with approximately 3-5 ml of ice-cold collagenase solution cannulation *via* the bile-pancreatic duct as previously described (136). Thereafter pancreas was dissected and carefully separated from the surrounding tissue and then placed in tube (20 ml) and in water bath (30 cycles/minute) at 37°C for 17 min.

Isolation of islets

The pancreatic islets were separated from the acinar tissue by vigorous shaking in ice-cold Hank's solution for several minutes. After sedimentation for about 10-15 min the islets were handpicked under a stereomicroscope at room temperature and then subjected to different experimental procedures.

In vitro experiments

Freshly isolated islets were preincubated for 30 min and incubated for 60 min in an incubation box (30 cycles/minute) at 37°C in Krebs-Ringer bicarbonate buffer (KRB), pH 7.4, supplemented with 10 mmol/l N-2-hydroxyethylpiperazine-N-2-ethanesul-fonic acid (HEPES), 0.1 % bovine serum albumin (BSA) and 1.0 mmol/l glucose. Each incubation vial contained 12 islets in 1.0 ml of buffer solution and was gassed with 95%

O_2-5% CO_2 to obtain constant pH and oxygenation. An aliquot of the medium was removed immediately after incubation and frozen for the subsequent assays.

BIOCHEMICAL AND RADIOIMMUNOLOGICAL ANALYSIS

Determination of insulin and glucagon

The concentrations of insulin and glucagon were determined by RIA (137) Short description, in both assays ^{125}I– labeled hormone competes with non-radioactive hormone in sample or standard for the binding of the hormone specific antibody. Separation of free and bound radioactivity is accomplished by centrifugation after addition of the secondary antibody. The radioactivity in the resulting pellet is inversely proportional to the amount of hormone in the sample/control and is measured in a γ-counter.

Determination of protein

Protein was determined according to Bradford (138).

High-performance liquid chromatography (HPLC) analysis of NOS activity

Freshly isolated pancreatic islets were washed and collected in ice-cold buffer (200µl) containing HEPES (20 mmol/l), EDTA (0.5 mmol/l) and DL-dithiothreitol (DTT) (1 mmol/l), pH 7.2, and thereafter stored at -20°C for subsequent NOS analysis.

In brief, after sonication on ice, the buffer solution containing the islet homogenate was supplemented to contain also $CaCl_2$ (0.45 mmol/l), NADPH (2 mmol l/l), calmodulin (25 U/ml), and L-arginine (0.2 mmol/l) in a total volume of 1 ml. For the determination of iNOS activity both Ca^{2+} and calmodulin were omitted from the bufffer described (125). The crude homogenate was than incubated at 37°C under constant air bubbling (1.0 ml/min) for 2 h. Aliquots of the incubated homogenate (200 µl) were mixed with an equal of O-phtaldialdehyde reagent solution in glass vial and passed through a 1-ml Amprep CBA cation-exchange column for determination of L-citrulline by HPLC analysis and it was measured in a Hitachi F1000 fluorescence spectrophotometer (Merk, Darmstadt, Germany) as previously described (125). Since L-citrulline and NO are generated in equimolar amounts, and since L-citrulline is stable whereas NO is not, L-citrulline is the preferred parameter when measuring NO production. Protein concentration was determined according to Bradford (138) on samples from the original homogenate.

Western blot analysis

Approximately 250 islets were collected in Hanks' buffer (150 µl) and sonicated on ice (3x10s). The protein content of the supernatant was determined according to Bradford. Homogenate samples representing 10 µl of total protein from islet were then run on 7.5 % SDS-polyacrylamide gel (Bio-Rad, Hercules, CA, USA). After electrophoresis, proteins were transferred to nitrocellulose membranes for 10-15 V, 60min (Bio-Rad). The membranes were blocked in LS-buffer (10 mmol/l Tris, pH 7.4, 100 mmol/l NaCl, 0.1 % Tween-20) containing 5 % non-fat dry milk powder for 40 min at 37°C. Incubation was performed with rabbit anti-mouse ncNOS (N-7155) and iNOS (N-7782) (1:2000; Sigma, St Louis, MO, USA). After three washings in LS-buffer the membranes were finally incubated with a horseradish peroxidase-conjugated goat anti-rabbit antibody (1:500). Immunoreactivity was detected using an enhanced chemiluminescence reaction (Pierce, Rockford, IL, USA). The intensities of the bands were quantified by densitometry (Bio-Rad GS-710 Densitometer). SAPK/JNK, p38 and p42/44 activation were determined using a phosphorylated SAPK/JNK, p38 and p42/44 assay kit (Cell Signaling Technology, Inc, TX, USA) according to the manufacturer's instructions.

PI hydrolysis

Hydrolysis of phosphatidyl inositol (PI) was assayed in the HEK293 and MIN6c4 cells. Cells were pre-loaded with myo-[3H]inositol (Perkin Elmer, Boston, MA) for 16-20 h and then thoroughly washed and incubated in the KRB-buffer +/- different additives for 30 min. After the incubation the cells were lysed with formic acid on ice and the inositophosphates were isolated using anion exchange chromatography. The PI hydrolysis were expressed as PI hydrolysis per well.

IMMUNOFLUORESCENCE AND CONFOCAL MICROSCOPY

Isolated islets were fixed with 4% formaldehyde, permeabilized with 5% Triton X-100, and unspecific sites blocked with 5% normal donkey serum (Jackson Immunoresearch Laboratories, Inc.). iNOS was detected with rabbit-raised polyclonal anti-iNOS antibody (1:100) in combination with Cy2-conjugated anti-rabbit IgG (1:150) (StressGen Biotechnologies Corp, Victoria, BC, Canada). GPR40 was detected with a polyclonal antibody (1:100) in combination with Cy2-conjugated anti-mouse IgG (1:150). The receptor specific antibody was raised in rabbit against the C-terminal peptide: NH2-CVTRTQRGTIQK-COOH. For staining of insulin, islets

were incubated with a guinea pig-raised anti-insulin antibody (1:1000) followed by an incubation with a Cy5-conjugated anti-guinea pig IgG antibody (1:150) (Eurodiagnostica, Malmö, Sweden). For the staining of glucagon, islets were incubated with a guinea pig-raised anti-glucagon (1:600) (Eurodiagnostica, Malmö, Sweden) antibody followed by incubation with a Cy5-conjugated anti-guinea pig IgG antibody (1:150) (Jackson Immunoresearch Laboratories Inc, West Grove, PA). For the staining of somatostatin, islets were incubated with a mouse-raised anti-somatostatin (1:500) (Novo-Nordisk, Bagsvaerd, Denmark) antibody followed by incubation with a Cy5-conjugated anti-mouse IgG antibody (1:150) (Jackson Immunoresearch Laboratories Inc, West Grove, PA).The fluorescence was visualized with a Zeiss LSM510 confocal microscope by sequentially scanning at (excitation/emission) 488/505-530nm (Cy2) and 633/>650nm (Cy5).

BIOPHYSICAL METHOD

Fluorescent measurements of intracellular $[Ca^{2+}]_i$ concentrations

$[Ca^{2+}]_i$ in the intact islets was measured using a dual-wavelength microfluorimetry with fura-2 as indicator dye. The islets were loaded with 3 μmol/l fura-2 (30 min) and then transferred to 15ºC experimental chamber and were kept in place by a heat-polished glass pipette. The thermostatically controlled chamber was continuously perfused with a buffer solution containing: NaCl (140 mmol/l), KCl (3.6 mmol/l), $NaHCO_3$ (2 mmol/l), NaH_2PO_4 (0.5 mmol/l), $MgSO_4$ (0.5 mmol/l), HEPES (5 mmol/l), $CaCl_2$ (2.6 mmol/l) and the test substances.

Fluorescence signals were recorded by using a microfluorimeter system (D104, PTI, Monmouth Junction, NJ, USA) with an emission wavelength of 510 nm at alternate 350/380 nm excitation wavelength and calibrated into $[Ca^{2+}]_i$ values using the equation previously described (139). The fluorescence (F) ratio F_{350}/F_{380} was determinated at a final ratio frequency of 10 Hz. $[Ca^{2+}]_i$ was then estimated using the equation and a K_d of 224 nM. The maximum ratio (R_{max}) was achieved using ionomycin (60 μmol/l). Background subtraction was performed after quenching the fluorescence signal with $MnCl_2$ (1 mmol/l). To reduce the noise in detected signals we used IGOR-program.

FLUOROMETRIC METHOD

Caspase-3 activity

The activity of caspase-3 was determined by monitoring the cleavage of a specific fluorogenic caspase-3 substrate Ac-DEVD-AMC (ac-Asp-Glu-Val-Asp-AMC (Upstate cell signalling solutions, NY, USA). The cells were washed with PBS and then lysed with the lysis buffer. Thereafter the homogenates were allowed to react with the fluorogenic caspase-3 substrate in a 96-well plate (3 µl to each well) in a reaction buffer containing HEPES (20 mmol/l), glycerol (10 %) and DTT (2 mmol/l). The mixtures were maintained at 37° C for 60 min (darkness) and subsequently analysed in a fluorometer (FLUO Star) equipped with excitation wavelength of 390 nm and emission wavelength of 460 nm. The results were correlated to the protein concentration of each well measured according to Bradford (138). The effect of palmitate on the caspase-3 activity was compared to the effect induced by a triple of cytokines (IL-1β: 30 ng/ml, TNFα: 150 ng/ml and INFγ: 150 ng/ml). The same procedure was also performed with isolated islets.

CELL CULTURE AND TRANSFECTION

Culturing of cells

A subclone of the MIN6 cell line, MIN6c4, was grown in DMEM with Glutamax-1 (Invitrogen, Paisley, UK) supplemented with 15% heat-inactivated FBS (Invitrogen), β-mercaptoethanol (60 Mm), penicillin (50 U/ml) and streptomycin (50 µg/ml). HEK293 cells were grown in DMEM with Glutamax-1 supplemented with 3% FBS, penicillin (50 U/ml) and streptomycin (50 µg/ml). All cells were maintained in a 37 °C incubator with 7% CO_2.

Transfection of cells

The mouse GPR40 ORF (Genbank accession number AB095745) was amplified with PCR (forward primer, 5' GCCAAGCTTACCATGGACCTGCCCCCACAGCTCTCCT- TCG 3';reverse primer, 5'GGCGAATTCCTACTTCTGAATTGTTCCTCTTTGAGTC 3'), subcloned into the pEAK12 expression vector (Edge Bio Systems, Gaithersburg, MD), and then transfected into HEK293 cells using lipofectamine 2000 (Invitrogen), according to the manufacturer's instructions. Total time of transfection was 6 h and the cells were assayed 48 h later.

Antisense intervention

Isolated islets were incubated for 30 min with palmitate (1 mmol/l) in KRB solution. After washing islets were then cultured for 48 h in the absence or presence of M40 morpholino oligonucleotide (1.4 μmol/l). At day 2 (after 24 h culture) palmitate (1 mmol/l) was added to the culture medium and the islets were cultured for an additional period of 24 h. A nonspecific random-sequence morpholino was used as control (90). The morpholino oligonucleotide was loaded into the islets using the Gene-Tools special delivery system according to the manufacturers' instructions.

IN VIVO STUDIES

Freely fed animals were used throughout. Blood sampling was performed as previously described (125) for the determination of insulin and glucose. Plasma glucose concentrations were determined with a glucose oxidase method and insulin with a radioimmunoassay (RIA) (125).

STATISTICAL ANALYSIS

Probability levels of random differences were determined by analysis of variance followed by Tukey-Kramer's multiple comparisons test. Student's unpaired *t*-test was also used to detect the level of the significance for the difference between sets of data. Results were expressed as means ± S.E.M. $P < 0.05$ was considered statistically significant.

RESULTS AND DISCUSSION

1. Action of palmitate on β-cell function with special regard to activation of GPR40, MAPK, NOS, caspase-3 and the effects of the antidiabetic drug rosiglitazone (ROZ)

We examined which intracellular signals are induced when palmitate interacts with the GPR40 receptor. For this purpose we used two cell lines *i.e.* HEK293 and MIN6c4 as well as isolated pancreatic islets. As HEK293 cells transiently express GPR40, they show a marked PIP_2 turn over and IP_3 generation in response to FFA. A result of similar character was observed when MIN6c4 cells were used. The rapid turn over in phospholipid metabolism and generation of IP_3 in response to an FFA challenge in the β-cell is dependent on GPR40, since inhibition or ablation of GPR40 by specific drug or antisense M40 treatment, respectively, was associated with a similar decrease in the β-cell response to FFA. This result favours the concept that GPR40 plays a pivotal role in the FFA-stimulated insulin secretion. Our conclusion is based on the finding that FFA rapidly potentiated glucose-stimulated increase in $[Ca^{2+}]_i$. It should be noted that the involvement of GPR40 in the acute stimulatory effects of FFA fits well with the results from other groups showing that the stimulatory effects of FFA on the insulin secretion is markedly reduced in GPR40-KO mice (140; 141).

Common pathophysiological signalling molecules that contribute to the development of β-cell failure are often associated with an increased ER-stress (142). FFA are known to increase β-cell stress that might contribute to insulin secretory defects. A sustained and long-term-stimulated increase in the IP_3 generation by FFA may in part explain the link between FFA and ER-stress. Moreover, many stress- and inflammation related kinases such SAPK/JNK and p38 have been shown to be involved in the β-cell dysfunction and function as pro-apoptotic signals (143). Our data provided the first evidence that FFA through GPR40-coupled signalling pathways increase the activities of p42/44, SAPK/JNK and p38 within 30 min of incubation. It has been shown that p42/44 is involved in cell proliferation, differentiation and mainly functions as anti-apoptotic signal (144). The role of FFA in regulating MAPK pathways with subsequent activation of

downstream biological functions is largely undefined. Previous investigations have shown that an elevated level of iNOS-derived NO induced by lipopolysaccharide (LPS) would cause the stimulation of p38 and SAPK/JNK (145-147). In contrast to these studies, we found an increased activity of SAPK/JNK, p38 and p42/44 after a short time of FFA-stimuli, where no iNOS was expressed. The data presented here indicates, that an increased $[Ca^{2+}]_i$ by FFA, pressed the increased activities of p42/44, SAPK/JNK and p38 showing the importance of $[Ca^{2+}]_i$ for the activation of these MAPK. A long-term (24h) stimulus of islets with FFA was accompanied by induction of iNOS and surprisingly suppression of only p42/p44 activity. Thus the suppression of p42/p44 might be a consequence of an exaggerated iNOS-derived NO production, since a recent study has shown that the activity of p42/p44 is inhibited by large amounts of NO (148). In other tissues SAPK/JNK and p38 are involved in apoptotic pathways stimulated by cytokines (149). Our results showed that prolonged exposure to FFA might damage the β-cell by activating noncytokine induced apoptotic signals.

Another feature of this toxic effect of FFA on the β-cell function was our finding of an increased NO production due to both an increased activity of ncNOS and also the induction of iNOS. Increased islet iNOS-derived NO production might be a common denominator in both glucotoxicity and lipotoxicity. It should be kept in mind that the expression of iNOS was induced during long-term culturing of islets with palmitate. This confirms the data of previous observations, showing that the ability of FFA to induce iNOS activity is time-dependent and can not be observed during short-term exposure of the islets to FFA (56; 57; 150). This effect of palmitate is different from glucose, since glucose has been shown to induce iNOS expression in isolated islets during short-term incubation (60-90 min) (55; 126). These data indicate that iNOS expression and activity in β-cells is regulated through different or partially different mechanisms after m exposure to palmitate *vs* glucose. It has been shown previously that iNOS is involved in cytokine-induced β-cell dysfunction (151). Using iNOS inhibitors, it has been shown that the cytotoxic effect of pro-inflammatory cytokines which is partially mediated by iNOS could be at least to some extent reversed by the inhibitor (125; 126). Our present results

confirmed that a highly increased NO generation suppresses glucose-stimulated insulin release after islet culture with palmitate.

Previous studies have indicated that activated SAPK/JNK and p38 interact with the caspase cascade in controlling the apoptotic process induced by combinations of cytokines such as IL-1β, TNF-α, IFN-γ (152; 153). Our present data show that FFA stimulates the activity of caspase-3 *i.e.* one of the member of the caspase family by a non-inflammatory pathway. Since the caspase-3 pathway is required for cytokine-induced β-cell apoptosis, the present data suggest that caspase-3 plays an important role in lipotoxicity-induced β-cell dysfunction.

Finally, another most prominent finding of the present study is that the deleterious action of FFA on the β-cells is counteracted by the thiazolidinedione drug ROZ. We could show that ROZ acutely inhibited GPR40 transduction mechanisms and abrogated both the stimulatory action FFA on the potentiation of glucose-stimulated enhancement of $[Ca^{2+}]_i$ and insulin release. In contrast, ROZ didn't influence pure glucose-stimulated increase in $[Ca^{2+}]_i$ and insulin secretion. It is well documented that $[Ca^{2+}]_i$ plays an important role in both glucose-induced insulin release as well as in FFA-mediated potentiation of glucose-stimulated insulin release (51; 66; 154; 155). Our finding that ROZ had a suppressive action on palmitate-induced $[Ca^{2+}]_i$ and also on palmitate-stimulated insulin release suggests that the interaction of ROZ with the secretory processes exerted at a step which is not showed by the secretory pathway for glucose. It is tempting to speculate that in the long run the increased $[Ca^{2+}]_i$ by FFA might in turn enhance the activities of wide variety of MAPKs among which SAPK/JNK and p38 are most interesting since they function as pro-apoptotic signals. Importantly in long-term experiments we showed that ROZ also did counteract the stimulatory effects of FFA on the MAPKs, iNOS and caspase-3 activity. The protective effect of ROZ on β-cell function through the interaction of these parameters was reflected in a normal β-cell response to a subsequent FFA/glucose challenge.

Concluding remarks. The present data strongly suggest that ameliorating the effect of FFA on β-cell function by the anti-diabetic drug ROZ or by interference with receptor expression (M40) is a consequence of a blockade of GPR40-coupled signalling pathways. These experiments thus suggest that ROZ protects the β-cells through, at least in part, counteracting FFA-induced activation of GPR40 followed by suppression of the sustained and exaggerated increase in $[Ca^{2+}]_i$, paralleled by suppression of MAPKs, iNOS and finally suppression of caspase-3. The ability to manipulate pharmacologically the dynamics of GPR40 expression in the islet β-cells may herald a new strategy for designing drugs that efficiently prevent FFA-related β-cell dysfunction and successfully prevent obesity and T2D. We are suggesting that thiazolidinediones are beneficial for β-cell function and survival in hyperlipidemia and lipotoxic conditions.

Based on our present findings, a schematic illustration of how FFA *via* GPR40 regulates the p42/p44, SAPK/JNK and p38 signal transduction pathway in β-cells is shown in Figure 3.

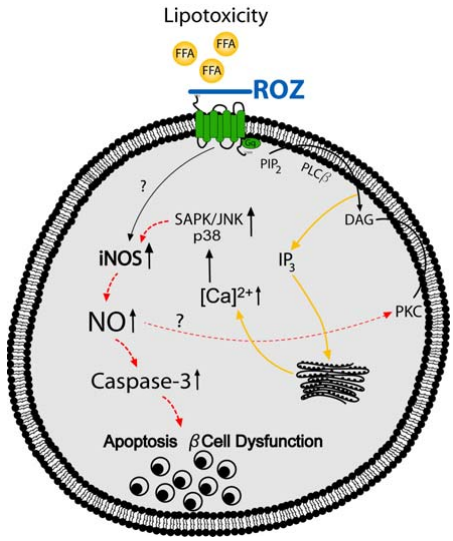

Figure 3. Schematic illustration of the regulation of SAPK/JNK, p38 and iNOS signal transduction pathways by FFA *via* GPR40 in β-cells

2. Palmitate-stimulated hormone secretion in relation to GPR40 expression in pancreatic islets of spontaneous obesity and type 2 diabetes in rats (paper III)

The present results strongly provide evidence for a causal link between the expression levels of GPR40 and FFA-induced modulation of insulin, glucagon and somatostatin secretion. Notably we found that not only the insulin and glucagon cells but also the somatostatin cells were equipped with GPR40. According to the observed data the low expression of GPR40 in the islets of GK rat and the high expression of GPR40 in the islets of the ZDF rats were associated with a concordant hormone releasing response to FFA by these islets. The decreased expression of GPR40 in the GK rats could not be attributed to any major changes in the contribution of β-cells to total islet cell mass, since at this age, islet insulin content and the serum glucose, TG, cholesterol, HDL and LDL levels, were almost of the same magnitude as in Wistar controls. Interestingly, the reduction in GPR40 expression in the islets of GK rats coincided with a markedly low response of these islets to palmitate stimulus. The low expression of GPR40 in the islets of GK rats share key characteristic features with certain effects when ROZ was present. The important point here is that although an islet of the GK rat normally exhibits a wide variety of defects (77-79; 156), an adequate expression of GPR40 seems to be of utmost importance for the proper response of islet cells to FFA.

In contrast to GK islets, the islets of ZDF rats, a model of obesity secondary to genetic unresponsiveness to leptin (157; 158) show a markedly increased expression of GPR40 accompanied by an exaggerated response of these islets to FFA. An exaggerated insulin response to enhanced circulating levels of FFA by the islets of ZDF rats seems to be a possible explanation to why these rats become rapidly obese. The data of the present study are thus in good accordance with a recent study showing a weak cause-effect relationship between the enzymes involved in islet FFA oxidation and FFA-stimulated insulin release (90; 159; 160). We used animals at the age of 6-8 weeks to study the hyperlipidemic ZDF rats in the preobese, prehyperglycemic state. The blood parameters of metabolic signals such as TG, cholesterol, HDL and LDL were of the same magnitude in GK and Wistar control rats except for blood glucose which was increased in the GK

rats. On the other hand, the blood glucose level was within the normal range in the ZDF rats whereas blood TG, cholesterol and HDL were slightly elevated while LDL was slightly reduced. The reason for the extremely low expression of GPR40 in the GK islets is presently unclear but it is not inconceivable that it might be a consequence of the high blood glucose levels.

Increased plasma concentrations of glucose are associated with defects exerted by the increased metabolic and secretory activities of the β-cells (2; 77; 126; 161). The subsequent oxidative stress evoked by the metabolic and secretory pathways has been proposed to play a pathogenic role in many cellular anomalies involved in the induction of an abnormal β-cell function (162). However, the cellular mechanisms for the lower or higher expression of GPR40 in the GK and ZDF islets irrespectively, remain unclear. In an attempt to distinguish between genetically responsible factors in contrast to environmental *i.e.* a high ambient glucose or FFA level, the islets of Wistar rats with a normal expression pattern of GPR40 were cultured in the presence of both low and high glucose concentrations as well as low and high FFA. These data support a particularly important role for high glucose to suppress the expression of GPR40 and mildly increased FFA to stimulate an increased expression of the receptor.

In conclusion, by using different approaches it was found that GPR40 expression in the islets is highly related to FFA-stimulated insulin and glucagon secretion as well as to FFA-induced suppression of somatostatin secretion from pancreatic islets isolated from two highly different animal models of T2D. The data thus strongly suggest the GPR40 mediates not only insulin and glucagon responses but also the somatostatin response to FFA-stimulation.

3. Excessive islet NO generation in type 2 diabetic GK rats coincides with abnormal hormone secretion and is counteracted by GLP-1 (paper IV)

Previous reports have shown that NO derived from iNOS is cytotoxic and implicated in the autoimmune-mediated destruction of pancreatic β-cells during the development of type 1 diabetes (163). It was now of interest to study whether ncNOS/iNOS expression and activities in the islets of a mildly diabetic T2D animal model, the GK rat, are also associated with the defective insulin secretory response reported previously (79). Since GLP-1 has gained considerable attention as insulinotropic agent in the treatment of T2D, it was of interest to investigate whether the impaired glucose-stimulated insulin release in the GK rat could be improved by GLP-1 and whether such an improvement could be related to modulation of NO generation.

The present results showed a rich occurrence of iNOS protein in both β-cells and α-cells of islets isolated from the hyperglycemic and GK rat. This observation thus raised the question whether long-term hyperglycemia might contribute to non-immunogenic diabetes by inducing iNOS in the pancreatic islets. An exaggerated NO production is known to affect multiple sites within the cell, mainly acting through S-nitrosylation (120; 164-166). Such effects of NO have been demonstrated in brain tissue, where a number of metabolic, structural, and signaling proteins were found to be afflicted by NO through S-nitrosylation processes (166). Notably very young GK rats were used in the present study to avoid any complications of diabetes on the islet cell mass (167). Thus a more or less continuous generation of NO through the action of iNOS might have deleterious effects on the β-cell function in the long run which is in good agreement with earlier studies showing that β-cell numbers were diminished in 6-months old GK rats (168). The effect of hyperglycemia on induction of iNOS in the islets of the GK rat is in accordance with previous observations showing that incubation of mouse pancreatic islets at high glucose for even short time periods (60-90 min) result in iNOS expression (55; 126). Moreover, in agreement with the hyperglycemia-induced iNOS expression seen in the GK islets, the present data also show that moving islets from normoglycemic *in vivo* milieu (Wistar rat) to a medium containing high glucose concentration (16.7 mmol/l) for a period as short as

2 h induces the expression of iNOS. In this context it should be mentioned that an abnormally increased iNOS-derived NO production also exists after *in vitro* incubation of GK rat islets at low glucose thus suggesting that moving the islets from an *in vivo* hyperglycemic milieu to a medium of a low "hypoglycemic" glucose concentration (3.3 mmol/l) for a period of ~ 2 h does not reduce or abolish iNOS expression and activity. These data thus suggest that islet iNOS activity might persist also during periods of low blood glucose in the GK rat.

It has previously been shown (55; 56; 169; 170) that a protective mechanism against the NO-mediated negative effects on β-cell function might be exerted through the cyclic AMP system. GLP-1 a suitable and potent cyclic AMP-generating incretin was used to test this hypothesis in the GK rat. The *in vitro* experiments showed that GLP-1 counteracted the impaired insulin response to glucose in islets of GK rats in association with a marked suppression of islet NOS activities. This effect seems to be exerted through the cAMP/PKA system since in previous experiments with rat islets (55) in the presence of the PKA inhibitor H-89, GLP-1 did not suppress iNOS expression. The proteasome system however, did not interact with the GLP-1-induced suppression of iNOS since the proteasome inhibitor MG132, did not appreciably influence this suppressive effect of GLP-1. In contrast to the modulatory effect of GLP-1 on insulin secretion which was dependent on the ambient glucose concentration the suppressive effect of GLP-1 on islet NOS activities did not seem to be glucose-dependent. Our *in vivo* experiments showed that a combination of GLP-1 and glucose induced a fairly good insulin response in GK rats, while the response to glucose alone was abrogated. These results agreed with our *in vitro* data. These effects of GLP-1 are most likely attributed to its ability to increase the islet cAMP/PKA system as previously suggested from our data in healthy animals (55-57; 150).

The suppressive effect of GLP-1 on the abnormal hypersecretion of glucagon from GK islets was observed at both low and high glucose *in vitro*. The abnormal secretion of glucagon observed *in vivo* in GK rats was only partially restored by administration of GLP-1. The data thus illustrate that GLP-1 has a good amplifying effect on glucose-stimulated insulin release but might be less efficient in strengthening glucose-induced

glucagon suppression *in vivo* in this model of T2D. Hence the present data strongly suggest that an abnormally increased NO production in α-cells might contribute to the abnormal glucagon hypersecretion in diabetes.

In conclusion, we believe that the importance of the deleterious effects of excessive islet NO production explain some if not all of the impairments of islet hormone secretion and β-cell survival. The present data show that abnormally increased expression and activities of islet NOS isoenzymes coincide with increased glucagon secretion and decreased glucose-stimulated insulin release and thus that an excessive NO production is an important contributing factor for the diabetic condition. GLP-1 can, only in part, counteract these abnormalities through activating the cyclic AMP/PKA system. Our data also suggest that high concentrations of circulating glucagon is important for the diabetic condition even in the presence of GLP-1, although this incretin hormone contributes to an increased insulin release through suppressing the NOS activities in the β-cell. These novel data on an NO-generated glucotoxic action in GK islets might be applicable, at least in part, to human T2D and hopefully pave the way for new therapeutic interventions to reduce islet NO overproduction.

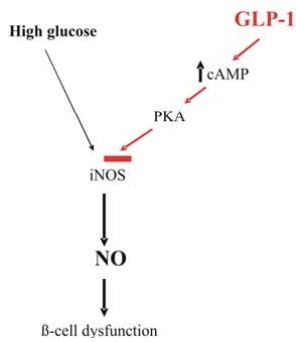

Figure 4. Schematic illustration of GLP-1 effects on the β-cell function

4. The imidazoline RX781024 effectively counteracts the negative impact of β-cell NO generation on insulin release stimulated by glucose (paper V)

Our investigation was performed to compare *in vitro* and *in vivo* the effects of the imidazoline derivative RX781024 (RX) on insulin release induced by glucose and carbachol and to explore the possibility whether RX interferes with islet NOS activities. RX has reportedly the ability not only to close the ATP-sensitive K^+ (K^+_{ATP}) channels but also to directly promote the exocytotic process in the β-cells (171; 172). Carbachol exerts a stimulatory effect on insulin secretion though G-protein-coupled receptors with subsequent increase in IP_3 generation and PKC activation (173), while glucose has multiple intracellular effects including closure of the K^+_{ATP} channels (61).

Our study shows, that the glucose-stimulated increase in ncNOS-derived NO production and glucose-induced expression of iNOS was markedly suppressed by RX. This effect of RX was associated with amplification of insulin release. A rapid generation of NO seems to be coupled, as a negative modulator, to the acute stimulation of the insulin secretory processes induced by both glucose and carbachol (76; 125; 126; 170; 174-176). The amplifying effects of RX seen on both glucose- and carbachol-induced insulin release (*in vitro* and *in vivo*) could well be coupled to the inhibition of NO production exerted by RX. Hence, the dynamic pattern of the *in vivo* insulin secretory responses following a glucose challenge after RX pretreatment is comparable to a similar experiment where the mice were pre-treated with the ncNOS inhibitor L-NAME (174). Further, previously it has been shown that pharmacological blockade of NOS activities resulted in an immediate amplification of insulin release by perfused isolated islets stimulated by glucose or carbachol (126; 176). Our data are also in good accordance with a previous report by Akesson and Lundquist, 1998 showing that blockade of NOS activities amplified insulin release induced by the phorbol ester TPA (Tetradecanoylphorbol Acetate). In contrast to carbachol, which is known to stimulate both the IP_3-Ca^{2+} and the DAG-PKC messenger system, TPA stimulates only PKC. Hence, PKC seems to be a main target for NO. This is conceivable since NO is known to inhibit many regulatory proteins through S-nitrosylation processes (177) and PKC is known to be equipped with

critical thiol-residues (178). This does not exclude that the influence of RX on the K^+_{ATP} channels makes it possible to reduce the threshold for the stimulatory action of carbachol. The *in vivo* experiments further confirmed that RX has an immediate amplifying effect on the insulin response following *i.v.* glucose or carbachol, which seems to be in good accordance with the rapid effects of these secretagogues on the generation of islet NO (126; 174; 179).

The suppressive effect of RX on islet NOS activities could also be observed during long-term culture of islets at high glucose. This protective and beneficial effect of RX was reflected in an amplifying insulin release after a subsequent glucose challenge. Moreover, these results suggest, that a progressive iNOS-derived NO generation being potentially destructive for the β-cells over time. However, our previous experiments (126; 174) have shown that, in contrast to ncNOS activity, which rises within minutes after exposure to high glucose, iNOS activity does not appear until the end of an incubation period of 60-90 min.

In conclusion, the present data bring evidence that the beneficial effects of the imidazoline derivative RX on insulin release induced by glucose and/or cholinergic muscarinic stimulation are coupled, at least in part, to an inhibitory action on the islet ncNOS- and iNOS-derived NO production. RX profoundly suppressed iNOS expression after both short-time and long-time exposure to high concentrations of glucose in association with an amplifying action on glucose-stimulated insulin release. The results underline the notion that imidazoline derivatives are potential candidates for treatment of β-cell dysfunction in hyperglycemia and T2D.

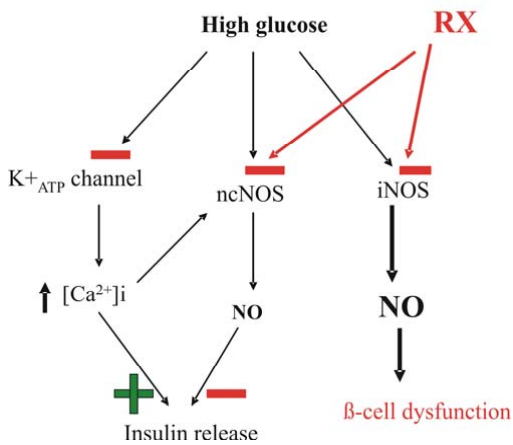

Figure 5. Schematic presentation of imidazolin effect on the β-cell function

GENERAL CONCLUSIONS

Modulation of lipotoxicity:

1. Chronic exposure of islets to FFA for 24h impair the β-cell insulin response to a further FFA-challenge

2. The insulin response to a subsequent glucose challenge is also diminished when the islets are cultured with FFA for 24h

3. Rosiglitazone protects β-cells from adverse signals induced by FFA. This thiazolidinedione drug modulates β-cell protection as partial agonist through the GPR40 receptor.

Modulation of glucotoxicity:

1. The imidazoline derivative RX has great amplifying action on glucose stimulated insulin secretion by profoundly suppressing islet NO production.

2. The incretin hormone GLP-1 has a positive impact on glucotoxic action in the β-cell, by effectively suppressing iNOS that in turn negatively modulates β-cell function.

POPULÄRVETENSKAPLING SAMMANFATTNING

Blodsocker rivån regleras av hormonerna insulin och glucagon som frisåttts fråm vissa celler i bukspottkörteln (pancreas). Insulinet som frisätts från β-celler i de Langerhanska öarna är den enda hormon som har en blodsocker sänkande effekt genom att underlätta socker upptaget i olika värnaler i kroppen. Glucagonet däremot har det motsatta effkten till insulin dvs den höjer blodsockret genom att påverka levern för att producera socker. En annan intressant hormon som också frisälts från en tredje cell typ i de Langerhanska öarna är somatostatin. Somatostatinet i sig har ingen effekt på socker upptang i vävnader eller socker produktion i levern men den kan hämma kraftigt frisättninggen av insulin och glukagon. En sänkning av somatostatinfrisättningen kan följakttligen leda till en ökad sekretion av både insulin och glukagon.

En nedsatt insulin frisättning (relativ insulinbrist) under en längre period kan orsaka förhöjda blodsockernivåer. Höga halter av sockre kan utöva en toxist effekt på alla celler i kroppen (glukotoxisitet). En sädan tillstönd utan behandling kan leda till folksjukdomen typ 2 diabetes (åldersdiabetes).
Ett flertal vetenskapliga studier har visat att risken att drabbas av åldersdiabetes är starkt kopplad till fetma (speciallt bukfetma) och sjukdomsutvecklingen också påskyndas vid förhöjda blodfetter (cirkulerande fria fettsyror). Sålädes förhöjda halter av fria fettsyror under en längre period kan vara toxiskt för olika celler i kroppen. Detta fenomen i vetenskaplinga sammanhang kallas lipotoxicitet. Under normal förhållande fungerar fria fettsyror i blodet inte bara som föda åt kroppens olika celler utan också fungerar de som signalmolekyler.

Följande upptäkt har gjorts under avhandlingsarbetet:
Vi har funnit att fria fettsyrors effekt på insulin, glukagon och somatostatin frisättning går för det mesta dels genom ett membran receptor (motagre) avs GPR40.

ACKNOWLEDGEMENTS

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Palmitate-Induced β-Cell Dysfunction Is Associated with Excessive NO Production and Is Reversed by Thiazolidinedione-Mediated Inhibition of GPR40 Transduction Mechanisms

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Abstract

Background: Type 2 diabetes often displays hyperlipidemia. We examined palmitate effects on pancreatic islet function in relation to FFA receptor GPR40, NO generation, insulin release, and the PPARγ agonistic thiazolidinedione, rosiglitazone.

Principal Findings: Rosiglitazone suppressed acute palmitate-stimulated GPR40-transduced PI hydrolysis in HEK293 cells and insulin release from MIN6c cells and mouse islets. Culturing islets 24 h with palmitate at 5 mmol/l glucose induced β-cell iNOS expression as revealed by confocal microscopy and increased the activities of ncNOS and iNOS associated with suppression of glucose-stimulated insulin response. Rosiglitazone reversed these effects. The expression of iNOS after high-glucose culturing was unaffected by rosiglitazone. Downregulation of GPR40 by antisense treatment abrogated GPR40 expression and suppressed palmitate-induced iNOS activity and insulin release.

Conclusion: We conclude that, in addition to mediating acute FFA-stimulated insulin release, GPR40 is an important regulator of iNOS expression and dysfunctional insulin release during long-term exposure to FFA. The adverse effects of palmitate were counteracted by rosiglitazone at GPR40, suggesting that thiazolidinediones are beneficial for β-cell function in hyperlipidemic type 2 diabetes.

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Introduction

The nutrients glucose and free fatty acids (FFA) are known to have a great impact on the function of pancreatic β-cells [1,2,3]. Although glucose is the major stimulus for insulin secretion, its effects are highly modulated by FFA. Insulin secretion might thus be acutely amplified or chronically inhibited by FFA-derived signals [1,2,3]. Although interaction between FFA and β-cells plays an important role in insulin secretion, the intimate targets responsible for FFA actions on β-cells are under debate and FFA and cytokines have been claimed to induce β-cell apoptosis by different mechanisms [3]. The acute stimulatory effects have been linked to the action of long chain acyl-CoA molecules on a variety of metabolic sites involved in the insulin secretory pathways [1,2,3]. This concept has recently been challenged since the stimulatory action of FFA on insulin secretion, at least in part, was shown to be mediated through a membrane-bound FFA receptor, the G protein-coupled receptor 40 (GPR40) [4,5,6]. Notably, the peroxisome proliferator-activated receptor γ (PPARγ), a member of the nuclear receptor superfamily, is involved in islet FFA metabolism. PPARγ is modulated by *e.g.* prostaglandin J2, leukotrine B4 and by a number of recently developed synthetic agents (thiazolidinediones) like rosiglitazone (ROZ) [7,8,9,10]. Since FFA are involved in developing insulin resistance, synthetic agonists of PPARγ have been used clinically to improve glucose tolerance by enhancing insulin sensitivity of adipocytes to suppress lipolysis thus reducing the metabolic burden to liver and muscle that in turn improves glucose homeostasis [7,8,9,10].

Since we have shown that long-term intralipid infusion in rats is accompanied by expression of inducible nitric oxide synthase (iNOS) in pancreatic islets [11,12,13], and since excessive NO generation derived from both iNOS and neuronal constitutive NOS (ncNOS) seems involved in impairment of glucose-stimulated insulin release and β-cell dysfunction [14,15,16,17, 18,19,20], we found it essential to explore in more detail the effects of FFA on pancreatic islet function. Hence the aim of the present investigation was to study both acute and especially long-term effects of palmitate and its interaction with the PPARγ agonist ROZ on the activities of islet NOS isoenzymes in relation to GPR40 and insulin secretion and thus to further elucidate whether

the thiazolidinedione drugs would be of possible therapeutic value for the function of the β-cell in dyslipidemic type 2 diabetes.

Results

Acute effects of palmitate and ROZ on PI hydrolysis in GPR40-transfected HEK293 cells and their interaction with PI hydrolysis and insulin release in MIN6c4 cells, as well as palmitate-induced effects on islet NOS activities and effects of ROZ and diazoxide on insulin release from isolated islets

We first tested the acute action-interaction of palmitate in relation to ROZ on PI hydrolysis in HEK293 cells transiently expressed with mouse GPR40. HEK293 cells do not express endogenous GPR40 [21] and is well suited to explore the immediate response to GPR40 ligands after transient expression of the receptor. **Fig. 1A** shows that PI hydrolysis after 30 min incubation of GPR40-transfected HEK293 cells in presence of 1 mmol/l palmitate is highly increased compared with nontransfected controls and that ROZ by itself has a significant agonistic action. **Fig. 1B**, on the other hand, shows that ROZ has an inhibitory action on the palmitate-stimulated PI hydrolysis in transfected cells.

Next we used MIN6c4 insulinoma cells, known to endogenously express GPR40 [21] to study whether ROZ interacts with the reported FFA-stimulated activation of PLC *via* GPR40 in insulin-producing cells. MIN6c4 cells were challenged with palmitate for 30 min in absence or presence of ROZ, and PI hydrolysis was analyzed. **Fig. 1C and D**, shows that palmitate-stimulated PI hydrolysis and insulin release in fact were dose-dependently suppressed by ROZ.

To test possible involvement of NOS enzyme activities in relation to palmitate-induced insulin release in acute experiments with primary β-cells, freshly isolated islets were incubated for 60 min. **Fig. 1E** shows basal and acute palmitate-modulated NOS activities and insulin release at 5 mmol/l glucose. ncNOS activity was increased but iNOS was not detectable. Notably, a restraining action of palmitate-induced insulin release through ncNOS-derived NO was revealed by showing a greatly amplified insulin release (**Fig. 1F**) in the presence of the ncNOS inhibitor L-NAME. Hence ncNOS-generated NO abrogated a major part of the palmitate-induced insulin release. Finally, **Fig. 1G** shows that the acute amplifying effect of palmitate on glucose-stimulated insulin release was greatly suppressed by ROZ. In comparison the K_{ATP}-channel opener diazoxide was even more efficient (**Fig. 1G**). A similar, although not identical pattern was seen for palmitate-induced insulin release at low glucose (**Fig. 1G**).

Pattern of insulin secretion and islet insulin content during long-term culturing with palmitate or glucose in the absence or presence of ROZ or diazoxide

Long-term experiments with palmitate or glucose were performed to study effects of ROZ or for comparison, diazoxide, on insulin secretion and insulin content in islets cultured for 24 h in absence or presence of either agent. **Fig. 2** shows that both palmitate and a high concentration of glucose (20 mmol/l) not only increased insulin release into the culture medium (**Fig. 2A**) but also slightly increased islet insulin content (**Fig. 2B**). ROZ suppressed insulin secretion during culture with palmitate, while slightly amplifying insulin secretion in presence of high glucose (**Fig. 2A**). Diazoxide suppressed insulin secretion during culture with high glucose and only marginally inhibited it in presence of palmitate. ROZ or diazoxide did not affect islet insulin content (**Fig. 2B**).

Effects of islet culturing with palmitate in presence and absence of ROZ or diazoxide on islet NOS activities and glucose-stimulated insulin secretion

Islets cultured for 24 h at 5 mmol/l glucose (basal) or 5 mmol/l glucose+palmitate in absence or presence of ROZ or diazoxide were analyzed for ncNOS and iNOS activities. **Fig. 3A** shows that palmitate did not only induce a marked increase in ncNOS activity but also an exclusive activity of iNOS. The stimulatory effect of palmitate on ncNOS and iNOS activities was markedly attenuated when ROZ was present during the culture period. Diazoxide only slightly influenced the stimulatory effect of palmitate on NOS activities (**Fig. 3A**).

A major part of the cultured islets from the same culturing batches were thereafter washed, preincubated in 1 mmol/l glucose for 30 min and incubated at 1 or 20 mmol/l glucose for 60 min. **Fig. 3B** illustrates that insulin release was slightly increased at low glucose when islets had been cultured with palmitate but not when ROZ or diazoxide was present. Conversely, glucose-stimulated insulin release was markedly attenuated after culturing with palmitate. This suppressive effect by palmitate was completely reversed when ROZ was present during the culture period but only slightly so when diazoxide was present.

Confocal microscopy

The cellular distribution of iNOS protein was examined with confocal microscopy. **Fig. 4** shows that after culture with palmitate (D–F) or high glucose (J–L) iNOS immunoreactivity was expressed in most islet cells, which also displayed insulin immunoreactivity. No iNOS immunoreactivity was detected in islets cultured at basal glucose (A–C). Addition of ROZ to culture medium suppressed palmitate-induced iNOS expression in β-cells (G–I), whereas glucose-induced expression of iNOS induced by high glucose was not affected (M–O).

Suppression of GPR40 by antisense M40 in cultured pancreatic islets abrogated palmitate-induced expression of iNOS and suppressed the activities of ncNOS and iNOS as well as insulin secretion

To explore whether GPR40 is involved in both palmitate-induced expression of islet iNOS and palmitate stimulation of insulin release during culture with 5 mmol/l glucose we used an antisense (M40) targeting the sequence important for the GPR40 transcript in islets. After culturing, islets were thoroughly washed and processed for the measurement of ncNOS and iNOS activities, insulin release into medium, and detection of iNOS protein. **Fig. 5A** shows abrogation of palmitate-induced iNOS activity, reduction of ncNOS activity, and inhibition of insulin secretion into culture medium in M40-treated islets. Confocal microscopy showed that palmitate-induced iNOS expression was colocalized with GPR40 (**Fig. 5B, A**–C) and abrogated together with GPR40 expression after M40 treatment (D–F). The colocalization of insulin, GPR40 and iNOS is shown in single β-cells (**Fig. 5C**) (A–D). The loss of GPR40 and iNOS proteins after M40 treatment is also shown (E–H).

Discussion

It is known that FFA are positive modulators of insulin secretion in short-time perspective but become toxic to β-cells when chronically present in elevated levels (lipotoxicity) [1,2,3,8,9,10]. Thus prolonged exposure of β-cells to FFA leads to increased basal but suppressed glucose-stimulated insulin secretion [1,2,8,9,10]. Since many type 2 diabetic patients exhibit elevated plasma levels

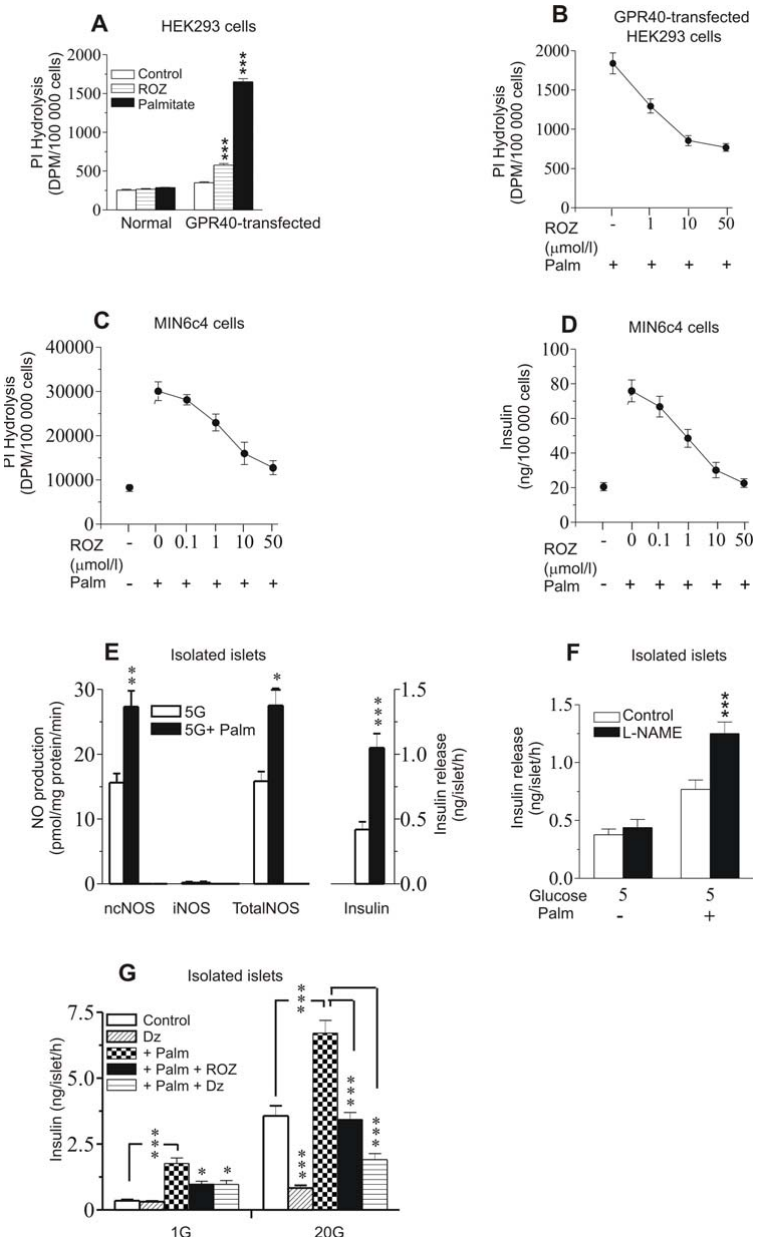

Figure 1. Short-time effects of palmitate and rosiglitazone (ROZ) on phosphatidyl inositol (PI) hydrolysis, in HEK293 and MIN6c4 cells, insulin release in MIN6c4 cells as well as NO production and insulin release in isolated islets. (A) PI hydrolysis in nontransfected and GPR40-transfected HEK293 cells in response to palmitate (1 mmol/l) and ROZ (50 μmol/l) (n = 8–12) and **(B)**; dose-dependent effect of ROZ on palmitate-induced PI hydrolysis (n = 8-12). **(C, D)** PI hydrolysis and insulin release in MIN6c4 cells in response to palmitate (1 mmol/l)±ROZ at different concentrations (n = 6). **(E)** NO production from neuronal constitutive nitric oxide synthase (ncNOS), inducible NOS (iNOS) and total NOS as well as insulin release in response to palmitate (1 mmol/l) after 60 min incubation of freshly isolated mouse islets at 5 mmol/l glucose (5G) (n = 4). **(F)** Effect of the NOS inhibitor L-NAME on insulin release induced by palmitate (1 mmol/l) in the presence of 5 mmol/l glucose (n = 8). **(G)** Effects of palmitate±ROZ or diazoxide on insulin release from freshly isolated mouse islets incubated at low (1 mmol/l) or high (20 mmol/l) glucose for 60 min. The concentrations of the different test agent were; palmitate (1 mmol/l), ROZ (1 μmol/l), diazoxide (dz) (250 μmol/l) (n = 8-12). Values are mean±s.e.m for. ** $p<0.01$; *** $p<0.001$.

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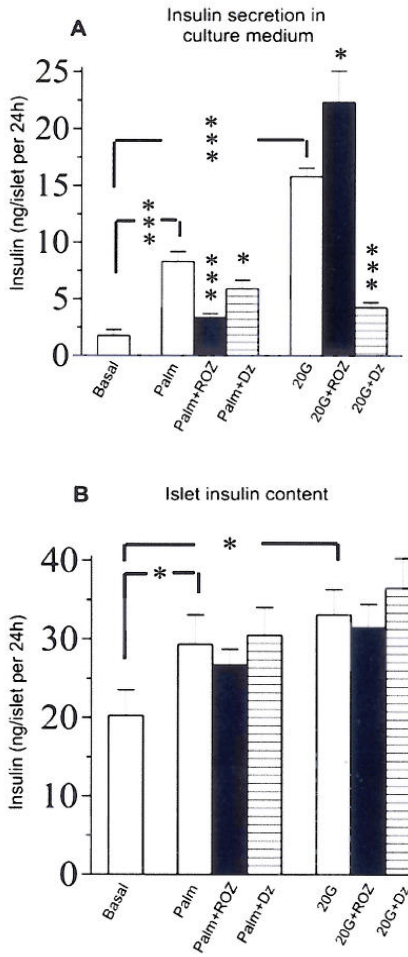

Figure 2. Insulin secretion and islet insulin content after culturing with palmitate or high glucose. Insulin secretion into culture medium (**A**) and islet insulin content (**B**) from isolated islets cultured for 24 h at a basal glucose concentration of 5 mmol/l (5G), 5G+palmitate (1 mmol/l) (Palm), 5G+palmitate+rosiglitazone (1 μmol/l) (Palm+ROZ) or 5G+palmitate+diazoxide (250 μmol/l) (Palm+Dz) as well as at high glucose concentration (20 mmol/l) (20G), 20G+rosiglitazone (1 μmol/l) (20G+ROZ) or 20G+diazoxide (250 μmol/l) (20G+Dz). The means±s.e.m for 10-12 batches of islets in each group are shown. Asterisks denote probability level of random difference. * $P<0.05$; ***$P<0.001$.

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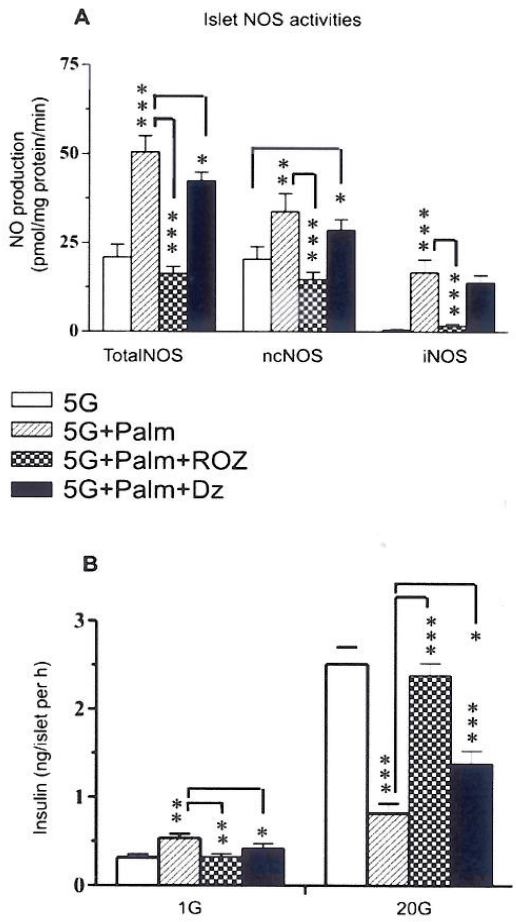

Figure 3. Islet NO generation from neuronal constitutive nitric oxide synthase (ncNOS), inducible NOS (iNOS), and total NOS after culturing with palmitate for 24 h as well as insulin release at basal and high glucose after a subsequent incubation. (A) Total NOS as well as ncNOS and iNOS activities in isolated islets cultured at a basal glucose concentration of 5 mmol/l (5G), 5G+palmitate (1 mmol/l) (Palm), 5G+palmitate+rosiglitazone (1 μmol/l) (Palm+ROZ) or 5G+palmitate+diazoxide (250 μmol/l) (Palm+Dz) (n = 8). **(B)** Insulin release at low (1 mmol/l) glucose or stimulated by high (20 mmol/l) glucose from islets incubated for 60 min after culturing and washing. The means±s.e.m for 10-12 batches of islets in each group are shown. Asterisks denote probability level of random difference. * $P<0.05$; ** $P<0.01$; ***$P<0.001$.

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of FFA and/or increased glucose levels, the underlying mechanisms for the deleterious action of long-term elevation of FFA on β-cells and glucose-induced insulin release would be highly important to elucidate. The present results favor the view that the adverse effects of chronic exposure to FFA is exerted mainly through the newly discovered FFA receptor GPR40 by inducing expression and activity of β-cell iNOS, a highly increased NO generation, and suppression of glucose-stimulated insulin release. Moreover, we show that this deleterious action of FFA on the β-cells is counteracted by the thiazolidinedione drug ROZ, which inhibits GPR40 transduction mechanisms and thus abrogates iNOS expression and in addition restores glucose-stimulated insulin release.

In our initial acute experiments we verified the reported acute activation of PLC and PI hydrolysis by GPR40-mediated

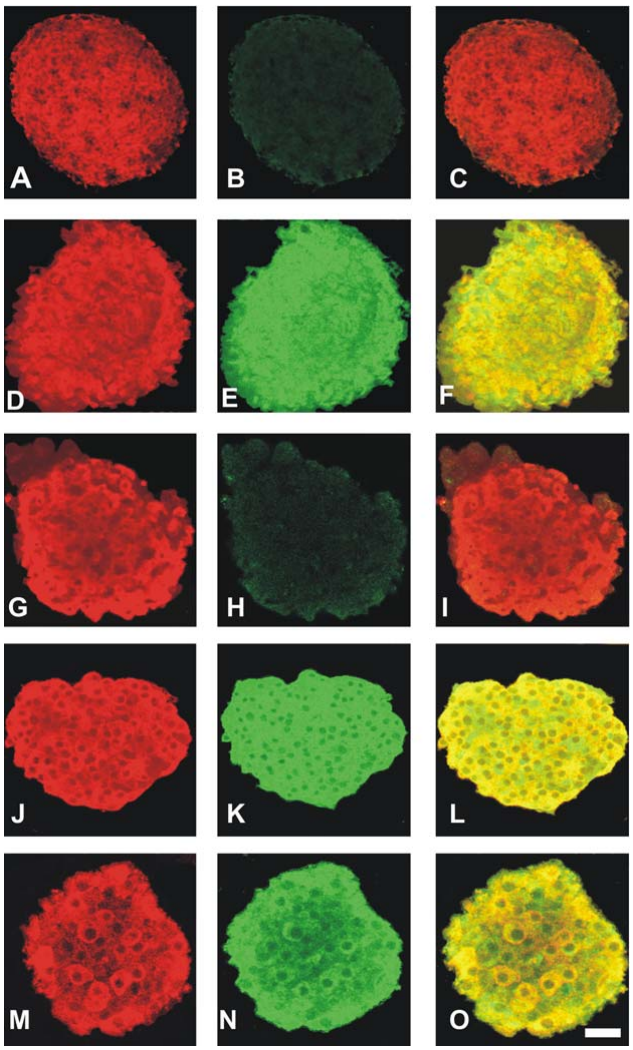

Figure 4. Confocal microscopy of mouse islets. Isolated islets were cultured for 24 h at a basal glucose concentration of 5 mmol/l (A–C); 5G+palmitate (1 mmol/l) (D–F); 5G+palmitate+rosiglitazone (1 μmol/l) (G–I) or 20G (J–L) and 20G+rosiglitazone (1 μmol/l) (M-O). After the culturing period the islets were double-immunolabelled for insulin (appears as red) (A, D, G, J and M) and iNOS (appears as green) (B, E, H, K and N) and analyzed by confocal microscopy. Co-localization of insulin/iNOS is seen as a yellowish fluorescence (C, F, I, L and O). Fluorescence intensity data of iNOS (green) were normalized to 100% as measured in E (5G+palmitate) and gave the following results (n = 12). B = 1.50±0.75; E = 100.6±2.52; H = 4.33±1.50; K = 99.0±2.96; N = 106.1±3.22.
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stimulation of FFA [5] and now show that this effect when induced by palmitate was inhibited by ROZ in HEK293 cells transiently transfected with GPR40 and in MIN6c4 insulin-producing cells with endogenously expressed GPR40, where ROZ was found to suppress palmitate-stimulated insulin release. These results appear to contradict earlier reports [21] suggesting ROZ to be a GPR40 agonist. However, the previous reports [21,22] were based on recombinant cell systems with high receptor densities. The most likely explanation is that ROZ is a partial agonist with high affinity and relatively low efficacy, and thus being more dependent on receptor density. We show now that ROZ served as a potent GPR40 inhibitor of palmitate-induced PI hydrolysis and that ROZ dose-dependently suppressed palmitate-stimulated PI hydrolysis in GPR40-transfected HEK293 cells as well as PI hydrolysis and insulin release in MIN6c4 cells. Moreover, we show that acute palmitate-stimulating effects on insulin release

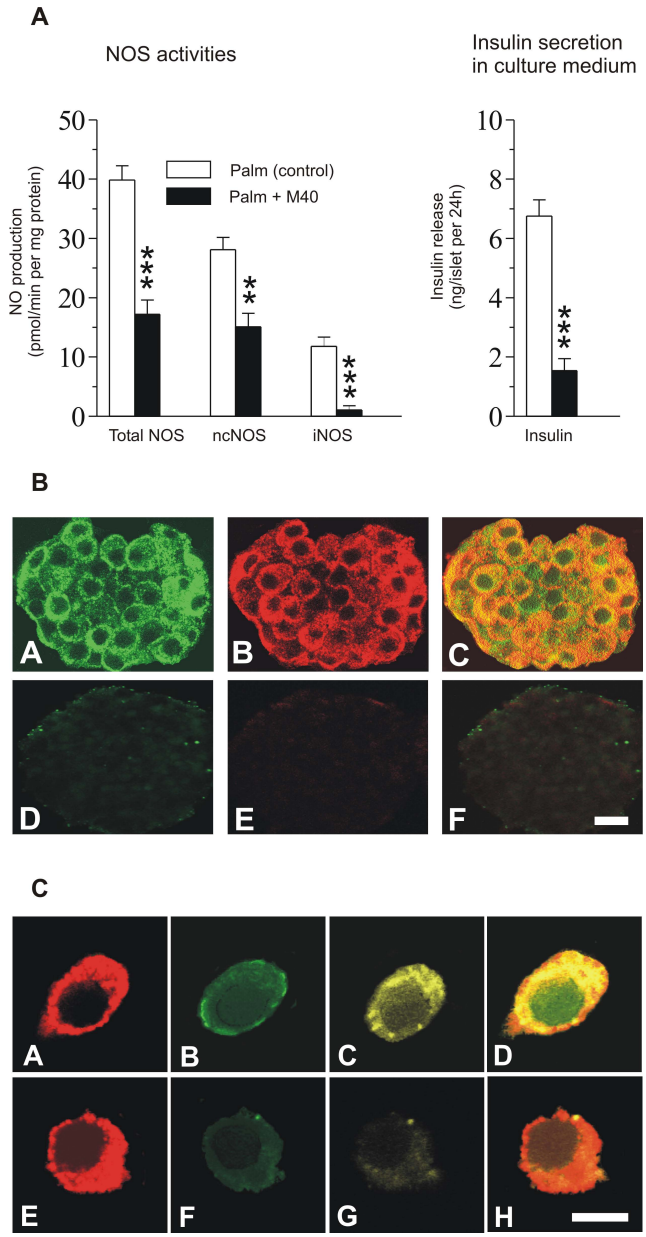

Figure 5. Islet NOS activities, insulin secretion, and expression of GPR40 and iNOS after treatment with palmitate and GPR40 antisense. Isolated islets were pretreated for 24 h with either the M40 antisense morpholino or a non-specific random sequence morpholino (control). The islets were incubated for 30 min in absence or presence of palmitate and then cultured with palmitate±M40 for a further 24 h. **(A)** M40 caused a marked suppression of palmitate-induced iNOS and ncNOS activities as well as a reduced insulin release (black bars). The results from the control morpholino are indicated by white bars. Values are mean±s.e.m for 4 different experiments performed at different occasions. ** $p<0.01$; *** $p<0.001$. **(B)** Expression of GPR40 (green) and iNOS (red) in islets cultured with palmitate (A-C) or palmitate+M40 (D-F). A and D = GPR40; B and E = iNOS, C and F = overlay. Bar indicates 5 μm. **(C)** Immunostaining and confocal images of formaldehyde-fixed β-cells. The expression pattern of insulin (red), GPR40 (green) and iNOS (yellow) from dispersed β-cells cultured with palmitate is shown. A = insulin, B = GPR40, C = iNOS and D = overlay. E–H show absence of expression of GPR40 (F) and iNOS (G) after M40 treatment. E = insulin, H = overlay. Bar indicates 5 μm.
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were associated with increased ncNOS-derived NO generation, the inhibition of which by the ncNOS inhibitor L-NAME resulted in marked amplification of palmitate-induced insulin response. This acute restraining action by ncNOS-derived NO on palmitate-induced insulin release thus agrees with our previous observations on acute glucose-induced insulin release [17,18,19,20].

We have postulated and presented suggestive data showing that ncNOS-derived NO is rapidly stimulated by glucose and exerts an acute negative feedback action on glucose-induced insulin release [18]. This effect is elicited within minutes and probably involved in creating the nadir separating first and second phase insulin response. This is in accordance with the notion that part of the ncNOS protein is confined to the insulin secretory granules [23]. We also found that the Ca^{2+}/calmodulin-dependent ncNOS activity was markedly inhibited by the ATP-sensitive K^+ channel opener diazoxide [18]. In contrast to the rapid action of glucose on ncNOS activity, there was no effect by glucose on iNOS expression until after ~1 h of exposure to high glucose *in vitro* and *in vivo* [18,20]. The Ca^{2+}/calmodulin-independent iNOS activity was not inhibited by diazoxide [18] and iNOS-derived NO reportedly exerts cytotoxic and apoptotic effects in β-cells [16,24]. Importantly, however, in contrast to glucose, the present data show that acute exposure to palmitate did not induce iNOS activity after short-time (~1 h) incubation suggesting distinct time-dependent mechanisms for palmitate *vs* glucose in iNOS induction and activity. Finally, the acute amplifying effect by palmitate on glucose-stimulated insulin release, which reportedly is linked to activation of GPR40 [5,6,25], we now found to be suppressed by ROZ, while the glucose-stimulated part of the release process was unaffected.

We have shown earlier that glucose-stimulated insulin release is disrupted in islets from long-term lipid-infused rats whose islets displayed increased NO production due to induction of iNOS expression [11,12,13]. Since we repeatedly found [17,18,19,20] that increased islet NO production in presence of high glucose inhibits insulin release and most likely is taking part in a process of glucotoxicity we are now inclined to ascribe increased iNOS-derived NO in islets cultured with palmitate as an important factor in FFA-induced lipotoxicity. Although there seems to be distinct differences in induction and prevention of iNOS expression and activity elicited by glucose *vs* FFA, increased islet iNOS-derived NO production might be a common denominator in both glucotoxicity and lipotoxicity.

Since ROZ, although debated, has been suggested to be an alternative drug in long-term therapy of type 2 diabetes we were particularly interested in the action of ROZ on long-term effects of FFA, as represented by palmitate, on islet activation of ncNOS and iNOS in relation to glucose-stimulated insulin release. After culturing with palmitate at basal glucose concentration insulin release into medium was enhanced 3-fold compared with basal glucose alone, while islets cultured at high glucose displayed almost 7-fold increase in insulin. Diazoxide abrogated this glucose-induced insulin response, whereas palmitate-induced insulin release was only slightly reduced, suggesting K^+_{ATP} channel-dependent Ca^{2+} influx mechanisms over time being less important for palmitate-stimulated insulin secretion during this long-term culturing compared with acute effects and possibly more influenced by intracellular Ca^{2+} perturbations [25]. Importantly, during culturing ROZ markedly reduced palmitate-induced but slightly increased glucose-induced insulin secretion into medium. These differences did not depend on islet insulin content, but are likely related to partially differential effects on insulin secretory mechanisms. PPARγ receptors in β-cells show reportedly a comparatively low expression [8] and hence other effects than directly on these receptors might also play a role in the effects of ROZ on insulin release as previously have been suggested [26]. Studies in models of type 2 diabetes have shown that thiazolidinediones enhance β-cell function by mobilizing fat out of the cells [27]. Moreover, it was very recently shown [28] that toxic accumulation of cholesterol in β-cells and associated defects in insulin release could be restored by ROZ through activation of the cholesterol efflux transporter Abca 1, which is upregulated by PPARγ activation. Hence, thiazolidinediones exert diverse beneficial effects on β-cell function as recently has been extensively discussed [9]. Now we show that islets cultured with palmitate displayed marked expression and activity of β-cell iNOS, increased ncNOS activity and a greatly reduced insulin response following a 60 min glucose challenge. After culturing with palmitate+ROZ, however, the increased NO production was abrogated and glucose-stimulated insulin release restored to normal suggesting that ROZ has a direct inhibitory effect on β-cell GPR40. Conversely, after culturing in high glucose+ROZ confocal microscopy showed no effect by ROZ on β-cell iNOS expression. These findings suggest, but do not definitely prove, that iNOS expression and activity in β-cells is regulated through different or partially different mechanisms after long-term exposure to palmitate *vs* glucose. It should be noted that when ROZ was replaced by diazoxide during palmitate culturing iNOS activity was unaffected and ncNOS activity and glucose-stimulated insulin release were less suppressed than after palmitate+ROZ. Attenuation of insulin release by K_{ATP} channel openers like diazoxide is ascribed to β-cell hyperpolarization, thereby providing β-cell rest. Notably, our present data suggest that such a protective effect on glucose-stimulated insulin secretion after diazoxide treatment [29] is not operating when high glucose is replaced by palmitate. Hence, although the preserving effect by diazoxide on glucose-stimulated insulin release is reportedly beneficial [29], our present data suggest that this "resting" effect is less important in presence of enhanced levels of FFA (palmitate).

The present data favor the idea that excessive and longstanding generation of NO derived from iNOS is an important player in FFA-induced β-cell dysfunction after long-term culturing. This is accordance with previous data obtained in isolated islets from prediabetic Zucker diabetic fatty rats [30]. In contrast Cnop et al [31] using isolated β-cells from normal rats did not detect any iNOS mRNA expression or nitrite production in their FFA experiments. However, Cnop et al did not measure the expression and activity of iNOS protein. It is known that a great deal of endogenously generated NO is trapped within the cell by *e.g.* S-nitrosylation [32] and thus nitrite production into the incubation medium might not truly mirror the intracellular situation. Interestingly a very recent report [33] showed that both (60 min) and long-time culturing (20 h) of rat islets with the thiazolidinedione troglitazone stimulated AMP-activated protein kinase (AMPK) activity, which was associated with a decrease of glucose-stimulated insulin release after short-time and an increase after long-time treatment. Although the experimental conditions in their study were not strictly comparable to our study these data show that AMPK might be involved in the thiazolidinedione action on the β-cell. In another study with isolated β-cells [34] FFA-induced cytotoxicity was found not to be decreased but instead increased after treatment with troglitazone. Since the experimental conditions in that study was different from ours, especially with regard to the use of isolated β-cells instead of islets, the data are not comparable. Notably, however, isolated islet β-cells lack the presence of islet glucagon and glucagon-induced stimulation of β-cell adenylate cyclase is expected to suppress iNOS expression and activity [12,13,20].

We finally explored the involvement of the FFA receptor GPR40 in the long-term action of palmitate to induce iNOS expression and influence palmitate-stimulated insulin release. It has recently been suggested that GPR40 is necessary but not sufficient for FFA stimulation of insulin secretion *in vivo* [35]. However, these results are at variance with another recent study [6] and because the data of these experimental studies were widely different further studies are needed to finally elucidate this issue. Our results suggest that knock-down of GPR40 suppressed both palmitate-induced activity of iNOS and insulin release. Confocal microscopy confirmed the antisense inhibition of both GPR40 and iNOS expression in the β-cells. Hence, GPR40 is a major regulator of iNOS expression and insulin release during long-term exposure to palmitate. The palmitate-induced NO generation and associated suppression of glucose-stimulated insulin release is counteracted by ROZ at the GPR40 receptor, and thus, ROZ and other thiazolidinedione drugs might be beneficial for β-cell function in hyperlipidemic type 2 diabetes..

Materials and Methods

Animals

Female mice of the NMRI strain (B&K, Sollentuna, Sweden) weighing 25–30 g were used. They were given a standard pellet diet (B&K) and tap water *ad libitum* throughout the experiments. All animals used for preparation of pancreatic islets were killed by cervical dislocation. The experimental procedures were approved by the Ethical Committee for Animal Research at University of Lund; Sweden.

Drugs and chemicals

Collagenase (CLS 4) was from Sigma Chemical Co (St Louis, MO, USA), HRP-conjugated goat anti-rabbit IgG was from Pierce Biotechnology, Rockford, IL, USA. Cy2-conjugated anti-mouse IgG and Cy5-conjugated anti-guinea pig IgG were from Jackson Immunoresearch Laboratories Inc, West Grove, PA, USA. Guinea pig-raised anti-insulin antibody was from Eurodiagnostica, Malmö, Sweden. Fatty acid free bovine serum albumin (BSA) was from Boehringer Mannheim, Germany. ROZ was a kind gift from Glaxo Smith-Kline, London, UK. N^G-nitro-L-arginine methyl ester (L-NAME) was from Sigma. The insulin radioimmunoassay kits were from Diagnostika, Falkenberg, Sweden. All other chemicals were from Merck AG, (Darmstadt, Germany) or Sigma.

Islet culturing and insulin secretion

Preparation of pancreatic islets from the mouse was performed by retrograde injection of a collagenase solution *via* the bile-pancreatic duct [14]. Islets were then isolated and hand-picked under a stereomicroscope at room temperature. After washing the islets were either used for short-term experiments and incubated as described previously [12] or used for culturing experiments. The islets were thereby cultured for 24 h in RPMI 1640 (SVA, Uppsala, Sweden) supplemented with 10% calf serum, 100 U/ml penicillin and 10 μg/ml streptomycin in the presence or absence of different test agents as indicated in the legends. After culturing the islets were preincubated for 30 min at 37°C in Krebs-Ringer bicarbonate buffer, pH 7.4, supplemented with 10 mmol/l HEPES, 0.1% BSA and 1.0 mmol/l glucose. After preincubation the buffer was changed and the islets were incubated at 1 or 20 mmol/l glucose for 60 min at 37°C unless otherwise stated. Each incubation vial contained 12 islets in 1.0 ml of buffer solution and was gassed with 95% O_2-5% CO_2 to obtain constant pH and oxygenation. All incubations were performed in an incubation box at 30 cycles/min. An aliquot of the medium was removed immediately after incubation and frozen for the subsequent assay of insulin. Palmitate was dissolved in ethanol (95%) with subsequent addition of stoichiometric amounts of NaOH. The stock solution was carefully evaporated under nitrogen gas. The dried residue was dissolved in water and thereafter heated to create a hot soap. The palmitate solution was stirred and fatty acid free BSA was added (10% w/v) and the pH was adjusted to 7.4 with NaOH. The solution was aliquoted and stored at –20 C. At the time of experiments, the stock solution of palmitate-BSA was diluted 1:10 in the KRB or RPMI 1640 buffer to achieve the desired concentration of palmitate. All components of the buffer used in the experiments were prepared to be 10% more concentrated to adjust for the addition of palmitate-BSA stock solution. Under control conditions, BSA (1% w/v) was always included. The procedure has been described in detail previously [36]. In some experiments the islets were cultured for 24 h in RPMI 1640 (SVA, Uppsala, Sweden) supplemented with 10% calf serum, 100 U/ml penicillin and 10 μg/ml streptomycin in the presence or absence of either a mouse GPR40 (mGPR40) specific antisense or a nonsense morpholino oligonucleotide (Gene-Tools) at a concentration of 1.4 μM [5].

Assay of islet NOS activities

After a culture period of 24 h, aliquots of the medium were removed for determination of insulin whereafter the islets were washed and collected in 200 μl buffer, containing 20 mmol/l HEPES, 0.5 mmol/l EDTA and 1/l mmol/l DL-dithiothreitol, and thereafter stored at −20°C. On the day of the assay, the islets were sonicated on ice and the buffer solution was enriched with 0.45 mmol/l $CaCl_2$, 2 mmol l/l NADPH, 25 U/ml calmodulin, and 0.2 mmol/l L-arginine. For the determination of iNOS activity both Ca^{2+} and calmodulin were omitted. The homogenate was incubated at 37°C under constant air bubbling with air, 1.0 ml/min for 2 h. Aliquots of the incubated homogenate (200 μl) were then passed through an 1 ml Amprep CBA cation-exchange column for determination of L-citrulline by high performance liquid chromatography (HPLC). The method has been described in detail [18]. Since L-citrulline and NO are generated in equimolar amounts, and since L-citrulline is stable whereas NO is not, L-citrulline is the preferred parameter when measuring NO production. Protein concentration was determined according to Bradford [37] on samples from the original homogenate.

Immunofluorescence and confocal microscopy

Culturing of the freshly isolated islets (24 h) in the presence of different agents were performed as stated above for the assay of islet NOS activities. An aliquot of the medium was removed for determination of insulin. The islets were then washed (3 times) and fixed with 4% formaldehyde, permeabilized with 5% Triton X-100, and unspecific sites were blocked with 5% Normal Donkey Serum (Jackson Immunoresearch Laboratories Inc, West Grove, PA). iNOS was detected with a rabbit-raised polyclonal anti-iNOS antibody (StressGen Biotechnologies Corp, Victoria, BC, Canada) (1:100) in combination with Cy2-conjugated anti-rabbit IgG (Jackson Immunoresearch Laboratories Inc, West Grove, PA) (1:150). For staining of insulin, islets were incubated with a guinea pig-raised anti-insulin antibody (Eurodiagnostica, Malmö, Sweden) (1:1000) followed by an incubation with a Cy5-conjugated anti-guinea pig IgG antibody (Jackson Immunoresearch Laboratories Inc, West Grove, PA) (1:150). For scoring of iNOS positive cells in islets multiple fields for each section were analysed under blind conditions. The mean fluorescence intensity of cellular iNOS was quantified using Zeiss LSM 5 analysis software. The methodology for detection of mouse GPR40 has recently been

described [21]. Briefly, a polyclonal antibody (1:100) in combination with Cy2-conjugated anti-mouse IgG (1:150) were used. The receptor specific antibody was raised in rabbit against the C-terminal peptide: NH2-CVTRTQRGTIQK-COOH. The fluorescence was visualized with a Zeiss LSM510 confocal microscope by sequentially scanning at (excitation/emission) 488/505–530 nm (Cy2) and 633/>650 nm (Cy5).

Cell culture and transfection

A subclone of the MIN6 cell line, MIN6c4, was grown in Dulbecco's modified Eagle's medium (DMEM) with Glutamax-1 (Invitrogen, Paisley, UK) supplemented with 15% heat-inactivated FBS (Invitrogen), 60 μM β-mercaptoethanol, 50 U/ml penicillin, and 50 μg/ml streptomycin. HEK293 cells were grown in DMEM with Glutamax-1 supplemented with 3% FBS, 50 units/ml penicillin and 50 μg/ml streptomycin. All cells were maintained in a 37°C incubator with 7% CO2. The mouse GPR40 ORF (Genbank accession number AB095745) was amplified with PCR (forward primer, 5′ GCCAAGCTTACCATGGACCTGCCCCCACAGC-TCTCCTTCG 3′; reverse primer, 5′ GGCGAATTCCTACT-TCTGAATTGTTCCTCTTTGAGTC 3′), subcloned into the pEAK12 expression vector (Edge BioSystems, Gaithersburg, MD), and then transfected into HEK293 cells using Lipofectamine 2000 (Invitrogen), according to the manufacturer's instructions. Total time of transfection was 6 h and the cells were assayed 48 h later.

PI hydrolysis

Receptor activation by FFA was assayed in the HEK293 and MIN6c4 cells by measuring hydrolysis of phosphatidyl inositol (PI) [38]. Briefly, MIN6c4 cells (approximately 100000 cells per well) were pre-loaded with myo-[3H]inositol (Perkin Elmer, Boston, MA) for 16–20 h and then thoroughly washed and incubated in the KRB-buffer at 8.3 mmol/l glucose in the absence or presence of palmitate (1 mmol/l)±ROZ (at different concentrations) for 30 min. After the incubation the cells were lysed with formic acid on ice and the inositol phosphates were isolated using anion exchange chromatography. The PI hydrolysis were expressed as PI hydrolysis per well. The coefficient of variation (interassay differences) was 7% for MIN6c4 cells.

Antisense intervention

Isolated islets (250 islets/vial) were incubated for 30 min with palmitate (1 mmol/l) in KRB solution. After washing islets were then cultured for 48 h in the absence or presence of 1.4 μmol/l of M40 morpholino oligonucleotide. At day 2 (after 24 h culture) palmitate (1 mmol/l) was added to the culture medium and the islets were cultured for an additional period of 24 h. A nonspecific random-sequence morpholino was used as control [5]. The morpholino oligonucleotide was loaded into the islets using the Gene-Tools special delivery system according to the manufacturers instructions.

Determination of insulin

Insulin secretion and insulin content of the islets were determined by radioimmunoassay [39].

Statistics

Results were expressed as means±s.e.m. The level of significance for the difference between sets of data was assessed using Student's unpaired *t*-test or analysis of variance followed by Tukey-Kramer's test whenever appropriate. $P<0.05$ was considered statistically significant.

Acknowledgments

The technical assistance of Britt-Marie Nilsson is gratefully acknowledged.

Author Contributions

Conceived and designed the experiments: AS. Performed the experiments: SM BO EF. Analyzed the data: AS SM BO IL JG. Contributed reagents/materials/analysis tools: AS SM. Wrote the paper: AS.

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Cellular and Molecular Life Sciences

Research Article

Rosiglitazone counteracts palmitate-induced β-cell dysfunction by suppression of MAP kinase, inducible nitric oxide synthase and caspase 3 activities

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Abstract. Chronic exposure of pancreatic islets to elevated levels of palmitate leads to β-cell dysfunction. We examined possible involvement of mitogen-activated protein kinases (MAPKs) and caspase-3 in palmitate-induced β-cell dysfunction and tested the influence of the anti-diabetic drug rosiglitazone (ROZ). Palmitate amplified glucose-stimulated augmentation of intracellular free calcium ($[Ca^{2+}]_i$) and insulin secretion in incubated islets. ROZ suppressed this amplification, whereas it modestly augmented glucose-induced increase in these events. ROZ suppressed short-term palmitate-induced phosphorylation of pro-apoptotic MAPKs, *i.e.*, SAPK/JNK and p38. Long-term islet culturing with palmitate induced inducible nitric oxide synthase (iNOS) and activated SAPK/JNK-p38. ROZ counteracted these effects. Both palmitate and cytokines activated caspase-3 in MIN6c4-cells and isolated islets. ROZ suppressed palmitate- but not cytokine-induced caspase-3 activation. Finally, after palmitate culturing, ROZ reversed the inhibitory effect on glucose-stimulated insulin release. We suggest that ROZ counteracts palmitate-induced deleterious effects on β-cell function *via* suppression of iNOS, pro-apoptotic MAPKs and caspase-3 activities, as evidenced by restoration of glucose-stimulated insulin release.

Keywords. Pancreatic islets, free fatty acids, insulin secretion, β-cell dysfunction.

Introduction

Pancreatic β-cells respond to free fatty acids (FFA) by integration of distinct and partially conflicting signals. Medium to long-chain FFA such as palmitate and oleate are known to have pleiotropic effects on the β-cell function [1, 2]. In contrast to the acute stimulatory action of palmitate on insulin release [2], long-term exposure of the endocrine pancreas to palmitate results in an adverse effect on β-cell function and survival [3, 4]. Previous studies have clearly demonstrated that an increase in intracellular generation of nitric oxide (NO) in the β-cell contributes to the development of several pathophysiological conditions [5–10]. It has been reported that inducible NO synthase (iNOS) in pancreatic islets is preferentially induced by macrophage-derived inflammatory agents such as various cytokines (IL-1β, TNF-α and IFN-γ). These agents are known to play a potential role in β-

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cell dysfunction and apoptosis in type 1 diabetes [9, 11–13]. Notably, we have presented evidence that also a noninflammatory and rather a metabolic induction of iNOS in the β-cell itself by elevated levels of glucose and/or FFA might be operating in otherwise healthy animals [4, 7, 8, 14–16]. Thus, we have found that an exaggerated NO production derived from islet iNOS is induced by the elevation of plasma FFA during long-term infusion of intralipids into healthy rats, and that this overproduction of NO might be implicated in the pathogenesis of β-cell dysfunction [4, 7, 8, 16]. These observations thus speak in favor of the idea that some signal(s) or metabolite(s) derived from FFA action could be involved in a noninflammatory induction of islet iNOS. It has previously been reported that hyperlipidemic patients display a markedly impaired insulin response to glucose and that hyperlipidemia is a prominent characteristic of type 2 diabetes [17]. However, the mechanisms by which FFA induce β-cell dysfunction and injury are not fully understood.

Recent studies have shown that two members of the mitogen-activated protein kinase (MAPK) superfamily, SAPK/JNK and p38, play an important role in cell dysfunction and apoptosis, whereas another member, p42/44, has an opposite effect and promotes cell proliferation, differentiation, and survival induced by a variety of cytokines or growth stimuli [18, 19]. Moreover, it has also been shown that various proinflammatory cytokines and many toxic compounds and chemotherapeutic agents might stimulate the activities of SAPK/JNK or p38, leading to cell death. In these processes caspase-3, a member of aspartate-specific cysteine proteases, is a well-known downstream executor [19–21]. The caspase families of enzymes are present within almost all cells as inactive forms and require a proteolytic process for activation [22] and thus their activation should be tightly controlled within the cell to avoid serious and deleterious consequences. Moreover, during recent years, a tight correlation between the activities of different caspases and β-cell dysfunction upon the presence of cytokines has been established [20, 21]. To our knowledge, the effects of palmitate on different MAPKs and their putative role in FFA-induced β-cell dysfunction have not been studied previously. Since it has been shown that PPARγ agonists such as the thiazolidinedione rosiglitazone (ROZ) might prevent the lipotoxic effect of FFA on the β-cells [23], we decided also to explore in more detail the influence of this drug on palmitate-induced β-cell signaling. In some initial experiments we studied short-term interactions between palmitate and ROZ by measuring intracellular Ca^{2+} ($[Ca^{2+}]_i$) and the activities of the MAPKs. We then examined long-time interactions between palmitate *versus* ROZ on the activities of MAPKs, iNOS and caspase-3 after 24-h culturing of islets or MIN6c4 cells. Finally, we tested the outcome of glucose- or glucose + palmitate-stimulated insulin release following the different culture conditions.

Materials and methods

Female mice of the NMRI strain (B & K, Sollentuna, Sweden) weighing 28–32 g were used throughout the experiments. They were given a standard pellet diet (B & K) and tap water *ad libitum*. All animals were housed in metabolic cages with constant temperature (22 °C) and 12-h light/dark cycles. The local animal welfare committee, Lund, Sweden, approved the experimental protocols.

Drugs and chemicals

Collagenase (CLS 4) was from Sigma (St. Louis, MO, USA). Fatty acid-free bovine serum albumin (BSA) was from Boehringer (Mannheim, Germany). The insulin radioimmunoassay kit was from Diagnostika (Falkenberg, Sweden). ROZ was kindly provided by GlaxoSmithKline (UK). All other chemicals were from Merck (Darmstadt, Germany) or Sigma. Palmitate was dissolved in ethanol (95 %). Before the experiments the stock solution was dissolved in the appropriate culture or incubation medium to achieve the desired concentrations of palmitate. According to the stepwise equilibrium method [24], the real concentration of palmitate in the presence of 1 % of fatty acid-free albumin is thus less than the given value in the figures. Due to a high binding capacity of BSA against FFA, the highest concentration of palmitate (1.0 mmol/l) gives rise to an approximate concentration of free palmitate of about 100 μmol/l [25].

Isolation of pancreatic islets

Preparation of mouse pancreatic islets was performed by retrograde injection of a collagenase solution *via* the bile-pancreatic duct [26]. Islets were then isolated and handpicked under a stereomicroscope at room temperature. The isolated islets were then subjected to different experimental procedures (see below).

Fluorescent measurements of $[Ca^{2+}]_i$ concentrations

As previously described [27], $[Ca^{2+}]_i$ in the intact islets was measured using a dual-wavelength microfluorimetry with fura-2 as indicator dye. Briefly, after an overnight culture period, the islets were loaded with 3 μmol/l fura-2 (30 min) and then transferred to a 15 °C experimental chamber and were kept in place by a heat-polished glass pipette. The thermostatically controlled chamber was continuously perfused with a

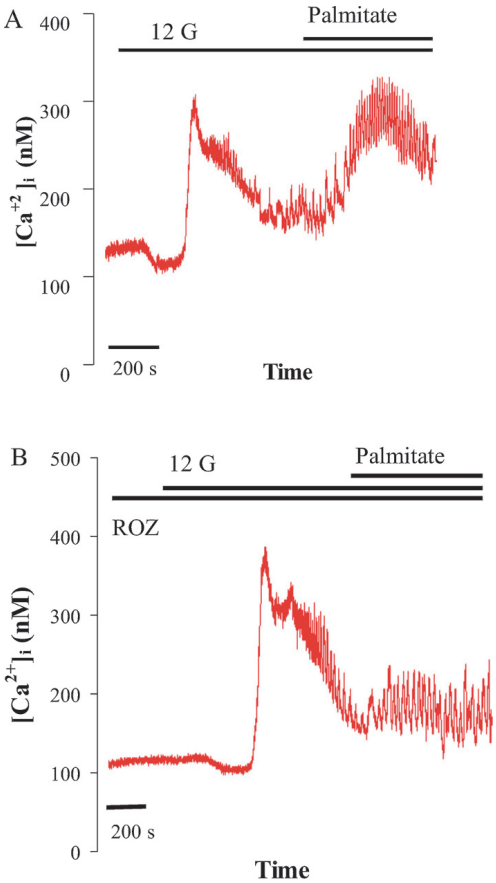

Figure 1. (*A*) Representative graph of glucose- and palmitate-stimulated increase in $[Ca^{2+}]_i$ in isolated intact islets. When glucose concentration was changed from 1 to 12 mmol/l a slight initial dip, followed by a prompt and rapid increase, in $[Ca^{2+}]_i$ was observed, which declined to a basal level a little higher than that of 1 mmol/l glucose. Addition of 1 mmol/l palmitate brought about a marked increase in $[Ca^{2+}]_i$ that, although gradually declining, was maintained at higher levels than with glucose alone. (*B*) The effects of glucose and palmitate on $[Ca^{2+}]_i$ when 1 µmol/l rosiglitazone (ROZ) was present during the experiments is shown. ROZ modestly amplified glucose-stimulated increase in $[Ca^{2+}]_i$, whereas it markedly suppressed the palmitate-stimulated amplification of $[Ca^{2+}]_i$.

buffer solution containing: 140 mmol/l NaCl, 3.6 mmol/l KCl, 2 mmol/l $NaHCO_3$, 0.5 mmol/l NaH_2PO_4, 0.5 mmol/l $MgSO_4$, 5 mmol/l HEPES, 2.6 mmol/l $CaCl_2$ and the test substances, *e.g.*, glucose and/or palmitate as indicated (pH was adjusted to 7.4).

Fluorescence signals were recorded using a microfluorimeter system (D104, PTI, Monmouth Junction, NJ, USA) with an emission wavelength of 510 nm at alternate 350/380 nm excitation wavelength and calibrated into $[Ca^{2+}]_i$ values using the equation previously described [27]. The fluorescence (F) ratio F_{350}/F_{380} was determined at a final ratio frequency of 10 Hz. $[Ca^{2+}]_i$ was then estimated using the equation given in [27] and a K_d of 224 nM. The maximum ratio (R_{max}) was achieved using 60 µmol/l ionomycin. Background subtraction was performed after quenching the fluorescence signal with 1 mmol/l $MnCl_2$. To work up the raw data in the detected signals we used the IGOR program.

Measurement of SAPK/JNK, p38 and p42/44 activities

Isolated islets (250 per vial) were incubated for 30 min in the presence of different test agents. After incubation, the islets were thoroughly washed and collected in ice-cold SDS buffer (200 µl) and stored at –20 °C for subsequent MAPK phosphorylation analysis. SAPK/JNK and p38 activation as well as p42/44 was determined using a phosphorylated SAPK/JNK, p38 and p42/44 assay kit including Western blots (Cell Signaling Technology, Inc, TX, USA) according to the manufacturer's instructions. Results were expressed as relative increase over GADPH in each tested group. In a parallel and similar experiment the islets were cultured for 24 h in the presence of the same agents prior to assay of SAPK/JNK, p38 and p42/44.

Measurement of iNOS activity

Isolated islets were either incubated for 2 h or cultured for 24 h. Thereafter, the islets (250 per vial) were thoroughly washed and collected in ice-cold DTT buffer (200 µl) and stored at –20 °C for subsequent analysis of iNOS activity. In brief, after sonication on ice, the buffer solution containing the islet homogenate was supplemented to contain 2.0 mmol/l NADPH and 0.2 mmol/l L-arginine in a total volume of 450 µl. The homogenate was then incubated at 37 °C under constant air bubbling (1.0 ml/min) for 3 h. Aliquots of the incubated medium (200 µl) were mixed with an equal volume of *o*-phthaldialdehyde reagent solution in a glass vial and then passed through an 1-ml Amprep CBA cation-exchange column for high-performance liquid chromatography (HPLC) analysis. The amount of L-citrulline formed (NO and L-citrulline are produced in equimolar concentrations) was then measured in a Hitachi F1000 fluorescence spectrophotometer (Merck) as previously described [15].

Protein

Protein was determined according to Bradford [28].

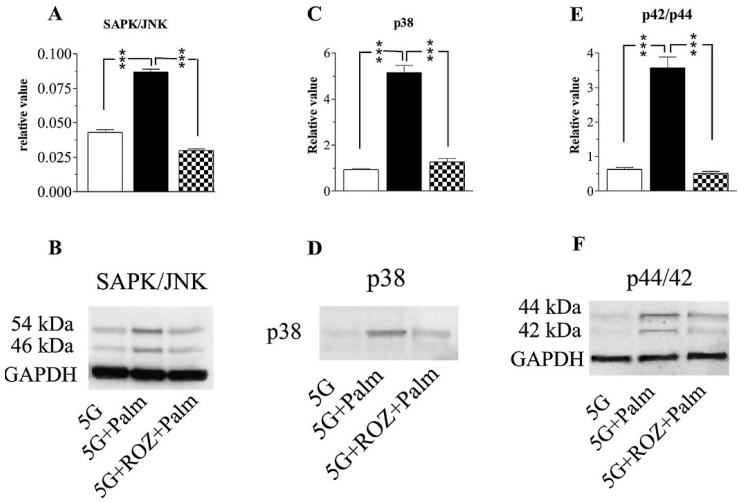

Figure 2. Suppressive effect of ROZ on short-term palmitate-induced activation of SAPK/JNK (*A*), p38 (C) and p42/44 (*E*). Pancreatic islets were preincubated with 5 mmol/l glucose (5G) alone or in combination with 1 μmol/l ROZ for 30 min, after which the medium was changed to an incubation medium containing either 5G, 5G + 1 mmol/l palmitate, or 5G + ROZ + palmitate and incubated for 30 min. After incubation, the islets were subjected to determination of SAPK/JNK, p38 and p42/44. The means ± SEM for five experiments in each group are shown. Asterisks (*) denote probability level of random difference; *** $p < 0.001$. Data for Western blots (*B*, *D* and *F*) are representative for five independent experiments in each group.

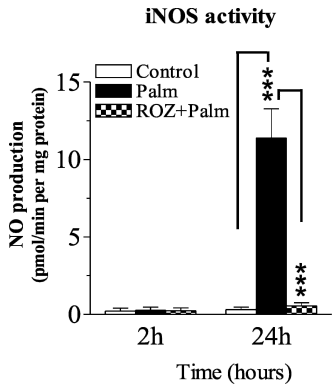

Figure 3. Inducible NO synthase (iNOS) activity measured as L-citrulline formation (pmol/min per mg protein) in isolated islets incubated for 2 h or cultured for 24 h at 5 mmol/l glucose (5G), 5G + 1 mmol/l palmitate, or 5G + 1 μmol/l ROZ + palmitate. The means ± SEM for five experiments in each group are shown. Asterisks (*) denote probability level of random difference; *** $p < 0.001$

Measurement of caspase-3 activity

The activity of caspase-3 was determined by monitoring the cleavage of a specific fluorogenic caspase-3 substrate Ac-DEVD-AMC (ac-Asp-Glu-Val-Asp-AMC; Upstate cell signaling solutions, NY, USA). The MIN6c4 cells were grown in monolayer culture with 10 % normal calf serum to 95 % confluence. The cells were thereafter washed with PBS prior to 24-h culture in culture medium containing either a basal glucose concentration of 5 mmol/l (5G), or 5G + palmitate (1 mmol/l) in the absence or presence of 1 μmol/l ROZ. After culturing, the cells were washed with PBS and then lysed with lysis buffer. Thereafter, the homogenates were allowed to react with the fluorogenic caspase-3 substrate in a 96-well plate (3 μl to each well) in a reaction buffer containing 20 mmol/l HEPES, 10 % glycerol and 2 mmol/l DTT. The mixtures were maintained at 37 °C for 60 min (darkness) and subsequently analyzed in a fluorometer (FLUO Star) equipped with excitation wavelength of 390 nm and emission wavelength of 460 nm. The results were correlated to the protein concentration of each well measured according to Bradford [28]. The effect of palmitate on the caspase-3 activity was compared to the effect induced by three cytokines (30 ng/ml IL-1β, 150 ng/ml TNF-α and 150 ng/ml IFN-γ). The same procedure was also performed with isolated islets.

Insulin secretion from MIN6c4 cells and isolated islets

Experiments with MIN6c4 cells. MIN6c4 cells were cultured in 48-well plates and, after reaching confluency, they were cultured for 24 h in the presence of different test agents as indicated in the figure legends. Thereafter the cells were washed and preincubated for 30 min at 37 °C in Krebs-Ringer bicarbonate buffer (pH 7.4), supplemented with 10 mmol/l HEPES, 1.0 % BSA and 1.0 mmol/l glucose. After preincubation the buffer was changed and the MIN6c4 cells were incubated at 1 or 12 mmol/l glucose ± 1 mmol/l palmitate for 60 min at 37 °C, to determine insulin secretion. Special care was taken to ensure that no cells were aspirated along with the collection of medium.

Experiments with isolated islets. Isolated islets were either used immediately after isolation and preincu-

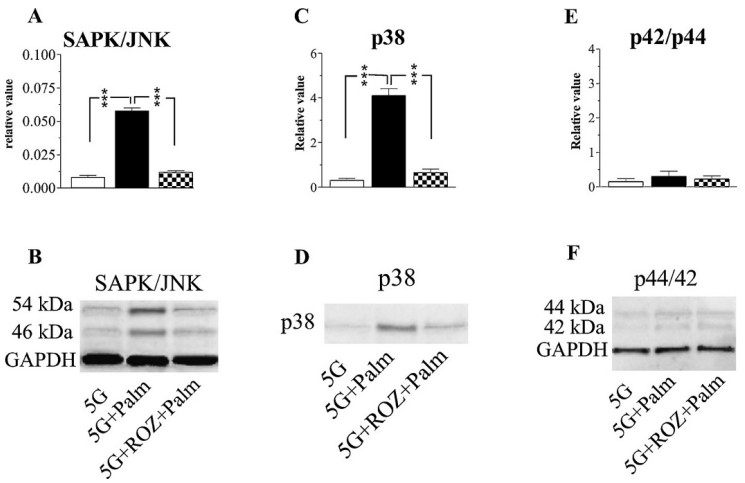

Figure 4. Suppressive effect of ROZ on palmitate-induced activation of SAPK/JNK (*A*), and p38 (*C*). p44/42 (*E*) was not affected by 24-h culturing with palmitate. Pancreatic islets were cultured for 24 h at 5 mmol/l glucose (5G), 5G + 1 mmol/l palmitate and 5G + 1 µmol/l ROZ + palmitate. Subsequently, the islets were thoroughly washed and subjected to determination of SAPK/JNK, p38 and p42/44. The means ± SEM for six experiments in each group are shown. Asterisks (*) denote probability level of random difference; *** $p < 0.001$. Data for Western (*B*, *D* and *F*) blots are representative for six independent experiments in each group.

bated (30 min) followed by incubation (30 min) or cultured for 24 h in the presence of different test agents as indicated in the figure legends. Thereafter, the cultured islets were washed and preincubated for 30 min at 37 °C in Krebs-Ringer bicarbonate buffer (as above) and 1.0 mmol/l glucose. After preincubation, the buffer was changed and the islets were incubated at 1 or 12 mmol/l glucose ± 1 mmol/l palmitate for 60 min at 37 °C. Each incubation vial contained 12 islets in 1.0 ml buffer solution and was gassed with 95 % O_2/5 % CO_2 to obtain constant pH and oxygenation. All incubations were performed in an incubation box at 30 cycles/min. An aliquot of the medium was removed immediately after incubation and frozen for the subsequent assay of insulin. The concentration of palmitate was chosen from ancillary dose-response studies in our laboratory showing a maximal effect at 1 mmol/l of this FFA, enabling us to safely detect significant changes of the measured parameters. A similar dose-response curve for another FFA, linoleic acid, was reported previously [2].

Statistics

The results are expressed as means ± SEM for the indicated number of observations or illustrated by an observation representative of a result obtained from different experiments. Probability levels of random differences were determined by analysis of variance followed by Tukey-Kramers' multiple comparisons test.

Results

Effect of ROZ on the increase in $[Ca^{2+}]_i$ and insulin secretion stimulated by glucose and palmitate

First, we wished to study the short-term influence of ROZ on palmitate-stimulated $[Ca^{2+}]_i$ and insulin release from isolated islets. As shown in Figure 1, when the islet was exposed to 12 mmol/l glucose, the prominent increase in $[Ca^{2+}]_i$ (both the initial peak and the following plateau of oscillations) was amplified when ROZ was present in the perfusion buffer. Quantification of repeated experiments showed that the Δ average $[Ca^{2+}]_i$, based on the calculation of Δ average time, was 112 ± 10 nmol/l for glucose alone *versus* 155 ± 15 nmol/l ($p < 0.05$; $n = 6$) when ROZ was included. As illustrated in Figure 1A, addition of 1 mmol/l palmitate to an islet already challenged with 12 mmol/l glucose resulted in a sustained increase in $[Ca^{2+}]_i$. Quantification showed that the Δ average $[Ca^{2+}]_i$, was 110 ± 10 nmol/l for glucose alone *versus* 185 ± 19 nmol/l ($p < 0.001$; $n = 6$) when palmitate was included. This effect of palmitate was markedly suppressed by ROZ, which was also confirmed by quantification of Δ average time calculations (194 ± 20 nmol/l for palmitate alone *versus* 116 ± 15 nmol/l ($p < 0.01$; $n = 6$) when ROZ was present (Fig. 1A and B). The action of ROZ to suppress the palmitate-stimulated elevation of $[Ca^{2+}]_i$ had its counterpart in a marked suppression of palmitate-induced insulin release. Glucose-stimulated insulin release, however, was amplified by ROZ. Thus, short-time insulin release (after 30-min incubation) at 12 mmol/l glucose was 0.36 ± 0.03 ng/islet in the absence and 0.51 ± 0.02 ng/islet in the presence of ROZ ($p < 0.05$; $n = 8$). In the presence of 12 mmol/l glucose,

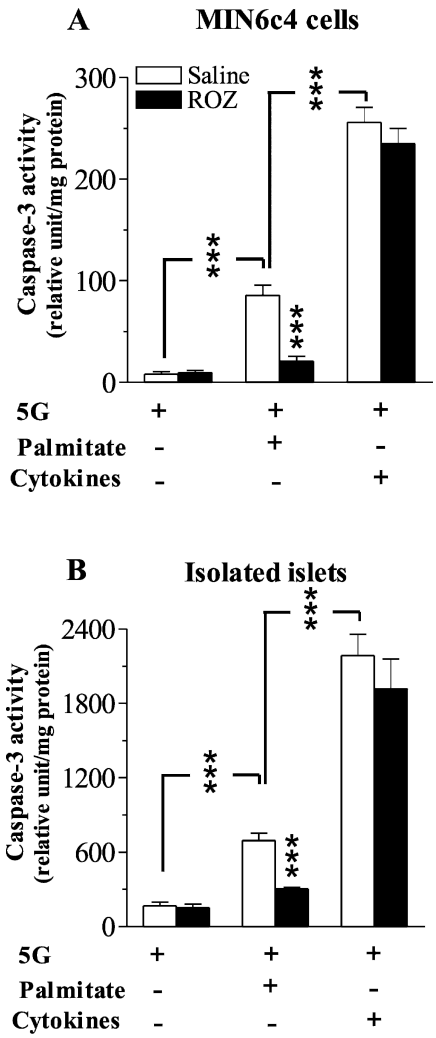

Figure 5. Caspase-3 activity in MIN6c4 cells (*A*) and isolated islets (*B*) cultured for 24 h. Effect of 1 mmol/l palmitate in absence or presence of 1 μmol/l ROZ at basal glucose concentration (5 mmol/l). For comparison, the effect of a triple-cytokine treatment (30 ng/ml IL-1β, 150 ng/ml TNF-α and 150 ng/ml IFN-γ), on caspase-3 activity is also shown. Caspase-3 activity in the cell or islet lysate was determined using caspase-3 assay kit. After the culture period the cells and the islets (175 islets/tube) were washed and lysed with lysis buffer on ice, and centrifuged at 2000 *g* at room temperature for 5 min. Supernatants were transferred to 96-well plates, and caspase-3 substrate (Ac-DEVD-AMC) and assay buffer were added. The plates were incubated at 37 °C and measured at excitation wavelength of 390 nm and emission wavelength of 460 nm. The values show caspase-3 activity–control blank per mg protein for each sample. Data are presented as means ± SEM. ($n = 8$–12). ***$p < 0.001$.

palmitate-stimulated insulin release 0.77±0.04, was markedly suppressed by ROZ to 0.54±0.02 ($p < 0.001$; $n = 8$ in each group).

Effect of palmitate on islet MAPK activities in the absence and presence of ROZ

We next investigated whether palmitate in short-time experiments (30 min) could influence the phosphorylation of SAPK/JNK, p38 and p42/p44 in isolated islets and to what extent this effect could be modulated by ROZ. Addition of 1 mmol/l palmitate to the incubation media (at a basal glucose concentration of 5 mmol/l) resulted in an increased activation of SAPK/JNK (Fig. 2A), p38 (Fig. 2C) and p42/p44 (Fig. 2E) in the islets. This effect was markedly suppressed by 1 μmol/l ROZ as also illustrated by Western blots (Fig. 2B, D and F).

Influence of ROZ on palmitate-induced islet iNOS activity

As seen in Figure 3, no iNOS activity could be detected during a 2-h incubation of islets with 1 mmol/l palmitate. However, when islets were cultured for 24 h with 1 mmol/l palmitate, a strong upregulation of iNOS activity was detected (Fig. 3). The palmitate-induced iNOS activity was markedly suppressed by ROZ (1 μmol/l) (Fig. 3).

Long-term interactions of palmitate and ROZ on islet MAPK activities

The long-term (24 h) effect of palmitate in the absence or presence of ROZ on the phosphorylation of SAPK/JNK, p38 and p42/p44 in the isolated islets was also investigated. The increased activities of SAPK/JNK (Fig. 4A) and p38 (Fig. 4C) were still evident after long-term culturing with 1 mmol/l palmitate, and also strongly suppressed by 1 μmol/l ROZ. The stimulatory effect of palmitate on p42/p44 (Fig. 4D) was, however, not observed after long-term culturing of islets with palmitate (Fig. 4). Associated Western blots are illustrated in Figure 4B, D and F.

Long-term effects of palmitate *versus* cytokines on caspase-3 activity in MIN6c4 cells and isolated islets in the absence or presence of ROZ

We used MIN6c4 insulinoma cells and isolated islets cultured for 24 h to study the effect of palmitate in the absence and presence of ROZ on caspase-3 activity. The ability of palmitate to stimulate caspase-3 activity was also compared to that of a mixture of cytokines (IL-1β, TNF-α and IFN-γ), which is known to induce caspase-3 activation [29]. As shown in Figure 5A and B, palmitate was capable of inducing a marked increase in caspase-3 activity in both MIN6c4 cells (Fig. 5A) and isolated islets (Fig. 5B), although this

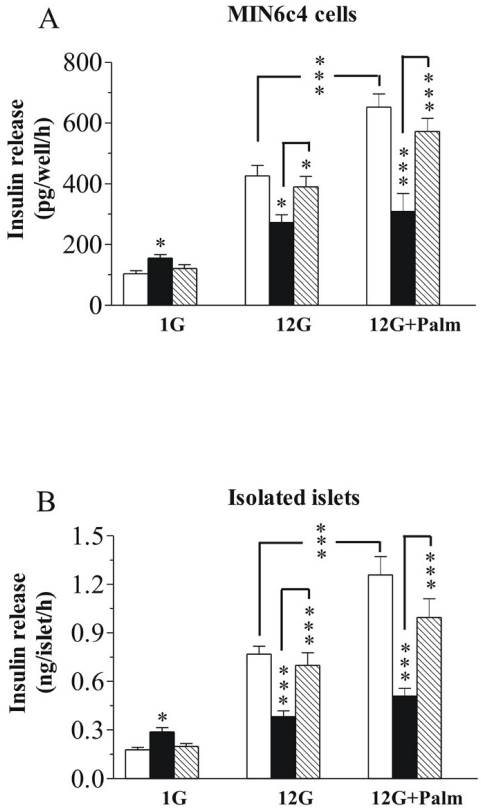

Figure 6. Effects of glucose (12 mmol/l) and palmitate (1 mmol/l) on insulin secretion from MIN6c4 cells (*A*) or isolated islets (*B*) that have been exposed to 1 mmol/l palmitate (black columns) or palmitate + 1 μmol/l ROZ (hatched columns) at 5 mmol/l glucose for 24 h. Basal controls (5 mmol/l glucose) are denoted by open columns. After culture, MIN6c4 cells and islets were washed and preincubated at 1 mmol/l glucose for 30 min followed by incubation for 60 min at 1 mmol/l glucose (1G) or 12 mmol/l glucose (12G) or glucose + palmitate (1 mmol/l). The values are means ± SEM for ten observations in each group for MIN6c4 cells (*A*) and eight observations in each group for isolated islets (*B*). Asterisks (*) denote probability level of random difference *versus* controls; * $p < 0.01$, *** $p < 0.001$.

effect was less pronounced compared to that of the cytokine mixture (Fig. 5). The effect of palmitate was markedly suppressed by 1 μmol/l ROZ, while the increase induced by the cytokines was unaffected. ROZ by itself had no appreciable effect on caspase-3 activity at basal glucose (5 mmol/l).

Effects of long-term culturing of MIN6c4 cells or isolated islets with palmitate with or without ROZ on insulin release challenging by glucose or glucose and palmitate

The insulin secretory response of MIN6c4 cells or isolated islets to glucose and palmitate after a culturing period for 24 h at 5 mmol/l glucose (5G), 5G + 1 mmol/l palmitate or 5G + 1 μmol/l ROZ + palmitate was studied. Figure 6A illustrates that insulin release was slightly increased at 1 mmol/l glucose when MIN6c4 cells had been cultured with palmitate but not when ROZ was present. The stimulatory action of glucose or glucose + palmitate on insulin release was markedly attenuated after MIN6c4 had been cultured with palmitate alone for 24 h but not when ROZ was present during the culturing period (Fig. 6A). The insulin secretory pattern of isolated mouse pancreatic islets to glucose or glucose + palmitate was similar, although more pronounced compared with that observed for MIN6c4 cells. As seen in Fig. 6B, the stimulatory action of glucose or glucose + palmitate on insulin release was markedly attenuated when islets had been cultured with palmitate for 24 h. This deleterious effect by palmitate was greatly reversed when ROZ was present during the culture period (Fig. 6B).

Discussion

Glucose tolerance tests performed in hyperlipidemic subjects often reveal a reduced insulin secretory response to glucose [30, 31]. Chronic elevation of plasma FFA has long been recognized as a major risk factor for β-cell dysfunction and the subsequent development of type 2 diabetes. Although β-cell dysfunction has been observed in pancreatic islets cultured with FFA in *in vivo* animal models of hyperlipidemia and in clinical observations of subjects with diabetes-prone obesity [1, 4, 7, 8, 16, 31], there is still controversy about the relevance of different target molecules in mediating lipotoxic effects of FFA in the β-cell that lead to the pathogenesis of type 2 diabetes. It has been claimed that lipotoxicity only in combination with glucotoxicity has a deleterious effect on islet hormone secretion [17, 32]. However, growing evidence including our own observations [4, 7, 8, 16] strongly suggests that lipotoxicity *per se* may contribute to β-cell dysfunction by several mechanisms. We have reported [4, 7, 8, 16] that hyperlipidemia induced experimentally by infusion of intralipid to rats for 8 days resulted in a markedly enhanced NO generation through a pronounced induction of iNOS expression and activity in the islets. Our present *in vitro* data confirm our previous *in vivo*

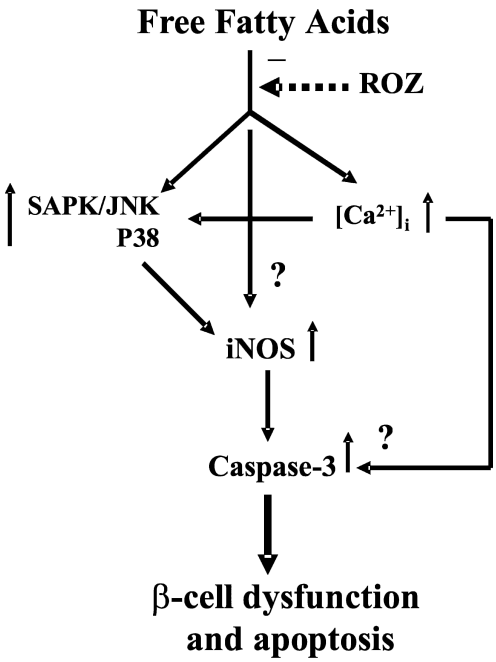

Figure 7. Proposed scheme suggesting an early step for the interaction of ROZ with the free fatty acid signaling pathways in the pancreatic β-cell leading to prevention of β-cell dysfunction/ apoptosis.

reports, and demonstrate that the ability of palmitate to induce iNOS activity is time dependent and cannot be observed during a short-term exposure of the islets to palmitate. Importantly, in the present study, we have shown that the thiazolidinedione ROZ markedly suppressed palmitate-induced iNOS activity in the islets, thus reducing a pathological level of NO generation. This is also of importance for the production of other related reactive molecules such as peroxynitrite ($ONOO^-$) that might adversely modulate β-cell function [13]. High levels of NO have been reported to induce apoptotic cell death by increasing the activities of caspases, *i.e.* caspase-9, caspase-8 and the most important member of the caspase family, caspase-3, in several cell types including pancreatic islets, macrophages, thymocytes, certain neurons and tumor cells [22]. The pro-apoptotic mechanisms of NO alone or in combination with $ONOO^-$ also include protein and DNA oxidation [13], lipid peroxidation [17, 33], protein S-nitrosylation [34] and endoplasmic reticulum stress with subsequent caspase activation [35]. Hence, NO might directly induce β-cell dysfunction through caspase activation [21, 35].

The importance of $[Ca^{2+}]_i$ as a signal molecule necessary for a normal insulin response to glucose or FFA is becoming increasingly clear [25, 36]. In this context it should be mentioned that a pulsatile rather than a sustained elevation of $[Ca^{2+}]_i$ plays a critical role for a proper insulin secretion by the β-cell [37]. Our present measurement of $[Ca^{2+}]_i$ showed that the glucose-stimulated increase of the $[Ca^{2+}]_i$ levels was markedly augmented after addition of palmitate. Interestingly, ROZ had a suppressive action on palmitate-induced $[Ca^{2+}]_i$ and also on palmitate-stimulated insulin release. Conversely, in the presence of glucose, ROZ induced an increase in $[Ca^{2+}]_i$ as well as in insulin release. These data strongly suggest that the interaction of ROZ with β-cell signaling generated by palmitate is distinct from that of glucose. A positive modulation of glucose-stimulated insulin release by ROZ has previously been reported from experiments in the perfused rat pancreas [38]. In contrast, long-term stimulation of the β-cell accompanied by a sustained increase in $[Ca^{2+}]_i$ might have adverse effects on the β-cell function. Hence a substantial body of evidence shows that an exaggerated increase in $[Ca^{2+}]_i$ is involved in the generation of other signal molecules important for the activation of a variety of target proteins, including phosphorylation of the different MAPKs [39]. Many stress- and inflammation-related kinases such as SAPK/JNK, p38, p42/44 and PKCs have been shown to mediate both inflammation and/or FFA-induced insulin resistance in hepatocytes, adipocytes and muscle tissues [40, 41]. The present investigation has identified SAPK/JNK and p38 as possible players not in short-term but in long-term palmitate-induced β-cell dysfunction. Our results show that a prolonged exposure of the β-cell to palmitate, which is reminiscent of the situation seen in obesity and obese diabetes, can induce β-cell dysfunction by activating SAPK/JNK and p38, while p42/44, which is growth-promoting, is unaffected. Hence our findings are consistent with the notion that the chronic presence of saturated FFA such as palmitate is important for fat-induced β-cell dysfunction. In addition, our results demonstrated for the first time that the pro-apoptotic MAPKs signaling pathway is suppressed by ROZ in islet tissue both after short-time and long-time incubation. Previous studies by other investigators have demonstrated that SAPK/JNK, p38 and p42/44, three members of the MAPK superfamily, are activated by cytokines [42]. However, activation of p42/44 (anti-apoptotic) and SAPK/JNK, p38 (pro-apoptotic) exerts opposite effects on cytokine-induced β-cell dysfunction. We found here that palmitate activated all three MAPKs in short-term experiments, while only SAPK/JNK and p38 were increased in long-term experiments. Importantly, however,

ROZ suppressed SAPK/JNK and p38 in both types of experiments, suggesting a marked protective capacity of this thiazolidinedione. Although the detailed mechanisms responsible for the regulation of SAPK/JNK, p38 and p42/44 activation in short-term palmitate-stimulated islets are not known, one can speculate that high levels of $[Ca^{2+}]_i$ alone or in combination with other palmitate-derived signals may exert a stimulatory effect on the phosphorylation of p42/44, SAPK/JNK and p38.

In previous studies reported by other investigators, almost a maximal SAPK/JNK, p38 and p42/44 activation was observed at ~30 min after incubation of either isolated islets or β-cell lines with different inflammatory agents, *i.e.*, IL-1 β, TNF-α and IFN-γ [29, 42]. The activities of SAPK/JNK and p38 remained elevated until appearance of apoptosis [39, 42, 43]. The time course of palmitate-stimulated activation of SAPK/JNK and p38 in our study might speak in favor of these signaling pathways being operated upstream of induction of iNOS expression in the islets since no iNOS activity could be detected in short-term incubation of islets with palmitate. In this context, it is interesting that we found that the increase in caspase-3 activity after 24-h culture of isolated islets and MIN6c4 cells in the presence of palmitate was markedly suppressed by ROZ, while ROZ had no effect on the increase induced by a mixture of cytokines (IL-1 β, TNF-α and IFN-γ). These results suggest that FFA-induced and cytokine-induced β-cell dysfunction are differentially regulated. This is in accordance with a recent report [44] suggesting that FFA and cytokines induce pancreatic β-cell dysfunction by different mechanisms. These authors also suggested that an apoptotic effect of FFA was independent of an increased iNOS-derived NO production, because they found only a marginal augmentation of iNOS mRNA. However, they did not measure the activity of the iNOS protein. Hence we propose that a post-translationally induced NO production is an important part of palmitate-induced β-cell dysfunction, although an additional NO-independent pathway cannot be excluded.

Finally, another important finding in the present study is that treatment with ROZ markedly improved β-cell function measured after a glucose challenge. Thus, a marked suppression of insulin release stimulated by glucose or glucose + palmitate in short-time incubations following 24-h culture with palmitate was greatly reversed when isolated islets or MIN6c4 cells were cultured with palmitate + ROZ. These experiments suggest that ROZ achieves its β-cell protection *via* suppression of an exaggerated and sustained increase in $[Ca^{2+}]_i$ induced by FFA and also by inhibition of MAPK activation as well as nitrosative stress. Moreover, data from a parallel investigation in our laboratory using G protein-coupled receptor 40 (GPR40) antisense treatment (unpublished) suggest that the protective effect of ROZ on FFA-induced β-cell dysfunction might be exerted at GPR40.

In summary, our present study demonstrates that palmitate-induced β-cell dysfunction is most likely achieved by increasing the induction of pro-apoptotic molecules, such as iNOS-derived NO, and pro-apoptotic signaling pathways, such as SAPK/JNK and p38 MAPKs, and finally increasing the activity of apoptotic enzymes such as caspase-3. Moreover, and most importantly our results clearly demonstrate that ROZ markedly suppressed these signals and restored β-cell function during its long-term presence together with a high level of palmitate. This suggests that ROZ has an essential and beneficial action on the pancreatic β-cell in addition to its anti-diabetic effects through its well-known cellular target PPARγ in peripheral tissues. A proposed scheme for the effects of FFA in inducing β-cell dysfunction as recorded in the present study is shown in Figure 7. Because ROZ counteracted all the deleterious parameters measured, we are inclined to suggest that ROZ elicits its effect through interaction with an early step in the FFA signaling pathway (Fig. 7).

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III

Palmitate-stimulated hormone secretion in relation to GPR40 expression in pancreatic islets of spontaneous obesity and type 2 diabetes in rats

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Abstract

Aim. The role of the G-protein-coupled receptor 40 (GPR40) in free fatty acid (FFA)-induced β-cell signalling and pathogenesis of diabetes is unclear. We examined GPR40 expression in relation to palmitate-induced hormone release in isolated islets from two rat models of spontaneous type 2 diabetes predominantly displaying either hyperlipidemia (fa/fa) or hyperglycemia (GK).

Methods. GPR40 expression was analysed by immunolabelling (confocal microscopy), Western blot and RT-PCR in islets from young prediabetic Zucker diabetic fatty (ZDF, fa/fa) rats or diabetic Goto-Kakizaki (GK) rats and their healthy controls, respectively. Dose-response studies of palmitate-induced secretion of insulin, glucagon and somatostatin from isolated islets were performed and then determined with radioimmunoassay.

Results. Confocal microscopy with immunolabelling of GPR40, insulin, glucagon and somatostatin in islets from control rats showed abundant expression of GPR40 protein not only in insulin but also in glucagon and somatostatin cells. The GPR40 expression was strongly increased in insulin, glucagon and somatostatin cells of prediabetic fa/fa islets. This increased expression had its counterpart in a marked increase in palmitate-stimulated release of both insulin and glucagon. Further, the palmitate-induced inhibition of somatostatin release was accentuated. Conversely, both insulin, glucagon and somatostatin cells in GK islets displayed an extremely faint expression of GPR40. This weak GPR40 expression was functionally reflected in an almost abolished hormone response to palmitate stimulation of GK islets. In accordance with the immunolabelling data, Western blot and mRNA expression of GPR40 showed a strong increase in fa/fa islets and a marked decrease in GK islets as compared to control islets.

Conclusion. GPR40 is abundantly expressed in islet cells of the rat and has a profound influence on the regulation of insulin, glucagon and somatostatin secretion by palmitate. A mild hyperlipidemia increases GPR40 expression and palmitate-induced effects on hormone secretion, whereas hyperglycemia, in the absence of hyperlipidemia, seems to reduce GPR40 expression and abrogate palmitate-induced secretory effects. GPR40 could be a target for a therapeutic approach in certain types of obesity and type 2 diabetes.

Introduction

A few years ago the orphan G-protein-coupled receptor 40 (GPR40) was identified as a receptor for medium to long-chain free fatty acids (FFA) (1-4). These studies showed an abundant if not exclusive expression of GPR40 in the pancreatic β-cells (2; 3). Acute activation of β-cell GPR40 with various FFA was found to amplify glucose-stimulated insulin release and thus challenged previous data suggesting that such an effect was mediated by the intracellular metabolism of FFA generating various lipid-derived signals resulting in augmentation of the release process (1-3; 5). Recently we (6) and others (7; 8) showed that long-time exposure of the β-cells to elevated levels of FFA leads to a marked impairment of glucose-stimulated insulin release and that this FFA-induced impairment most likely was transduced through GPR40-related events. These results also suggested that β-cell GPR40 during chronic exposure of pancreatic islets to elevated levels of FFA, both in the absence and in the presence of elevated levels of glucose, might play an important role in the pathogenesis of obesity and type 2 diabetes. Hence, GPR40 has been considered a putative target for therapeutic intervention in obesity and type 2 diabetes (3; 6; 9; 10), and very recently we found that the thiazolidinedione derivative rosiglitazone strongly counteracted palmitate-induced β-cell dysfunction at the GPR40 level (6; 9). However, genetic ablation of GPR40 in mice has given controversial results showing on one hand that such animals were as sensitive to long-time deleterious effects by FFA as controls (5) and on the other hand that this deletion of GPR40 protected the islets from FFA-induced dysfunction (7). Hence the aim of the present investigation was to perform a basic study of the relation between palmitate-induced hormone secretion and the expression pattern of GPR40 in insulin, glucagon and somatostatin cells in spontaneous obesity/type 2 diabetes using young (6-8 weeks) hyperlipidemic, normoglycemic (ZDF, fa/fa) rats, which are not diabetic until after ~10 weeks of age and in contrast to this young hyperglycaemic, normolipidemic GK rats (11; 12).

2. Materials and methods

Male Zucker diabetic fatty fa/fa rats (Charles River) and GK rats (Möllegård, Denmark) with their controls weighing 150-175 g (6-8 weeks) were used for the experiments. They were given a standard pellet diet (B&K) and tap water *ad libitum*. All animals were housed in metabolic cages with constant temperature (22º C) and 12-h light/dark cycles. The local animal welfare committee, Lund, Sweden, approved the experimental protocols and all procedures using animals.

Drugs and Chemicals

Collagenase (CLS 4) was from Sigma St. Louis, MO, USA. Fatty acid free bovine serum albumin (BSA) was from Boehringer Mannheim, Germany. The insulin, glucagon and somatostatin radioimmunoassay kits were from Euro-Diagnostica, Malmö, Sweden. All other chemicals were from Merck AG (Darmstadt, Germany) or Sigma (USA).

Isolation of pancreatic islets

Preparation of rat pancreatic islets was performed by retrograde injection of a collagenase solution *via* the bile-pancreatic duct (13). Islets were then isolated and handpicked under a stereomicroscope at room temperature. The isolated islets were then subjected to different experimental procedures (see below).

Detection of mRNA for GPR40 by quantative real-time PCR

Total RNA from islets was isolated using TRIzol (Invitrogen) according the manufacture's instructions with slight modifications *i.e.* after phase separation, the upper aqueous phase was incubated in -20ºC over night with 10 µg glycogen (Invitrogen) and centrifuged for 15min at 12000 x g, 4ºC. Each individual RNA pellet was dissolved in RNas-free water and converted into cDNA using TaqMan Reverse transcription (Applied Biosystems, CA, USA) according to the manufacturer's instructions.

Confocal microscopy

The islets were fixed with 4% formaldehyde, permeabilized with 5% Triton X-100, and unspecific sites were blocked with 5% Normal Donkey Serum (Jackson Immunoresearch Laboratories Inc, West Grove, PA). Mouse GPR40 was detected with polyclonal antibody (1:50) in combination with Cy3-conjugated anti-rabbit IgG (1:100) (Jackson Immunoresearch

Laboratories Inc, West Grove, PA). The GPR40 antibody was raised in rabbit against the C-terminal peptide: NH2-CVTRTQRGTIQK-COOH (Innovagen, Lund, Sweden). The specificity of this antibody was tested in a previous study (3). For staining of insulin, islets were incubated with a guinea pig-raised anti-insulin antibody (1:1000) (Eurodiagnostica, Malmö, Sweden) followed by an incubation with a Cy5-conjugated anti-guinea pig IgG antibody (Jackson Immunoresearch Laboratories Inc, West Grove, PA) (1:150). For the staining of glucagon, islets were incubated with a guinea pig-raised anti-glucagon antibody (1:600) (Eurodiagnostica, Malmö, Sweden) followed by incubation with a Cy5-conjugated anti-guinea pig IgG antibody (1:150) (Jackson Immunoresearch Laboratories Inc, West Grove, PA). For the staining of somatostatin, islets were incubated with a mouse-raised anti-somatostatin antibody (1:500) (Novo-Nordisk, Bagsvaerd, Denmark) followed by incubation with a Cy5-conjugated anti-mouse IgG antibody (1:150) (Jackson Immunoresearch Laboratories Inc, West Grove, PA).

The fluorescence was visualized with a Zeiss LSM510 confocal microscope by sequentially scanning at (excitation/emission) 505-530nm (Cy3) and 633/>650nm (Cy5).

Western Blot analysis

Approximately 400 islets were handpicked in Hank's buffer under a stereomicroscope and then suspended in 100 µl of SDS-buffer (Tris-HCl, 50 mmol/l; EDTA 1 mmol/l) containing a mixture of a protease inhibitor cocktail, frozen and sonicated on ice on the day of analysis (14). The protein content of the homogenates was determined according to the Bradford. Homogenate samples representing 20 µg of total protein were run on 7.5 % SDS-polyacrylamide gel (Bio-Rad, Hercules, CA, USA). After electrophoresis, proteins were transferred to a nitrocellulose membrane (Bio-Rad). The membrane was blocked in LS-buffer (10mmol/l Tris, pH 7.4, 100 mmol/NaCl, 0.1% Tween-20) containing 5% non-fat dry milk powder for 40 min at 37ºC. Subsequently the membranes were incubated over night with a polyclonal anti-GPR40 antibody (1:150) at room temperature. After washing (three times) in LS-buffer the membrane were finally incubated with a horseradish peroxidase-conjugated anti-rabbit antibody (1:500) (StessGen Biotechnologies Corp, Victoria, BC, Canada). Immunoreactivity was detected using an enhanced chemiluminescence reaction (Pierce, Rockford, IL, USA). The results were quantified by densitometric analysis using the Bio-Rad software (15).

Incubation of pancreatic islets

The freshly isolated islets were preincubated for 30 min at 37°C in Krebs-Ringer bicarbonate buffer, pH 7.4, supplemented with 10 mmol/l HEPES, 0.1 % bovine serum albumin and 1.0 mmol/l glucose. After preincubation the buffer was changed and the islets were incubated at 5 mmol/l glucose ± different concentrations of palmitate for 60 min at 37°C unless otherwise stated i.e. in one set of experiments 250 islets from Wistar rats were cultured at 5 or 16.7 mmol/l glucose or 5 mmol/l glucose + palmitate (50 μmol/l or 1 mmol/l). Each incubation vial contained 12 islets in 1.0 ml of buffer solution and was gassed with 95% O_2-5% CO_2 to obtain constant pH and oxygenation. All incubations were performed in an incubation box at 30 cycles/min. Immediately after incubation an aliquot of the medium was removed and frozen for subsequent assay of insulin, glucagon and somatostatin.

Statistics

The results are expressed as means ± SEM for the indicated number of observations or illustrated by an observation representative of a result obtained from different experiments. Probability levels of random differences were determined by analysis of variance followed by Tukey-Kramers' multiple comparisons test.

3. Results

Expression of GPR40 in relation to palmitate-induced hormone release in islets from fa/fa rats and their controls

Figure 1 shows the expression pattern of GPR40 protein in control **(a)** and fa/fa **(b)** islets as determined by immunolabelling and confocal microscopy. In control islets **Figure 1 (a)**, GPR40 is abundantly expressed in the insulin-producing β-cells (A-C) but also in glucagon cells (D-F) and somatostatin cells (G-I). In fa/fa islets **Figure 1(b)** the expression pattern was markedly increased in insulin (J-L), glucagon (M-O) and somatostatin (P-R) cells. Fluorescence intensity measurements comparing GPR40 fluorescence in control *vs* fa/fa islets showed the following results in arbitrary units taking the intensity in control islets as 100%; β-cells X + X; α-cells X + X and δ-cells (n=X in each category).

Figure 2 shows the expression pattern of GPR40 mRNA and protein in whole islets as determined with qRT-PCR and Western blot, respectively. Both these parameters were greatly increased in fa/fa islets compared with controls (**Figure 2**). Regarding the Western blots, densitometric quantitative analysis of GPR40 bands from fa/fa islet homogenates compared to

the bands from control islets showed an enhanced expression level of GPR40 protein in the fa/fa islets. (38.8 ± 2.1 and 24.2 ± 1.6 Units/mm^2 for fa/fa and control respectively) (**Figure 2**). To correlate these morphological and biochemical observations with FFA-induced hormone secreting capacity of control and fa/fa islets we performed a dose-response study with palmitate as secretagogue. **Figure 3** shows that palmitate-stimulated release of both insulin and glucagon was greatly increased in fa/fa islets compared with the control islets. Moreover, the palmitate-induced inhibition of somatostatin release was more pronounced in fa/fa compared with control islets.

Expression of GPR40 in relation to palmitate-induced hormone release in islets from GK rats and their controls

Figure 4 illustrates the confocal microscopy picture of the expression pattern of GPR40 protein in Wistar controls **(a)** and GK **(b)** islets after immunolabelling. The distribution of GPR40 in insulin (A-C), glucagon (D-F) and somatostatin (G-I) cells in the Wistar control islets (**Figure 4**) was abundant and almost identical to the pattern seen in fa/fa control islets (**Figure 1a**). In GK islets, however, GPR40 expression was almost abolished in insulin (J-L), glucagon (M-O) and somatostatin (P-R) cells (**Figure 4b**). Comparing GPR40 fluorescence intensity in Wistar control *vs* GK islets showed the following results in arbitrary units taking the intensity in Wistar control islets as 100%; β-cells X + X; α-cells X + X and δ-cells (n=X in each category). The results of examining the expression of GPR40 mRNA and protein in whole islets are shown in **Figure 5**. Both mRNA and protein expression of GPR40 were greatly reduced in GK islets compared with Wistar control islets. Densitometric quantitative analysis of GPR40 bands from GK islet homogenates compared to that from Wistar islets showed a reduced expression level of GPR40 protein in the GK islets. (27.0 ± 1.7 and 8.5 ± 1.8 Units/mm^2 for GK and Wistar, respectively) (**Figure 5**). A dose-response study with isolated islets showed that palmitate-induced release of insulin and glucagon seen in Wistar control islets was almost abolished in GK islets and that the basal secretion of somatostatin in GK islets was extremely low and not influenced by palmitate (**Figure 6**).

Discussion

We show here for the first time that the FFA receptor GPR40 is located not only to the insulin producing β-cells (2; 3) and glucagon-producing α-cells (16) but also to the somatostatin producing δ-cells in the pancreatic islets. Moreover, we also show for the first time that young hyperlipidemic, normoglycemic fa/fa rats display a markedly increased expression of GPR40 in all three types of these pancreatic endocrine cells compared with their healthy controls, while the young normolipidemic, hyperglycaemic GK rats were almost devoid of GPR40 expression in their islets. There was no apparent difference in GPR40 expression pattern between the two categories of control rats *i.e.* fa/fa control and Wistar control. Finally, dose-response studies with palmitate-stimulated release of insulin, glucagon and somatostatin showed a striking correlation between the secretory pattern and the GPR40 expression pattern.

The importance of GPR40 for insulin secretion and β-cell function is a matter of controversy especially with regard to long-time effects of FFA (5-7; 9). Thus using different genetic manipulations in mice Steneberg *et al* (2005) have provided evidence that GPR40 mediates both acute and chronic effects of FFA on β-cells, while Latour *et al* (2007) claim that GPR40 contributes to half of the acute insulin response to FFA but does not play a role for the chronic FFA effects on β-cell function (5). In accordance with Steneberg *et al* (2005) we recently found, in normal mouse islets, that palmitate-induced effects on β-cell function are highly dependent on GPR40 both in short-term and long-term aspects (6; 9). We also found that long-term effects of palmitate to impair β-cell function via GPR40 include excessive generation of NO and a marked activation of proapoptotic MAPKs i.e. SAPK/JNK and p38 as well as also caspase-3 activity (9). Furthermore, we showed that these deleterious effects on the β-cell could be counteracted by the anti-diabetic drug rosiglitazone, which was found to serve as a GPR40 antagonist, and thus thiazolidinedione derivatives might be considered of therapeutic and preventive value in the treatment of FFA-induced impairment of β-cell function in hyperlipidemia-linked type 2 diabetes.

It was shown several years ago (17) that the preobese, prediabetic male ZDF (fa/fa) rat displayed increased circulating levels of FFA and triglycerides at the age of 6-7 weeks, while the blood glucose level did not start to rise until at the age of ~9 weeks. Hence, by using fa/fa rats between 6-8 weeks of age we could study GPR40 expression in islet endocrine cells in the hyperlipidemic, normoglycemic, prediabetic stage of an animal model under the early

development of spontaneous type 2 obese diabetes. The present results speak in favour of GPR40 being highly important for FFA-induced hormone secretion in these rats. Not only insulin secretion but also glucagon and somatostatin secretion seemed to be markedly dependent on the extent of GPR40 expression. Hence our data strongly suggest but do not definitely prove that GPR40 is a most important player in FFA-induced hormone secretion from these three types of pancreatic endocrine cells. Such a hypothesis was further strengthened by our finding that an almost total absence of GPR40 protein expression in insulin, glucagon and somatostatin cells of hyperglycaemic GK rat was associated with an almost total absence of any effect of palmitate on the secretion of their hormone. Hence there is a question whether hyperglycemia by itself and not only hyperlipidemia is an important regulator of GPR40 expression because the plasma lipids in the GK rats are within the same normal range as in their Wistar controls (). Of course, much further work is to be done to settle this complex question. Hopefully our initial studies presented here on spontaneous type 2 diabetes in two highly different animal models might pave the way for future more detailed studies concerning the interaction of lipids and glucose on FFA-induced activation of GPR40 in pancreatic endocrine cells.

Legends to the figures

Figure 1. *Confocal microscopy of freshly isolated islets from control* ***(a)*** *and fa/fa rat* ***(b)***.

The islets from control (a) or fa/fa rats (b) were double-immunolabelled for insulin (A and J), glucagon (D and M), somatostatin (G and P) and GPR40 (B, E, H, K, N and O). Insulin, glucagon and somatostatin stainings appear as *red* and GPR40 staining appears as *green* fluorescence. Co-localization of insulin/GPR40, glucagon/GPR40 and somatostatin/GPR40 is seen as *orange-yellowish* fluorescence. Bars indicate lengths (20 μm).

Figure 2. *Relative mRNA quantification of freshly isolated islets from control and fa/fa rats* ***(a)*** *and GPR40 protein expression* ***(b).***

Expression of GPR40 mRNA (a) and GPR40 protein (b) in islets isolated from control (white bar) and fa/fa rat (black bar). Data are presented as relative expression of GPR40 to GAPDH for 6 different observations (a). A representative example of Western blots of islets taken from control or fa/fa rats and incubated with GPR40 antibody is also shown (b). The blots were performed with 25 μg of islet protein on each lane. Arrow indicates the molecular weight of GPR40 (~ 32 kDa).

Figure 3. *Dose-dependent modulation of hormone release by palmitate from freshly isolated islets from control and fa/fa rats.*

Dose-response effect of palmitate on insulin (a), glucagon (b) and somatostatin (c) secretion measured from freshly isolated islets from control or fa/fa rats incubated for 60 min. The medium glucose concentration was 5 mmol/l. Means ± SEM for 10 incubated batches of islets on each point are shown.

Figure 4.*Confocal microscopy of freshly isolated islets from Wistar* ***(a)*** *as well as GK rats* ***(b)***.

The islets from Wistar (a) or GK rats (b) were double-immunolabelled for insulin (A and J), glucagon (D and M), somatostatin (G and P) and GPR40 (B, E, H, K, N and O). Insulin, glucagon and somatostatin stainings appear as *red* and GPR40 staining appears as *green* fluorescence. Co-localization of insulin/GPR40, glucagon/GPR40 and somatostatin/GPR40 are seen as *orange-yellowish* fluorescence. Bars indicate lengths (20 μm).

Figure 5. *Relative mRNA quantification of freshly isolated islets from Wistar and GK rats* ***(a)*** *and GPR40 protein expression* ***(b).***

Expression of GPR40 mRNA (a) and GPR40 protein (b) in islets isolated from Wistar (white bar) and GK rats (black bar). Data are presented as relative expression of GPR40 to GAPDH for 6 different observations (a). A representative example of Western blots of islets taken from Wistar (control) or GK rats and incubated with GPR40 antibody is also shown (b). The blots were performed with 25 µg of islet protein on each lane. Arrow indicates the molecular weight of GPR40 (~ 32 kDa).

Figure 6. *Dose-dependent modulation of hormone release by palmitate from freshly isolated islets from Wistar and GK rats.*

Dose-response effect of palmitate on insulin (a), glucagon (b) and somatostatin (c) secretion measured from freshly isolated islets from Wistar or GK rats incubated for 60 min. The medium glucose concentration was 5 mmol/l. Means ± SEM for 10 incubated batches of islets on each point are shown.

Table 1. Concentrations of serum glucose, triglycerides, cholesterol, HDL and LDL in control and fa/fa rats. Means ± SEM of 4-5 rats in each group. *p<0.05 *vs* controls.

	Glucose mmol/l	**Triglycerides mmol/l**	**Cholesterol mmol/l**	**HDL mmol/l**	**LDL mmol/l**
Control	6.1 ± 0.32	0.86 ± 0.07	1.70 ± 0.08	1.22 ± 0.04	0.59 ± 0.07
fa/fa	5.7 ± 0.34	1.25 ± 0.07 *	2.10 ± 0.04 *	1.49 ± 0.09 *	0.35 ± 0.06 *

Table 2. Concentrations of serum glucose, triglycerides, cholesterol, HDL and LDL in Wistar control and GK rats. Means ± SEM of 5-7 rats in each group. ***p<0.001 *vs* Wistar.

	Glucose mmol/l	**Triglycerides mmol/l**	**Cholesterol mmol/l**	**HDL mmol/l**	**LDL mmol/l**
Wistar	5.7 ± 0.3	0.99 ± 0.05	1.88 ± 0.11	1.15 ± 0.07	0.55 ± 0.07
GK	11.0 ± 0.7 ***	0.88 ± 0.03	1.81 ± 0.06	1.09 ± 0.05	0.53 ± 0.05

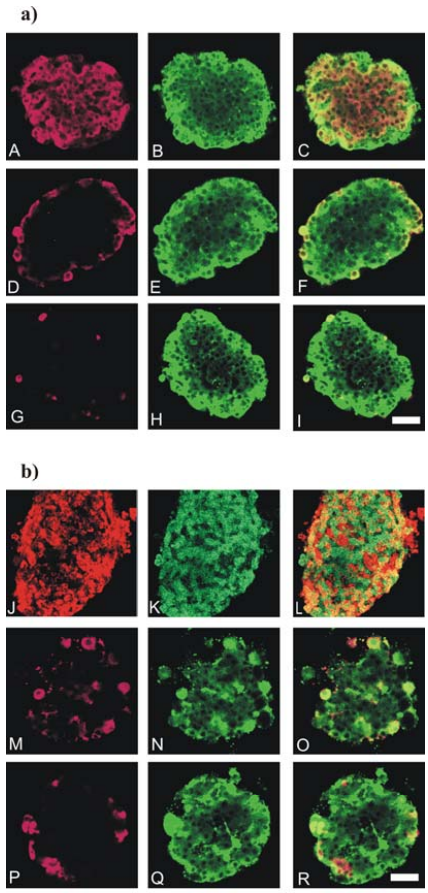

Figure 1

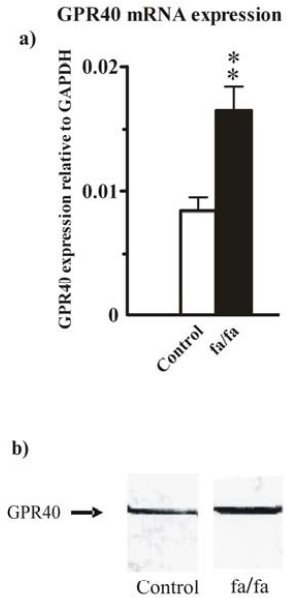
GPR40 mRNA expression
a)
GPR40 expression relative to GAPDH
0.02
0.01
0
**
Control
fa/fa
b)
GPR40
Control
fa/fa

Figure 2

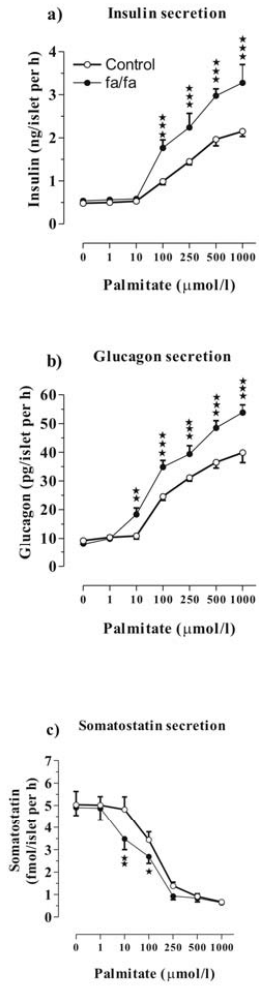
a)
Insulin secretion
Control
fa/fa
Insulin (ng/islet per h)
Palmitate (µmol/l)
b)
Glucagon secretion
Glucagon (pg/islet per h)
Palmitate (µmol/l)
c)
Somatostatin secretion
Somatostatin (fmol/islet per h)
Palmitate (µmol/l)

Figure 3

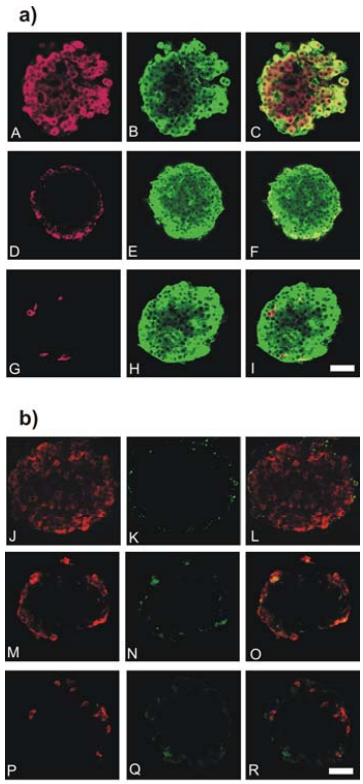

Figure 4

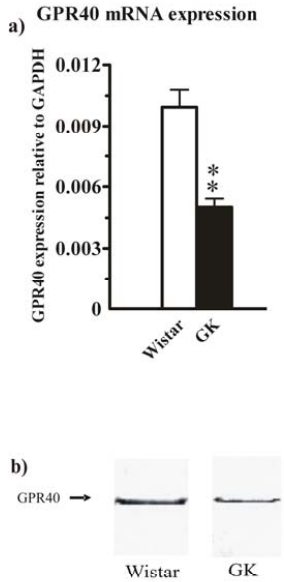
a)
GPR40 mRNA expression
GPR40 expression relative to GAPDH
0.012
0.009
0.006
0.003
0
**
Wistar
GK
b)
GPR40
Wistar
GK

Figure 5

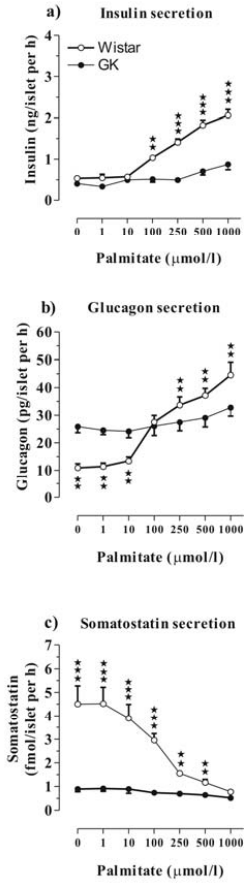
a)
Insulin secretion
Wistar
GK
Insulin (ng/islet per h)
Palmitate (μmol/l)
b)
Glucagon secretion
Glucagon (pg/islet per h)
Palmitate (μmol/l)
c)
Somatostatin secretion
Somatostatin (fmol/islet per h)
Palmitate (μmol/l)

Figure 6

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IV

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Excessive Islet NO Generation in Type 2 Diabetic GK Rats Coincides with Abnormal Hormone Secretion and Is Counteracted by GLP-1

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Abstract

Background: A distinctive feature of type 2 diabetes is inability of insulin-secreting β-cells to properly respond to elevated glucose eventually leading to β-cell failure. We have hypothesized that an abnormally increased NO production in the pancreatic islets might be an important factor in the pathogenesis of β-cell dysfunction.

Principal Findings: We show now that islets of type 2 spontaneous diabetes in GK rats display excessive NO generation associated with abnormal iNOS expression in insulin and glucagon cells, increased ncNOS activity, impaired glucose-stimulated insulin release, glucagon hypersecretion, and impaired glucose-induced glucagon suppression. Pharmacological blockade of islet NO production by the NOS inhibitor N^G-nitro-L-arginine methyl ester (L-NAME) greatly improved hormone secretion from GK islets suggesting islet NOS activity being an important target to inactivate for amelioration of islet cell function. The incretin hormone GLP-1, which is used in clinical practice suppressed iNOS and ncNOS expression and activity with almost full restoration of insulin release and partial restoration of glucagon release. GLP-1 suppression of iNOS expression was reversed by PKA inhibition but unaffected by the proteasome inhibitor MG132. Injection of glucose plus GLP-1 in the diabetic rats showed that GLP-1 amplified the insulin response but induced a transient increase and then a poor depression of glucagon.

Conclusion: The results suggest that abnormally increased NO production within islet cells is a significant player in the pathogenesis of type 2 diabetes being counteracted by GLP-1 through PKA-dependent, nonproteasomal mechanisms.

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Introduction

Diabetes type 2 is now a global health problem, characterized of both insulin resistance and impaired insulin response to glucose, defects that are regarded multifactorial in origin and are considered a result of both environmental and undefined genetic factors. We have shown [1,2,3,4,5,6,7,8] that islet nitric oxide (NO) derived from neuronal constitutive NO synthase (ncNOS) is a strong negative modulator of glucose-stimulated insulin release. This enzyme resides abundantly in β-cells [9]. Moreover, ncNOS is in part associated with insulin granules [10] making it a suitable regulator of the secretory process. We have also found, in healthy animals, that exposure of islets to high concentrations of glucose or lipids induces expression and strong activity of inducible NOS (iNOS) in their β-cells concomitant with reduced insulin response to glucose [2,3,7,11]. These data suggested to us that glucose-induced "glucotoxicity" as well as lipid-induced "lipotoxicity" in the β-cell, at least in part, might be due to possible nonimmunogenic deleterious effects of iNOS-derived NO. Hence there is reason to believe that a sustained elevation of islet iNOS activity and the ensuing excessive production of NO might be of significant importance in the development of type 2 diabetes.

Nutrient ingestion stimulates secretion of gut hormones such as glucagon-like peptide-1 (GLP-1) and gastric inhibitory polypeptide (GIP), which serve to amplify glucose-stimulated insulin release [12,13]. GLP-1 is also known to decrease glucagon release [12,13]. Both effects are glucose-dependent and GLP-1 is considered therapeutically useful to reduce elevated blood glucose [12,13]. Therefore, GLP-1 has received great attention as treatment for type 2 diabetes [12,13,14]. We found recently that GLP-1 restored the impairment of glucose-stimulated insulin release in islets taken from lipid-infused rats and that this effect was linked to increased cyclic AMP levels and suppression of islet activities of both ncNOS and iNOS [3]. Since surprisingly even short-time (~60 min) exposure of islets to high glucose in healthy animals was associated with enhanced production of iNOS-derived NO [7,11], the present investigation was undertaken to study islet NOS activities and the influence of GLP-1 on an animal model of spontaneous type 2

diabetes and "glucotoxicity", the mildly diabetic Goto-Kakizaki (GK) rat, which is considered a good model of human type 2 nonobese diabetes [15,16,17,18]. Because the glucagon-producing α-cells harbor the ncNOS enzyme [9], which apparently is an important regulator of glucagon release [1,2,3,4,6,18,19,20,21], and because raised plasma levels of glucagon is a common feature of and contribute to human type 2 diabetes [22] we also performed parallel studies on glucagon secretion.

Results

Confocal microscopy of iNOS expression in islets directly isolated from GK rats

To investigate whether isolated islets express iNOS protein we performed a confocal microscopic study in GK islets. Islets were simultaneously immunolabeled for insulin or glucagon to investigate β- and α-cell specific expression of iNOS. As shown in **Fig. 1a**, immunoreactivity for iNOS was detected in insulin immunoreactive β-cells (A–C) and in glucagon immunoreactive α-cells (D–F) (orange-yellowish fluorescence in the overlay pictures). No iNOS immunoreactivity was found in Wistar control islets (**Fig. 1b**). Insulin immunoreactive β-cells (G–I) and glucagons immunoreactive α-cells (J–L) are shown.

NOS activities in islets directly isolated from GK and Wistar rats

Fig. 1c shows that islets isolated from GK rats displayed increased ncNOS activity. Moreover, while iNOS activity in Wistar islets was negligible, GK islets displayed high iNOS activity. Hence total NO generation was impressively increased in GK compared with Wistar islets (**Fig. 1c**).

Effect of the NOS inhibitor L-NAME on insulin and glucagon secretion from isolated Wistar and GK islets at low and high glucose and in vivo effect of L-arginine

To directly study the basic involvement and regulatory role of islet NO production for insulin and glucagon release we incubated isolated islets from GK and Wistar rats at low and high glucose in the absence and presence of the NOS inhibitor L-NAME (5 mmol/l). **Table 1a** shows that glucose-stimulated insulin release from both GK and Wistar islets is markedly amplified in the presence of L-NAME. This amplification is more pronounced in GK islets (90%) compared with Wistar islets (38%). No effect of L-NAME on insulin release was seen at low glucose (**Table 1a**). Moreover, NOS inhibition by L-NAME greatly suppressed the glucagon hypersecretion from GK islets incubated at low glucose (**Table 1b**). Such an inhibition of glucagon release from GK islets in the presence of L-NAME could also be seen at high glucose. Further, L-NAME did decrease glucagon secretion also from Wistar islets at low glucose, but had no significant effect at high glucose (**Table 1b**). Finally, to further test a regulatory role of ncNOS for a stimulated acute release of insulin and glucagon *in vivo* we injected L-arginine, which is known to stimulate both insulin and glucagon secretion, in the absence and presence of L-NAME pretreatment. **Table 1c and d** shows that NOS inhibition amplified the insulin but decreased the glucagon response following L-arginine injection in both GK and Wistar rats..

Influence of GLP-1 on basal insulin and glucagon release in relation to islet ncNOS and iNOS expression and activities in incubated islets from GK and Wistar rats

Since not only pharmacological blockade [5,6,8,10,11] but also cyclic AMP stimulating agents are known to suppress islet NO production in healthy animals[3,7], we tested possible beneficial effects of GLP-1 on islet NOS activities in the GK rat. **Fig. 2 a, b** and **Table 2a** show the effect of GLP-1 on islet NOS activities and protein expression (Western blot) as well as insulin and glucagon secretion in islets from GK and Wistar rats incubated at low glucose (3.3 mmol/l). We used a concentration of 100 nmol/l of GLP-1, having maximal stimulating effect on glucose-induced insulin release in isolated rat islets [3]. Total NO generation was markedly increased in GK islets (**Fig. 2a**). This was mainly due to iNOS activity. No significant iNOS expression and activity was detectable in Wistar islets (**Fig. 2a, b**). ncNOS activity was modestly upregulated in GK islets (**Fig. 2a**). GLP-1 induced pronounced suppression of iNOS expression and activity in GK islets and suppressed ncNOS activity in both types of islets (**Fig. 2a, b** and **Table 2a**). Basal insulin secretion in GK and Wistar islets was similar at low glucose and GLP-1 had no effect (**Fig. 2a**). Glucagon secretion was impressively increased in GK islets *vs* Wistar islets (33.2±2.4 pg/islet per h *vs* 19.8±1.7 pg/islet per h; $p<0.01$) (**Fig. 2a**). GLP-1 suppressed glucagon secretion to 17.2±1.3 pg/islet per h in GK islets and to 12.8±1.1 pg/islet per h in Wistar controls. Notably, GK islets still hypersecreted glucagon after GLP-1 treatment (**Fig. 2b**). The densitometric analysis showed that GLP-1 induced a pronounced suppression of both ncNOS and iNOS expression in GK islets. A marked suppression of ncNOS expression was found also in Wistar islets, while no iNOS expression could be detected (**Table 2a**).

Confocal microscopic study of the effects of GLP-1, the PKA inhibitor H-89 and the proteasome inhibitor MG 132 on iNOS expression in GK islets incubated at low glucose

The suppressive effect by GLP-1 on iNOS protein expression in islets from healthy rats incubated at high glucose is PKA-mediated [7]. Because iNOS expression was present already in directly isolated GK islets next experiments were conducted at low glucose to avoid interference with *in vitro* glucose-induced iNOS stimulation [7]. Thus immunolabeling of iNOS expression at 3.3 mmol/l glucose was performed and effects of GLP-1 and the PKA inhibitor H-89 were recorded. GK islets were simultaneously immunolabeled for insulin to identify β-cell specific iNOS expression. As shown in **Fig. 3** A–C, iNOS immunoreactivity was detected in insulin-immunoreactive β-cells. GLP-1 (100 nmol/l) greatly suppressed the expression of iNOS (**Fig. 3** D–F) and the PKA inhibitor H-89 (2 μmol/l) reversed this suppression (**Fig. 3** G–I). **Fig. 3** J–L shows that H-89 by its own has no apparent effect on iNOS expression. Finally we studied the possible involvement of the proteasome system, since the proteasome has been suggested to modulate iNOS expression in other cell types [23,24]. **Fig. 3** M–O shows that the suppressive effect of GLP-1 on iNOS expression (**Fig. 3** D–F) was not reversed by the proteasome inhibitor MG 132 (10 μmol/l), and **Fig 3** P–R shows, surprisingly, that the prominent iNOS expression in the β-cells (**Fig. 3** A–C) was abolished also after incubation with MG 132 itself. **Fig. 3** S–U shows that no iNOS expression was found in Wistar control islets.

Influence of GLP-1 on insulin and glucagon release in relation to islet ncNOS and iNOS expression and activities in islets from GK and Wistar rats incubated at high glucose

Fig. 4a, b and **Table 2b** shows the effect of GLP-1 on NOS activities and protein expression in islets incubated at high glucose (16.7 mmol/l). Again, NOS activities were increased in GK compared with Wistar islets. This was mainly due to increased iNOS activity (**Fig. 4a**). However, upregulation of NOS activities

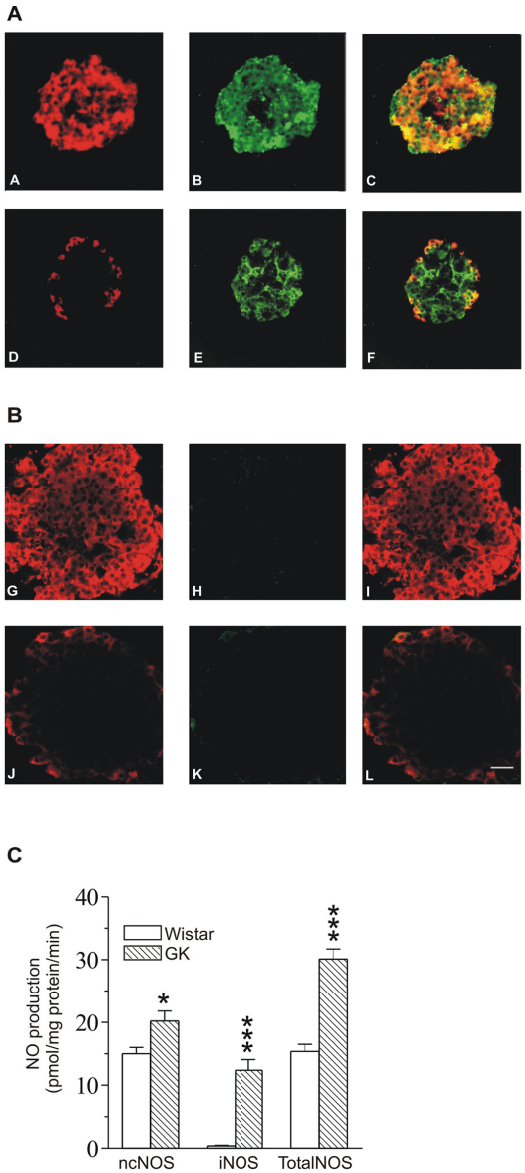

Figure 1. a Confocal microscopy of islets directly isolated ex vivo from the GK rat. The islets were double-immunolabelled for insulin or glucagon and iNOS and analysed by confocal microscopy. Insulin staining anc iNOS staining appear, respectively, as *red* (**A**) and *green* (**B**) fluorescence. Co-localization of insulin/iNOS is seen as *orange-yellowish* fluorescence (**C**). Similarly glucagon staining and iNOS staining appear, respectively, as *red* (**D**) and *green* (**E**) fluorescence. Co-localization of glucagon/iNOS is seen as *orange-yellowish* fluorescence (**F**). Bars indicate lengths (10 μm). **b** Plates G–I and J–L show the absence of iNOS fluorescence in Wistar control islets (**H**, **K**). **c** *NOS activities in freshly isolated islets.* NO production from ncNOS, iNOS and total NOS in freshly isolated islets from Wistar control rats (open bars) and GK rats (hatched bars). Values are mean±s.e.m for n = 4–6 animals. *P<0.05; *** P<0.001
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Table 1. Effect of the NOS inhibitor L-NAME on insulin and glucagon release *in vitro* and *in vivo*.

		Wistar	GK
Insulin release (ng/islet per h)			
a)	1G	0.269±0.029	0.258±0.032
	1G+L-NAME	0.305±0.052	0.303±0.033
	16.7G	4.01±0.32	1.89±0.26
	16.7G+L-NAME	5.61±0.35 **	3.68±0.38 ***
Glucagon release (pg/islet per h)			
b)	1G	29.46±1.1	41.04±1.62
	1G+L-NAME	20.04±1.3 **	29.38±1.7 **
	16.7G	18.33±1.9	36.64±2.25
	16.7G+L-NAME	15.67±2.1	26.35±2.53 **
Plasma insulin response (pmol/l)			
c)	Saline+L-arg.	948±111	238±54
	L-NAME+L-arg.	1349±82 **	410±52 *
Plasma glucagon response (ng/l)			
d)	Saline+L-arg.	551±45	404±71
	L-NAME+L-arg.	331±67 *	214±36 *

Effect of pharmacological blockade of islet NO generation by the NOS inhibitor L-NAME on islet hormone secretion from Wistar and GK rats. **a)** Insulin release and **b)** glucagon release from isolated islets at low and high glucose in the absence or presence of L-NAME (5 mmol/l). n = 8 in each group. Asterisks denote significant effects of L-NAME at 1G or 16.7G.
*p<0.05;**p<0.01;***p<0.001.
c) Peak insulin response and **d)** Peak glucagon response in plasma at 2 min after an *i.v.* injection of L-arginine (L-arg.) (3.6 mmol/kg) following pretreatment with saline or L-NAME (1.2 mmol/kg). There were 4–7 animals in each group. Asterisks denote significant effects of L-NAME pretreatment.
*p<0.05;**p<0.01
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was not so pronounced in GK islets at high glucose as at low glucose possibly because NOS activities were already upregulated *in vivo* and in comparison high glucose increased NOS activities also in Wistar islets (**Fig. 4a, b**). NO production and Western blots show that GLP-1 reduced both expression and activities of iNOS and ncNOS (**Fig. 4a, b**, **Table 2b**). This suppression was associated with a prominent increase in glucose-stimulated insulin release and thus the impairment of glucose-induced insulin response in GK islets was efficiently counteracted (**Fig. 4a**). Moreover, while high glucose suppressed glucagon secretion in Wistar islets, negligible suppression by glucose was found in glucagon hypersecreting GK islets (compare **Fig. 2a** and **4a**). GLP-1 reduced glucagon secretion in both types of islets (**Fig. 2a**). However, compared to Wistar, GK islets still displayed elevated glucagon secretion even after combination of high glucose and GLP-1 (**Fig. 4a**). The densitometric analysis showed that GLP-1 induced a marked suppression of both ncNOS and iNOS expression in GK as well as Wistar islets (**Table 2b**).

Basal plasma levels of insulin, glucagon and glucose in GK and Wistar rats and effects of glucose and GLP-1 injections

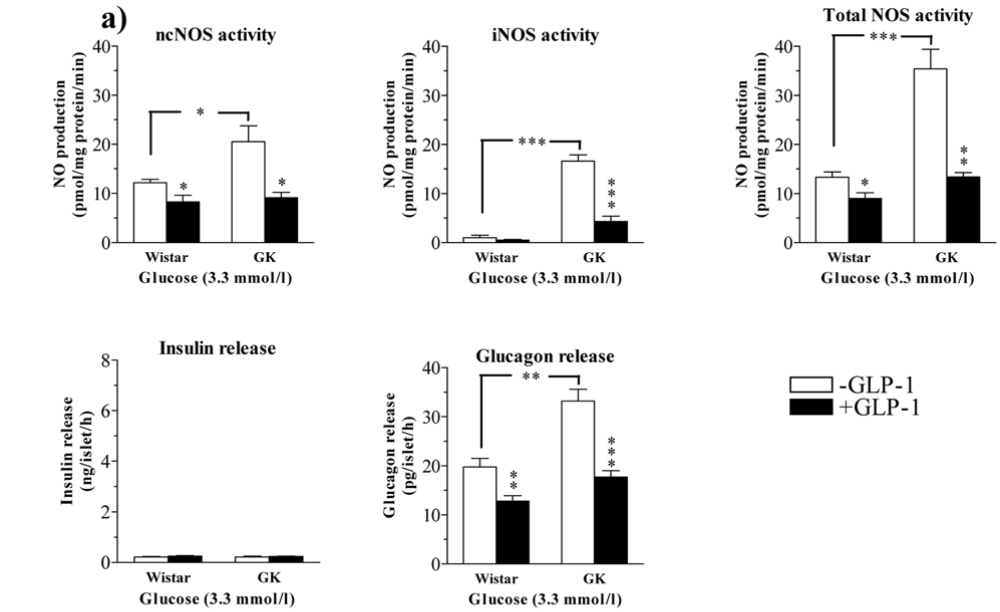
Since GK islets apparently hypersecreted glucagon we measured circulating levels of glucagon, insulin and glucose *in vivo*. In addition we measured islet content of glucagon and insulin. Plasma levels of glucagon and glucose were elevated in GK rats, while insulin levels were not appreciably different. However, calculating the basal insulinogenic index, *i.e.* dividing circulating insulin concentrations with circulating glucose concentrations, revealed that the insulin response to basal glucose was impaired in GK *vs* Wistar rats (data not shown). The plasma levels were as follows, Wistar *vs* GK: Insulin (pmol/l) 100±8 *vs* 81±6.4 (NS); Glucagon (ng/l) 254.5±10.2 *vs* 311.5±12.9 (p<0.001); Glucose (mmol/l) 7.3±0.2 *vs* 11.0±0.2 (p<0.001). There were 28 animals in each group. Islet content of insulin and glucagon at 6 weeks of age was similar in GK and Wistar rats. Insulin content Wistar *vs* GK was 5.5±0.6 and 5.6±0.9 nmol/mg protein and glucagon content 1.02±0.09 and 0.89±0.09 μg/mg protein (n = 8 in each group). Because the action of GLP-1 is known to be highly glucose-dependent [12,13] we examined *in vivo* insulin and glucagon responses to GLP-1 mixed with glucose. **Fig. 5a–b** shows the effects of an *iv* injection of this mixture and a control experiment with a high dose of glucose alone (**Fig. 5d–e**). The insulin response to the combination of GLP-1 and glucose was modestly lower in GK compared with Wistar. However, there was an abnormal transient increase in glucagon response followed by a slow suppression, which did not reach the low levels of glucagon recorded in Wistar. After injection of glucose alone the insulin response in GK rats was abrogated, and the glucagon response showed an initial normal suppression but a marked rebound (**Fig. 5d–e**). An impaired glucose tolerance curve after glucose alone (**Fig. 5f**) was still impaired in the GK rat even after addition of GLP-1 (**Fig. 5c**).

Discussion

Previous studies have suggested that high amounts of NO derived from iNOS activity are cytotoxic and implicated in the autoimmune-mediated dysfunction and destruction of islet β-cells during development of type 1 diabetes [25]. This iNOS-stimulated NO production is not only mediated by invading macrophages but also by the β-cells themselves [25]. On the other hand, the role of islet ncNOS, which produces small amounts of NO in a signaling way, is still unclear [1,2,3,4,5,6,7,8,10,11,18,19,20,26,27,28,29,30]. We have found that inhibition of ncNOS activity is accompanied by increased insulin release induced by glucose, L-arginine and to a lesser extent by cholinergic stimulation, while secretion induced by agents directly stimulating the cyclic AMP system is not inhibited and might even be slightly increased by NO [1,2,3,4,5,6,7,8,11,19,20,29]. Moreover, islet NOS activities are efficiently counteracted by the cyclic AMP/PKA system [2,3,7,29].

Based on these observations we proposed that ncNOS-derived NO might serve as a physiological negative feedback inhibitor of acute glucose-stimulated insulin release [1,3,5,7,8,11]. Furthermore, we have shown in healthy mice that hyperglycemia lasting for only ~60 min results in islet iNOS expression and activity [11], a finding that raised the question whether the islet NO system, and especially iNOS, might be implicated also in the development of nonimmunogenic type 2 diabetes.

Our present results showed a rich occurrence of iNOS protein in both β-cells and α-cells of islets isolated from the diabetic GK rat. The abundance of iNOS expression in the α-cells of the GK rat was unexpected, since injection of large doses of iNOS-stimulating endotoxin did induce iNOS only in single α-cells while almost all β-cells were affected [9,29]. This observation thus raised the question whether long-term hyperglycemia might contribute to nonimmunogenic diabetes by inducing iNOS in the α-cells. Because NO not only inhibits insulin release but also stimulates glucagon release [1,2,3,6,19,20,21,29] this observation thus underlined a possible pathogenic role of the islet NO system in the development of type 2 diabetes. In accordance, GK islets displayed increased ncNOS activity and impressive iNOS activity compared with Wistar control islets. These findings might, at least in part, explain the defective



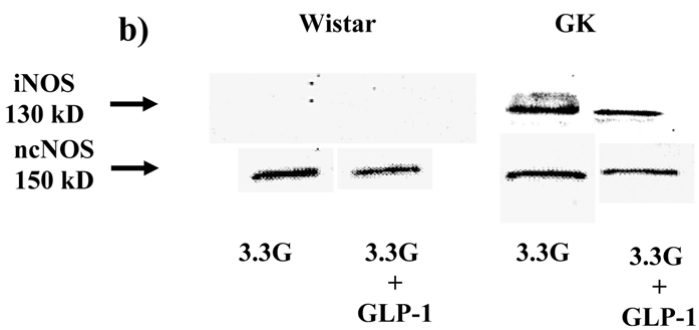


Figure 2. (a) NOS activities and hormone secretion in islets incubated at low glucose. Islet NO production from ncNOS, iNOS and total NOS as well as insulin and glucagon release from islets of Wistar or GK rats incubated at 3.3 mmol/l glucose in the absence (open bars) and presence (dark bars) of 100 nmol/l GLP-1. Values are mean±s.e.m for 5–9 batches of islets at each point. *P<0.05; ** P<0.01; *** P<0.001. (**b**) Representative examples of Western blots of iNOS and ncNOS protein in the absence and presence of GLP-1 are shown.
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insulin response to glucose and the glucagon hypersecretion in these rats and also a possible deleterious effect of iNOS-derived NO on the β-cells over time. Further, the present data showing an abnormally increased iNOS-derived NO production also after *in vitro* incubation of GK islets at low glucose suggest that moving the islets from an *in vivo* hyperglycemic milieu to a medium of a low "hypoglycemic" glucose concentration (3.3 mmol/l) for a period of ~2 h did not reduce or abolish iNOS expression and activity.

The NOS inhibitor L-NAME has previously been shown to serve as a potent inhibitor of islet ncNOS activity both *in vitro* and *in vivo* in the rat as well as in the mouse [5,6,8,10,11,19,27,31]. Such a pharmacological inhibition of β-cell ncNOS activity was now found to restore the impaired insulin response to glucose and also to restore, at least in part, the increase glucagons secretion in GK islets thus suggesting that the increased NO generation in these islets is indeed an important factor for β-cell dysfunction and α-cell hyper-responsiveness in this model of animal type 2 diabetes. These results were further strengthened by the *in vivo* L-arginine data showing that L-NAME amplified the insulin but decreased the glucagon response. This is in accordance with a recent study in a new rat model of type 2 diabetes induced by streptozotocin- nicotinamide [32]. These authors found that an impaired ncNOS expression was associated with insulin release hyper-responsiveness to L-arginine.

Table 2. Densitomeric analysis of iNOS and ncNOS protein expression.

	Wistar		GK	
a)	**3.3G**	**3.3G+GLP-1**	**3.3G**	**3.3G+GLP-1**
iNOS	0.2±0.09	0.13±0.05	18.7±3.3	8.5±1.1 ***
ncNOS	15.5±1.5	10.1±1.2 *	22.9±3.1	7.6±2.3 ***
b)	**16.7G**	**16.7G+GLP-1**	**16.7G**	**16.7G+GLP-1**
iNOS	16.3±2.05	6.1±0.91 ***	24.5±2.8	10.4±2.9 ***
ncNOS	25.2±2.4	15.05±1.7 **	40.5±4.1	11.3±2.01 ***

Densitometric analysis of Western blots for iNOS and ncNOS expression after incubation of Wistar and GK islets in the absence and presence of GLP-1 (100 nmol/l) at **a)** low glucose, 3.3 mmol/l (3.3G) and **b)** high glucose, 16.7 mmol/l (16.7 G). Asterisks denote significant effect of GLP-1 for n = 4 in each group.
*p<0.05;***p<0.001.
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From previous studies [3,6,7,29] we hypothesized that a protective mechanism against the NO-mediated negative effects on β-cell function in GK rats might be exerted through the cyclic AMP system. We therefore selected the potent cyclic AMP-generating incretin, GLP-1 as a suitable agent to test this hypothesis. In our present short-term experiments GLP-1 counteracted the impaired insulin response to glucose and induced a marked decrease in islet NOS activities. Since we have shown that increased islet endogenous NO production and addition of exogenous NO gas or an intracellular NO donor to incubated islets are accompanied by reduction of glucose-stimulated insulin response [1,2,3,5,8,11], we are now inclined to ascribe, at least in part, the beneficial effect of GLP-1 on the impairment of acute glucose-induced insulin release in the GK rat to its suppressive action on the islet NO system. Apart from its effect on the cyclic GMP system, NO is known to affect multiple targets within the cell, mainly acting through S-nitrosylation [33,34,35,36]. It is unclear whether cyclic GMP, the glutathione system and/or different regulatory proteins in the stimulus-secretion coupling are primarily affected [8,11,34,35,36,37] and regulation of NOS activities has been observed at all levels from gene transcription to covalent modification and allosteric regulation of the enzyme itself [33]. Interestingly, recent results from brain tissue have demonstrated that a number of metabolic, structural, and signaling proteins might be afflicted by NO through S-nitrosylation processes [36]. Notably the present study was performed in very young GK rats, and thus a more or less continuous activity of iNOS-derived NO might have further deleterious effects on the β-cell in the long run and earlier studies showed that β-cell numbers were diminished in 6-months old GK rats [15]. Such a notion is further underlined by previous data showing marked diabetes in mice with iNOS overexpression in their β-cells [38]. It should be recalled that the β-cell mass in the Stockholm GK colony is not affected in young rats [39].

The possible implication of proteasomal mechanisms in the loss of iNOS protein during short-time islet incubation with GLP-1 was explored by using the proteasome inhibitor MG 132. Unexpectedly, MG 132 did not reverse the GLP-1-induced suppression of iNOS expression but instead induced loss of iNOS protein and hence proteasomal inhibition did not prevent but stimulated degradation of iNOS, suggesting other control mechanisms regulating the cellular balance of this protein. A similar paradoxical effect of proteasomal inhibition was reported concerning (pro)insulin, the degradation of which was increased by

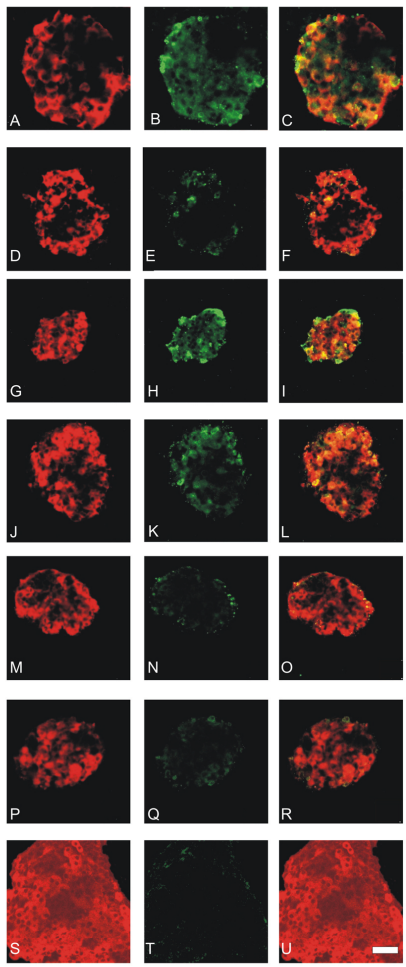

Figure 3. Confocal microscopy of incubated islets from the GK rat. Isolated islets were incubated for 90 min in the presence of; **A**, **B** and **C**) 3.3 mmol/l glucose; **D**, **E** and **F**) 3.3 mmol/l glucose+100 nmol/l GLP-1; **G**, **H** and **I**) 3.3 mmol/l glucose+100 nmol/l GLP-1+2 μmol/l H-89; **J**, **K** and **L**) 3.3 mmol/l glucose+2 μmol/l H-89; **M**, **N** and **O**) 3.3 mmol/l glucose+100 nmol/l GLP-1+10 μmol/l MG 132; **P**, **Q** and **R**) 3.3 mmol/l glucose+10 μmol/l MG 132. After incubation the islets were double immunolabeled for insulin and iNOS and analysed by confocal microscopy. Insulin and iNOS stainings appear, respectively, as *red* (**A**, **D**, **G**, **J**, **M** and **P**) and *green* (**B**, **E**, **H**, **K**, **N** and **Q**) fluorescence. Co-localisation of insulin/iNOS is seen as a *orange-yellowish* fluorescence (**C**, **F**, **I**, **L**, **O** and **R**). Plates **S**-**U** shows Wistar control islets at 3.3 mmol/l glucose. No iNOS expression could be detected (**T**). Bars indicate lengths (10 μm).
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the proteasome inhibitor lactacystin [40]. Similarly, β-cell iNOS induced by lipid infusion to rats was abolished by treatment of isolated islets from such rats with MG 132 [41]. A paradoxical effect of proteasomal inhibition by MG 132 was also reported in

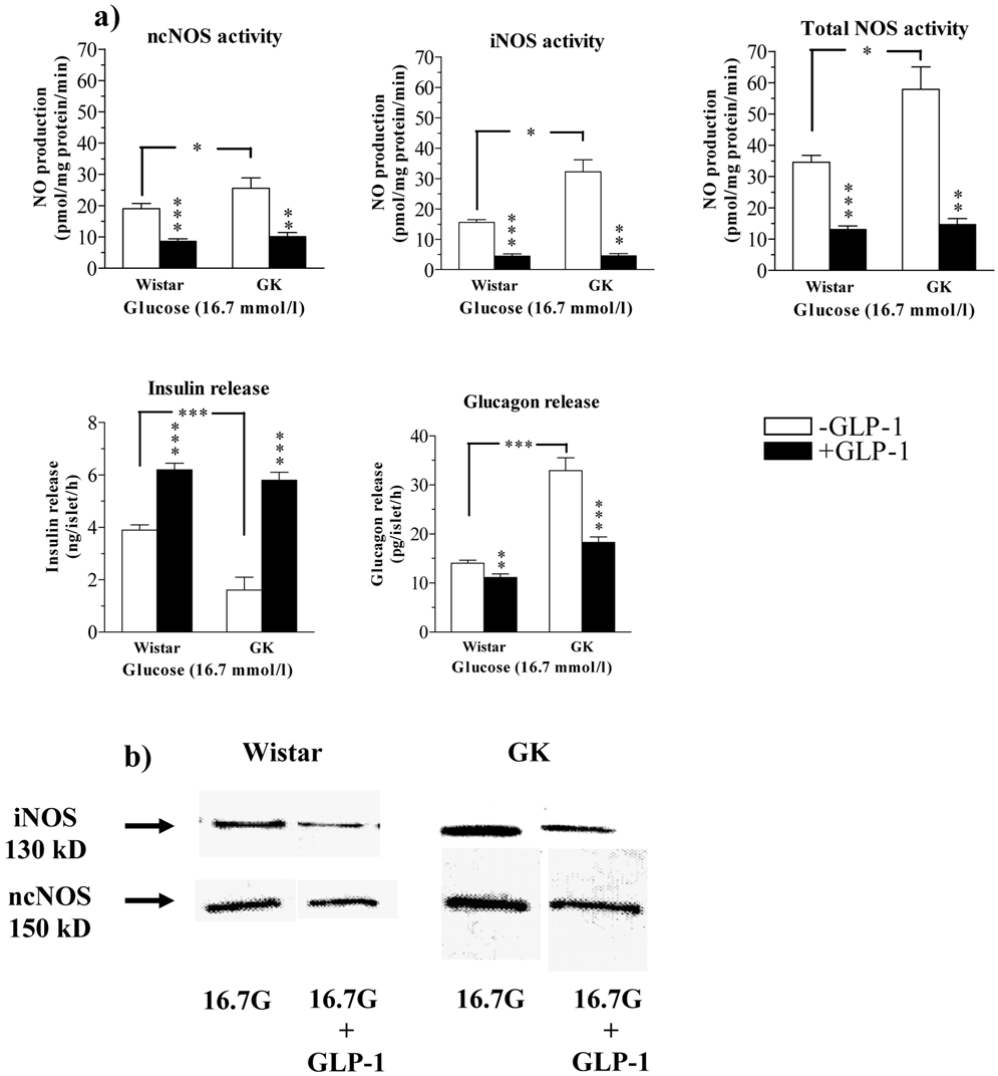

Figure 4. (a) NOS activities and hormone secretion in islets incubated at high glucose. Islet NO-production from ncNOS, iNOS and total NOS as well as insulin and glucagon release from islets of Wistar or GK rats incubated at 16.7 mmol/l glucose in the absence (open bars) and presence (dark bars) of 100 nmol/l GLP-1. Values are mean±s.e.m for 6–10 batches of islets at each point. *P<0.05; ** P<0.01; *** P<0.001. (**b**) Representative examples of Western blots of iNOS and ncNOS protein in the absence and presence of GLP-1 are shown.
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vascular smooth muscle [23]. These observations raised the question whether certain regulatory mechanisms modulating the expression and degradation of iNOS might be circumvented by the lack of appropriate signaling through the mere presence of a proteasome inhibitor or by other less well-understood mechanisms possibly including factors affiliated with ER stress [42,43]. In fact, very recent studies have shown a most complex series of events induced by various proteasomal inhibitors in different tissues[44,45]. Thus proteasome inhibition indeed seems to induce ER stress followed by activation of autophagic processes in *e.g.* β-cells [44], embryonic fibroblasts and different cancer cell lines [45], while HEK 293 cells and macrophage cell line RAW 264.7 were dependent on the proteasome degradation pathway [24]. The present observation that proteasome inhibition in GK β-cells stimulates the degradation of iNOS protein within 90 min raises further questions concerning the complex interaction between the

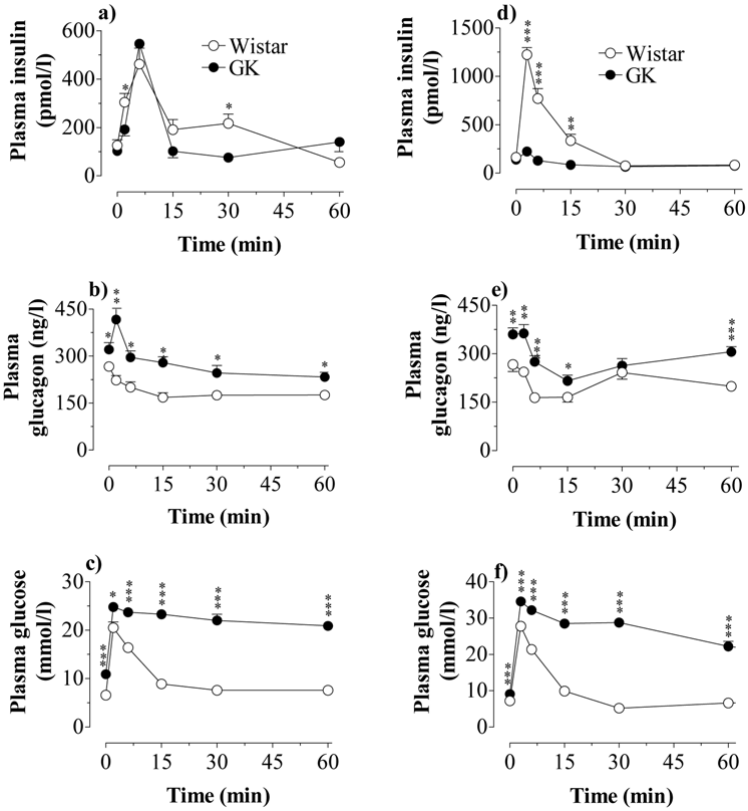

Figure 5. In vivo action of GLP-1 and glucose. Effect of an *iv* injection of GLP-1 (10 nmol/kg)+glucose (4.4 mmol/kg)(**a**–**c**) or glucose alone (11.1 mmol/kg)(**d**–**f**) on the plasma concentrations of insulin, glucagon and glucose in Wistar and GK rats. Values are mean±s.e.m for 8 animals in each group. *P<0.05; ** P<0.01; *** P<0.001.
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proteasomal and autophagic systems in the β-cell, which hopefully will be answered in future studies.

Our *in vivo* experiments showed that a combination of GLP-1 and glucose induced a fairly good insulin response in GK rats, while the response to glucose alone was abrogated. These results agreed with our *in vitro* data, showing a restraining action of GLP-1 on islet NOS activities, and a concomitant amplification of glucose-stimulated insulin release. These effects of GLP-1 are most likely attributed to its ability to increase the islet cyclic AMP/PKA system as previously suggested from our data in healthy animals [2,3,7]. Although also PKA-independent effects of cyclic AMP on distal steps in the stimulus-secretion coupling are described [46] our data favor PKA being of major importance for the regulation of islet NOS activities.

The basal plasma concentrations of glucagon in young GK rats were increased. Moreover, an abnormal secretion of glucagon in GK rats was detected after injecting GLP-1 plus glucose. An unexpected transient hypersecretory glucagon response was followed by a slight suppression, which was less pronounced compared with the marked decrease of glucagon observed in controls. Hence GLP-1 only in part restored the abnormal hypersecretion of glucagon in GK rats. Glucose alone, however, induced a normal initial transient fall of glucagon but this eas followed by a marked rebound at 60 min. An abnormal hypersecretion of glucagon from GK islets was also observed *in vitro*. Notably glucose had a negligible suppressive effect on glucagon secretion in isolated GK islets, while GLP-1 decreased glucagon release concomitant with decreased NO generation in both types of islets, although less efficient so in GK islets. These data thus suggest that GLP-1 has a good amplifying effect on glucose-stimulated insulin release but might be less efficient in strengthening glucose-induced glucagon suppression *in vivo* in this model of type 2 diabetes. NO is an important stimulator of glucagon secretion in healthy animals [1,2,3,6,19,20,21,29,47] and ncNOS resides in both β-cells and α-cells [9], while no expression of iNOS is observed. As mentioned above, in GK rats there is an abundance of peripherally located islet cells, which were iNOS positive and corresponded to glucagon cells showing, that iNOS indeed is highly expressed also in the α-cells of the diabetic rats. Increased levels of circulating glucagon are a common feature in human type 2 diabetes [22]. Hence the present data strongly suggest that an abnormally increased NO production in α-cells

might contribute to the abnormal glucagon hypersecretion in diabetes. The mechanisms of a less efficient effect by GLP-1 to suppress glucagon secretion in diabetes remain unclear but further underline the importance of restoring glucagon hypersecretion in this disease.

In conclusion, we believe that the importance of deleterious effects of excessive islet NO production in nonimmunogenic type 2 diabetes has been seriously overlooked and might explain some if not all of the impairments of islet hormone secretion and β-cell survival. The present data show that abnormally increased expression and activities of islet NOS isoenzymes coincide with increased glucagon secretion and decreased glucose-stimulated insulin release and thus that an excessive NO production is an important contributing factor for the diabetic condition. GLP-1 can, only in part, counteract these abnormalities through activating the cyclic AMP/PKA system. Our data also suggest that high concentrations of circulating glucagon is important for the diabetic condition even in the presence of GLP-1, although this incretin hormone contributes to an increased insulin release through suppressing the NOS activities in the β-cell. These novel data on an NO-generated glucotoxic action in GK islets might be applicable, at least in part, to human type 2 diabetes and hopefully pave the way for new therapeutic interventions to reduce islet NO production.

Materials and Methods

Animals

Young age-matched male GK rats of the Stockholm colony bred at the Karolinska Institute and Wistar control rats (commercially available from B&K, Sollentuna, Sweden) 5–7 weeks old were used in all experiments. They were fed a standard pellet diet (B&K) and tap water *ad libitum*. Notably it is known that certain colonies of Wistar rats might have a defective insulin response to glucose. Ancillary experiments in our laboratory, however, have shown that the actual B&K Wistar rats display an insulin response comparable to that of age-matched Sprague-Dawley rats. The experiments were approved by the Ethical Committee for Animal Research at the University of Lund, Sweden.

Chemicals

Bovine serum albumin was from ICN Biochemicals, High Wycombe, UK. Glucagon-Like Peptide-1 (7–36) amide (GLP-1) was from Peninsula Laboratories, Belmont, CA, USA. H-89 and MG 132 were from Calbiochem, La Jolla, CA, USA. The NOS inhibitor N^G-nitro-L-arginine methyl ester hydrochloride (L-NAME) and all other drugs and chemicals were from Sigma Chemicals, St Louis, MO, USA or Merck AG, Darmstadt, Germany. Polyclonal rabbit anti-iNOS and HRP-conjugated goat anti-rabbit IgG was from StressGen Biotechnologies Corp, Victoria, BC, Canada. Cy2-conjugated anti-rabbit IgG and Cy5-conjugated anti-guinea pig IgG were from Jackson Immunoresearch Laboratories Inc, West Grove, PA, USA. The radioimmunoassay kits for insulin and glucagon determinations were obtained from Diagnostika (Falkenberg, Sweden) and Eurodiagnostica (Malmö, Sweden), respectively.

Isolation of pancreatic islets

Preparation of isolated pancreatic islets was performed by retrograde injection of a collagenase solution *via* the bile-pancreatic duct as previously described [1]. Islets were then collected under a stereomicroscope at room temperature.

In vitro experiments

The freshly isolated islets were preincubated for 30 min at 37°C in Krebs Ringer bicarbonate (KRB) buffer, pH 7.4, supplemented with 10 mmol/l HEPES, 0.1% bovine serum albumin, and 3.3 mmol/l glucose. The composition of the KRB buffer was (in mmol/l); NaCl 120, KCl 4.7, $CaCl_2$ 2.54, KH_2PO_4 1.2, Mg SO_4 1.2 and $NaHCO_3$ 25. Each incubation vial contained 250 islets in 1.5 ml buffer solution (60 islets in 1.5 ml for confocal experiments) and was gassed with 95% O_2-5% CO_2 to obtain constant pH and oxygenation. After preincubation the buffer was changed to a medium containing the test agents, and the islets were incubated for 90 minutes. All incubations were performed at 37°C in an incubation box (30 cycles/min). Immediately after incubation, aliquots of the medium were removed for assay of insulin and glucagon [48,49,50] and the islets were prepared for measurement of NOS activities as described below.

Immunofluorescence and confocal microscopy

Islets freshly isolated or collected after incubation in KRB buffer as described above were fixed with 4% formaldehyde, permeabilized with 5% Triton X-100, and unspecific sites blocked with 5% Normal Donkey Serum (Jackson Immunoresearch Laboratories Inc, West Grove, PA, USA). iNOS was detected with a rabbit-raised polyclonal anti-iNOS antibody (StressGen Biotechnologies Corp, Victoria, BC, Canada) (1:100) in combination with Cy2-conjugated anti-rabbit IgG (Jackson Immunoresearch Laboratories Inc, West Grove, PA, USA). (1:150). For staining of insulin and glucagon, islets were incubated with a guinea pig-raised anti-insulin antibody (1:1000) and anti-glucagon antibody (1:200) (Eurodiagnostica, Malmö, Sweden) followed by an incubation with a Cy5-conjugated either anti-guinea pig IgG antibody (insulin) or Cy5-conjugated anti-rabbit IgG antibody (glucagon) (Jackson Immunoresearch Laboratories Inc, West Grove, PA, USA) (1:150). The fluorescence was visualized with a Zeiss LSM510 confocal microscope by sequentially scanning at (excitation/emission) 488/505-530 nm (Cy2) and 633/>650 nm (Cy5).

Measurement of NOS activities

Isolated islets (batches of 250 islets in 1.5 ml of buffer solution) were either prepared for direct assay of NOS activities or preincubated for 30 min and then incubated for 90 min in KRB buffer as described above. Thereafter an aliquot of the medium was taken for the assay of insulin and glucagon. Islets were thoroughly washed and collected in ice-cold buffer (200 µl) containing HEPES (20.0 mmol/l), EDTA (0.50 mmol/l) and D, L-dithiothreitol (DTT) (1.0 mmol/l), pH 7.2, and stored at −20°C for subsequent NOS analysis [1,11]. In brief, after sonication on ice, the buffer solution containing the islet homogenate was supplemented to contain also $CaCl_2$ (0.45 mmol/l), calmodulin (25 U/ml), NADPH (2.0 mmol/l) and L-arginine (0.2 mmol/l) in a total volume of 450 µl for determination of total NOS. For the assay of iNOS another portion of the homogenate was incubated in the absence of both calmodulin and $CaCl_2$ [11]. The homogenates were then incubated at 37°C under constant air bubbling (1.0 ml/min) for 180 minutes. Aliquots of the incubated medium (200 µl) were mixed with an equal volume of *o*-phtaldialdehyde reagent solution in a glass vial and then passed through an 1 ml Amprep CBA cation-exchange column for high-performance liquid chromatography (HPLC) analysis. The amount of L-citrulline formed (NO and L-citrulline are produced in equimolar concentrations) was then measured in a Hitachi F1000 fluorescence spectrophotometer (Merck, Darmstadt, Germany) as previously described [1,11]. The resulting activity for iNOS was subtracted from total NOS activity

to give the ncNOS activity [11]. Protein was determined with the Bradford method [51].

Western blot analysis

Islets incubated as stated above for the assay of islet NOS activities were analyzed for immunoblotting. After incubation the islets were washed in Hanks' buffer and then suspended in 150 µl of 10 mmol/l Tris lysis buffer, pH 7.4, containing 0.5% Triton X-100, 0.5 mmol/l EDTA and 0.2 mmol/l PEFA block, frozen and sonicated on ice on the day of analysis [11]. The protein content of the supernatant was determined according to Bradford [51]. Homogenate samples representing 10 µg of total protein were run on 7.5% SDS-polyacrylamide gel (Bio-Rad, Hercules, CA, USA). After electrophoresis, proteins were transferred to nitrocellulose membranes (Bio-Rad). The membranes were blocked in LS-buffer (10 mmol/l Tris, pH 7.4, 100 mmol/l NaCl, 0.1% Tween-20) containing 5% non-fat dry milk powder for 40 min at 37°C. Incubation was performed with rabbit anti-mouse ncNOS (N-7155) and iNOS (N-7782) (1:2000; Sigma, St Louis, MO, USA). After three washings in LS-buffer the membranes were finally incubated with a horseradish peroxidase-conjugated goat anti-rabbit antibody (1:50000). Immunoreactivity was detected using an enhanced chemiluminescence reaction (Pierce, Rockford, IL, USA). The intensities of the bands were quantified by densitometry (Bio-Rad GS-710 Densitometer).

In vivo studies

The rats were injected *i.v* with a mixture of GLP-1 (10 nmol/kg) and glucose (4.4 mmol/kg), or glucose alone (11.1 mmol/kg) and blood sampling was performed as described previously [17]. GLP-1 was dissolved in 0.9% NaCl-0.1% gelatine. In the L-arginine experiments L-NAME (1.2 mmol/kg) or saline (controls) was injected *i.v.* 10 seconds before the *i.v.* injection of L-arginine (3.6 mmol/kg). Both L-NAME and L-arginine were dissolved in 0.9% NaCl. The volume load was 5 µl/g rat.

Determination of insulin, glucagon and glucose

Insulin and glucagon were determined with radioimmunoassays [48,49,50]. Glucose was determined with glucose oxidase.

Statistics

Results are expressed as mean±s.e.m. Probability levels of random differences were determined by the unpaired Student's t-test or where applicable by analysis of variance followed by Tukey-Kramer's multiple comparison test.

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Author Contributions

Conceived and designed the experiments: AS. Performed the experiments: AS SM JJ. Analyzed the data: AS IL SM. Contributed reagents/materials/analysis tools: CÖ SE IL. Wrote the paper: AS.

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Imidazoline-induced amplification of glucose- and carbachol-stimulated insulin release includes a marked suppression of islet NO generation in the mouse

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Abstract

Aim: The role of islet nitric oxide (NO) production in insulin-releasing mechanisms is unclear. We examined whether the beneficial effects of the imidazoline derivative RX 871024 (RX) on β-cell function might be related to perturbations of islet NO production.

Methods: Experiments were performed with isolated islets or intact mice challenged with glucose or carbachol with or without RX treatment. Insulin was determined with radioimmunoassay, NO generation with high-performance liquid chromatography and expression of inducible NO synthase (iNOS) with confocal microscopy.

Results: RX treatment, in doses lacking effects on basal insulin, greatly amplified insulin release stimulated by the NO-generating secretagogues glucose and carbachol both *in vitro* and *in vivo*. RX also improved the glucose tolerance curve. Islets incubated at high glucose levels (20 mmol L^{-1}) displayed increased NO production derived from both neuronal constitutive NO synthase (ncNOS) and iNOS. RX abrogated this glucose-induced NO production concomitant with amplification of insulin release. Confocal microscopy revealed abundant iNOS expression in β cells after incubation of islets at high but not low glucose levels. This was abolished after RX treatment. Similarly, islets cultured for 24 h at high glucose levels showed intense iNOS expression in β cells. This was abrogated with RX and followed by an amplified glucose-induced insulin release.

Conclusion: RX effectively counteracts the negative impact of β-cell NO generation on insulin release stimulated by glucose and carbachol suggesting imidazoline compounds by virtue of NOS inhibitory properties being of potential therapeutic value for treatment of β-cell dysfunction in hyperglycaemia and type 2 diabetes.

Keywords cholinergic stimulation, glucose stimulation, imidazoline RX 871024, insulin release, islet nitric oxide synthase isoenzymes.

Over the last several years, a wide range of imidazoline compounds has been shown to have the capacity to amplify glucose-stimulated insulin release and thus being suggested as a new therapeutic approach to type 2 diabetes (reviewed by Morgan *et al.* 1999, Efendic *et al.* 2002). One of the most studied imidazoline derivatives is RX 871024 (hereafter called RX in the text) (Zaitsev *et al.* 1996, Mourtada *et al.* 1999, Efanov *et al.* 2001, 2002) which belongs to the so-called classical insulinotropic imidazolines possessing both the property to close the ATP-sensitive K^+ (K^+_{ATP}) channel activity and having additional effects to directly

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promote the exocytotic process in the β cells. The direct facilitating effect on exocytosis has been reported to be exerted mainly through activating the DAG–PKC system (Efendic *et al.* 2002). In previous studies, we have presented evidence that both glucose and the PLC–PKC-stimulating cholinergic muscarinic agonist carbachol induce a marked and immediate generation of islet NO production, which in turn induces a negative feedback action on the ensuing release of insulin (Panagiotidis *et al.* 1995, Salehi *et al.* 1996, Akesson & Lundquist 1998, 1999a, Akesson *et al.* 1999, Henningsson *et al.* 2002, Jimenez-Feltstrom *et al.* 2005). Thus, a suppressive effect through the action of RX on the islet NO-generating system might be a possible event contributing to the beneficial effects of RX on insulin secretory processes stimulated by glucose and carbachol. The aim of the present investigation was to compare *in vitro* and *in vivo* effects of RX on insulin release induced by glucose and carbachol and further to explore whether there is a direct relation between islet NO generation and insulin release after short-time and long-time incubations at high glucose levels in the absence and presence of RX, as hyperglycaemia is a decisive factor underlying the development of type 2 diabetes.

Materials and methods

Animals

Female mice of the NMRI strain (B&K, Sollentuna, Sweden), weighing 25–35 g, were used throughout the experiments. They were given a standard pellet diet (B&K) and tap water *ad libitum*, before both *in vivo* and *in vitro* experiments. The experimental procedures were approved by the Ethics Committee for Animal Research at University of Lund, Sweden.

Drugs and chemicals

RX 871024 [1-phenyl-2-(imidazoline-2-yl)benzimidazole] was obtained from Reckitt and Colman (UK). Collagenase (CLS-4) was purchased from Worthington Biochem., Corp., Freehold, NJ, USA. Carbachol was obtained from Sigma Chemicals, St Louis, MO, USA. All other drugs and chemicals were from British Drug Houses Ltd, Poole, UK or Merck AG, Darmstadt, Germany. The radioimmunoassay kits for insulin determinations were obtained from Diagnostica Ltd, Falkenberg, Sweden.

Experimental protocol

In vitro insulin release studies. Preparation of isolated pancreatic islets from the mouse was performed by retrograde injection of a collagenase solution via the bile-pancreatic duct (Gotoh *et al.* 1985). In batch incubation experiments, freshly isolated islets were pre-incubated for 30 min at 37 °C in Krebs–Ringer bicarbonate buffer, pH 7.4, supplemented with 10 mmol L^{-1} HEPES, 1 mmol L^{-1} glucose and 0.1% bovine serum albumin (Salehi *et al.* 1996). Each incubation vial was gassed with 95% O_2 and 5% CO_2 to obtain constant pH and oxygenation. After pre-incubation, the buffer was changed to a fresh KRB buffer supplemented with the different test agents and the islets (10–12 islets per 1.0 mL of medium in each incubation vial) were incubated for 60–90 min. All incubations were performed at 37 °C in an incubation box (30 cycles per min). Immediately after incubation aliquots of the medium were removed and frozen for subsequent assay of insulin (Salehi *et al.* 1996). In one set of experiments, the islets were cultured for 24 h in RPMI-1640 (SVA, Uppsala, Sweden) supplemented with 10% calf serum, 100 U mL^{-1} penicillin and 10 μg mL^{-1} streptomycin with and without RX as indicated in the legends. After culture and washing, the islets were pre-incubated and incubated as described above.

Assay of islet NOS activities

Pre-incubation and incubation of freshly isolated islets were performed as stated above with the exception that each incubation vial contained 250 islets in 1.5 mL (Henningsson *et al.* 2002) of buffer solution. After an incubation period of 90 min, aliquots of the medium were removed for determination of insulin. Thereafter, the islets were washed and collected in 200 μL of buffer, containing 20 mmol L^{-1} HEPES, 0.5 mmol L^{-1} EDTA and 1.0 mmol L^{-1} DL-dithiothreitol and stored at −20 °C. On the day of the assay, the islets were sonicated on ice and for measuring ncNOS activity the buffer solution was enriched with 0.45 mmol L^{-1} $CaCl_2$, 2 mmol L^{-1} NADPH, 25 U mL^{-1} calmodulin and 0.2 mmol L^{-1} L-arginine. For the determination of iNOS activity, both Ca^{2+} and calmodulin were omitted from the buffer (Henningsson *et al.* 2002). The homogenate solution was then incubated at 37 °C under constant air bubbling, 1.0 ml min^{-1} for 2 h. Aliquots of the incubated homogenate (200 μL) were then passed through an 1-mL Amprep CBA cation exchange column for determination of L-citrulline by high-performance liquid chromatography (HPLC). The method has been described in detail (Henningsson *et al.* 2002). As L-citrulline and NO are generated in equimolar amounts, and as L-citrulline is stable, whereas NO is not, L-citrulline is the preferred parameter when measuring NO production. Protein was determined according to Bradford (1976) on samples from the original homogenate.

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Immunofluorescence and confocal microscopy

In a parallel experiment, the freshly isolated islets were incubated for 90 min at 20 mmol L^{-1} glucose in the presence or absence of RX (10 μmol L^{-1}) with the same experimental procedure as stated above for the assay of islet NOS activities. A control experiment at 7 mmol L^{-1} glucose was also performed. After incubation, the islets were washed (three times) and fixed with 4% formaldehyde, permeabilized with 5% Triton X-100 and unspecific sites were blocked with 5% Normal Donkey Serum (Jackson Immunoresearch Laboratories Inc., West Grove, PA, USA). iNOS was detected with a rabbit-raised polyclonal anti-iNOS antibody (StressGen Biotechnologies Corp., Victoria, BC, Canada) (1 : 100) in combination with Cy2-conjugated anti-rabbit IgG (Jackson Immunoresearch Laboratories Inc.) (1 : 150). For staining of insulin, islets were incubated with a guinea pig-raised anti-insulin antibody (Eurodiagnostica, Malmö, Sweden) (1 : 1000) followed by incubation with a Cy5-conjugated anti-guinea pig IgG antibody (Jackson Immunoresearch Laboratories Inc.) (1 : 150). The fluorescence was visualized with a Zeiss LSM510 confocal microscope by sequentially scanning at (excitation/emission) 488/505–530 nm (Cy2) and 633/>650 nm (Cy5). In another experiment, the same procedure was used to detect the iNOS expression after 24-h culturing of islets at 5 or 20 mmol L^{-1} glucose in the presence or absence of RX (10 μmol L^{-1}). The expression of iNOS at a low substimulatory glucose concentration was also studied at both conditions (short-term incubation or long-term culture of islets). For scoring of iNOS-positive cells in islet tissue, multiple fields for each section were analysed under blind conditions. The mean fluorescent intensity of cellular iNOS was quantified using Zeiss LSM5 analysis software.

In vivo studies. RX 871024 was dissolved in 0.1 N HCl and the pH adjusted to 5.0 with 1 N NaOH. It was then diluted in 0.9% NaCl (saline). Freely fed animals were pre-treated with RX by an intraperitoneal injection (i.p.), as denoted in the figure legends. Controls received the solvent solution. Glucose and carbachol were dissolved in saline and injected into a tail vein (volume load 5 μL g^{-1} mouse). Blood sampling was performed as previously described (Salehi *et al.* 1996) for the determination of insulin and glucose. Plasma glucose concentrations were determined with a glucose oxidase method and insulin with a radioimmunoassay (RIA) (Salehi *et al.* 1996).

Statistics

Probability levels of random differences were determined by an analysis of variance followed by Tukey–Kramer's multiple comparisons test or Student's *t*-test for unpaired data with Welch correction where applicable.

Results

RX-induced insulin release from incubated islets: dose–response study at basal and elevated glucose concentrations and effect on carbachol-stimulated secretory response

The first experiment was designed to study the effects of increasing concentrations of RX on the insulin release pattern of incubated islets at basal (7 mmol L^{-1}) glucose. Figure 1a shows that RX concentrations up to 10 μmol L^{-1} did not affect basal insulin release at this glucose concentration. We have previously found that there is a steady-state basal insulin release from isolated mouse islets incubated at concentrations between 1 and 7 mmol L^{-1} glucose and that the maximal insulin response after incubation with the cholinergic muscarinic agonist carbachol at 7 mmol L^{-1} glucose is achieved at a concentration of 50 μmol L^{-1} (Akesson & Lundquist 1999a). In ancillary experiments, we found that the insulin response to 12 mmol L^{-1} glucose and 50 μmol L^{-1} carbachol, respectively, was of approximately the same magnitude. This is also shown here (Fig. 1a

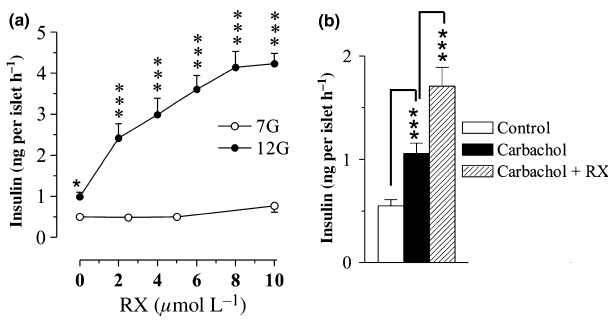

Figure 1 (a) Dose–response effects on insulin release from isolated islets by RX 781024 at 7 mmol L^{-1} glucose (open circles) and 12 mmol L^{-1} glucose (closed circles). (b) Effect of RX 781024 (10 μmol L^{-1}) on insulin release stimulated by carbachol (50 μmol L^{-1}) at basal glucose levels (7 mmol L^{-1}). Mean SEM for 8–13 incubation vials in each group is shown. $^{*}P < 0.05$, $^{***}P < 0.001$.

and b). To compare the effects of RX on insulin release induced by glucose vs. cholinergic stimulation, we first performed a dose–response study with RX at 12 mmol L^{-1} glucose. Figure 1a shows a pronounced amplification of glucose-stimulated insulin release already at an RX concentration of 2 μmol L^{-1} and a maximal response (fivefold increase) was recorded at 8–10 μmol L^{-1} RX (Fig. 1a). Figure 1b shows that also carbachol-stimulated insulin release is greatly increased by 10 μmol L^{-1} RX, although not to the same level as the glucose-stimulated insulin response.

Acute in vivo *effects of RX pre-treatment on insulin release induced by glucose and carbachol*

Figure 2 shows the acute insulin response following an i.v. injection of saline, glucose or carbachol with and without RX pre-treatment. The doses of glucose and carbachol in the absence of RX were adjusted to give an insulin response of comparable magnitude (Fig. 2). Injection of RX doses less than 66 μmol kg^{-1} of RX had no effect on basal plasma insulin levels (Fig. 2; saline). Pre-treatment with 16.5 μmol kg^{-1} of RX, a dose that did not influence basal insulin, was found to induce a maximal amplification of the insulin response to both glucose and carbachol (Fig. 2).

In vivo *dynamics of plasma levels of insulin and glucose following stimulation by glucose after pre-treatment with RX or saline*

Groups of mice were pre-treated with an i.p. injection of RX (33 μmol kg^{-1}) or saline (controls). After 10 min, the animals were injected i.v. with glucose and the dynamics of the insulin and glucose responses were recorded. Figure 3 shows that glucose-induced insulin release was greatly augmented by RX pre-treatment. This increase in insulin release was reflected in a marked improvement in the glucose tolerance curve (Fig. 3).

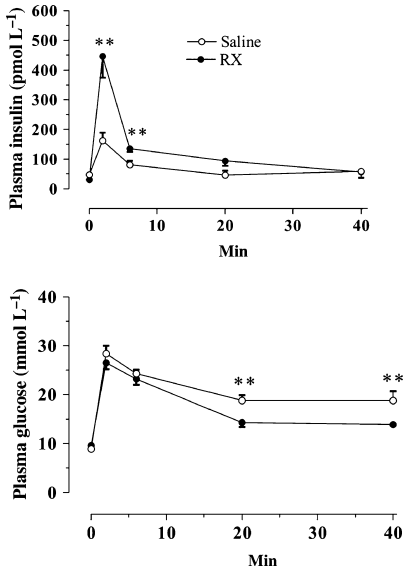

Figure 3 *In vivo* dynamics of plasma levels of insulin and glucose after an i.v. injection of glucose (3.3 mmol kg^{-1}). Pre-treatment with RX 781024 (33 μmol kg^{-1}) (closed circles) or solvent (open circles) was performed i.p. at −10 min. Mean ± SEM is shown. There were eight animals in each group. $^{*}P < 0.01$.

Confocal microscopy of islet iNOS expression after short-term incubation at low and high glucose levels as well as activities of islet NOS isoenzymes in relation to glucose-stimulated insulin release in the absence and presence of RX

Confocal microscopy showed abundant if not exclusive iNOS expression in the β cells after the incubation of the islets at high glucose levels (20 mmol L^{-1}) (Fig. 4d–f).

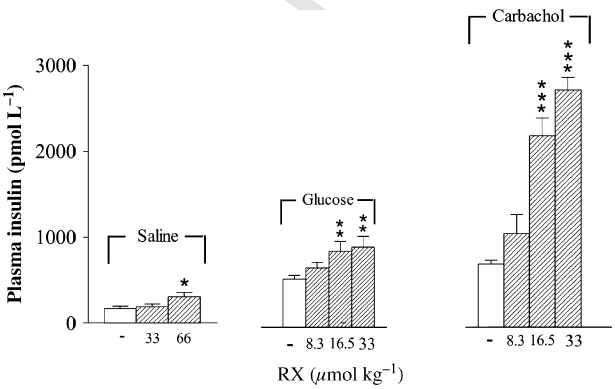

Figure 2 *In vivo* effect of different pre-treatment doses of RX 781024 on the acute insulin secretory response to glucose (3.3 mmol kg^{-1}), carbachol (0.16 μmol kg^{-1}) or saline (basal). RX 781024 (hatched columns) or solvent (white columns) was injected i.p. 10 min before an i.v. injection of glucose, carbachol or saline. Blood sampling was performed at 2 min after the i.v. injection. Mean ± SEM is shown. There were seven to 20 animals in each group. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

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COLOUR

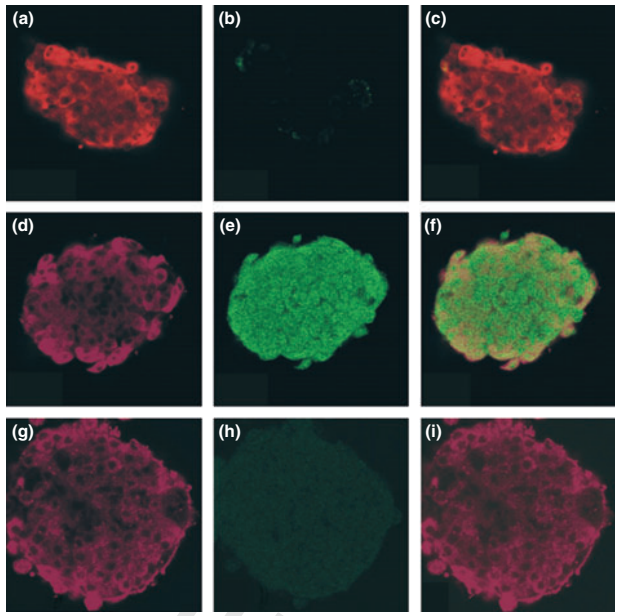

Figure 4 Confocal microscopy of islets incubated in the absence and presence of RX 781024. Isolated islets were incubated for 90 min in the presence of 7 mmol L^{-1} glucose (a,b,c); 20 mmol L^{-1} glucose (d,e,f); 20 mmol L^{-1} glucose + 10 μmol L^{-1} RX (g,h,i). After incubation, the islets were double immunolabelled for insulin and iNOS. Insulin and iNOS staining appear, respectively, as red (a,d,g) and green (b,e,h) fluorescence. Co-localization of insulin/iNOS is seen as an orange-yellowish fluorescence (c,f,i).

This expression of iNOS was abolished in the presence of RX (Fig. 4g–i). There was no expression of iNOS after islet incubation at 7 mmol L^{-1} glucose (Fig. 4a–c). Fluorescence intensity measurements showed the following results in arbitrary units (taking the intensity at 20 mmol L^{-1} glucose as 100%): basal (7 mmol L^{-1}) glucose 1.95 ± 0.57 (n = 12); high glucose 100.0 ± 2.8 (n = 12) and high glucose + RX 8.5 ± 2.4 (n = 12).

The next experiment was then designed to study the direct effect of RX on islet NO production. Figure 5 illustrates the results of short-term (90 min) incubation of isolated islets at high glucose levels (20 mmol L^{-1}) on the activities of ncNOS, iNOS as well as total NOS and insulin release in the absence and presence of RX. Basal controls at 7 mmol L^{-1} glucose are included. The data show that RX did induce a pronounced suppression of NO generation at high glucose levels in association with a marked amplification of glucose-stimulated insulin release.

Islet culture for 24 h at low and high glucose levels in the absence and presence of RX: confocal images of iNOS expression and effects on glucose-stimulated insulin release

Islets were cultured for 24 h at low (5 mmol L^{-1}) or high (20 mmol L^{-1}) glucose levels in the absence and presence of RX. All three categories of islets were then examined for the expression and localization of iNOS by confocal microscopy. Figure 6d–f shows that culture at high

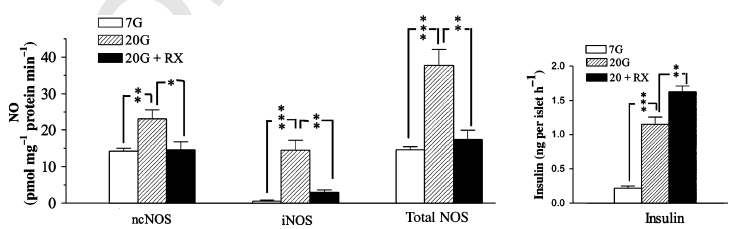

Figure 5 Activities of islet NOS isoenzymes in relation to glucose-induced insulin release after short-time incubation (90 min) of isolated islets in the absence and presence of RX 781024 (10 μmol L^{-1}). Islet NO production from ncNOS, iNOS and total NOS as well as insulin release from islets incubated at 7 mmol L^{-1} glucose (open columns), 20 mmol L^{-1} glucose (hatched columns) and 20 mmol L^{-1} glucose + RX (black columns) are shown. Mean ± SEM for six to seven incubation vials in each group is shown. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$.

COLOUR

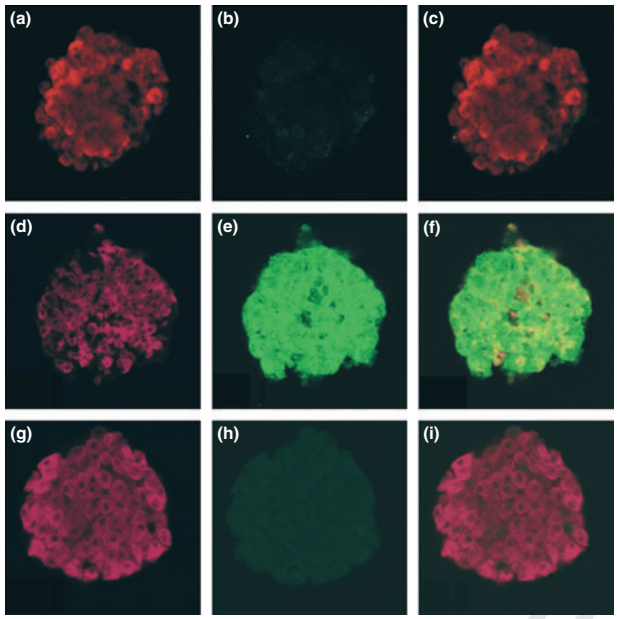

Figure 6 Confocal microscopy of islets cultured for 24 h in the absence and presence of RX 781024. Isolated islets were cultured for 24 h in the presence of 7 mmol L^{-1} glucose (a,b,c); 20 mmol L^{-1} glucose (d,e,f) and 20 mmol L^{-1} glucose + 10 μmol L^{-1} RX (g,h,i). After incubation, the islets were double immunolabelled for insulin and iNOS. Insulin and iNOS staining appear, respectively, as red (a,d,g) and green (b,e,h) fluorescence. Co-localization of insulin/iNOS is seen as an orange-yellowish fluorescence (c,f,i).

glucose levels induced a marked expression of iNOS, which was mainly colocalized to the insulin-producing β cells. The addition of RX to the culture medium abrogated iNOS expression (Figure 6g–i). No iNOS was expressed after culture at low glucose (Figure 6a–c). Fluorescence intensity measurements showed the following results in arbitrary units (taking the intensity at 20 mmol L^{-1} glucose as 100%); 5G: 3.4 ± 0.9 (n = 12); 20G: 100.0 ± 4.0 (n = 12) and 20G + RX: 12.5 ± 2.7 (n = 12). It is of interest that the intensity of iNOS fluorescence after culture in high glucose levels for 24 h was much higher than after incubation for 90 min suggesting a progressive iNOS-derived NO generation being potentially destructive for the β cells over time. Intensity measurements (arbitrary units) comparing exposure to high glucose levels for 24 h vs. 90 min taking the intensity after 24 h as 100% gave the following results: 24 h: 100.0 ± 4.0 and 90 min: 65.30 ± 4.1 (n = 10) (P < 0.001). The cultured islets were then incubated for 60 min in low (1 mmol L^{-1}) or high (20 mmol L^{-1}) glucose levels to test the insulin secretory capacity of the different categories of islets. Figure 7 shows that culture at 5 mmol L^{-1} of glucose in the presence of RX did not change either the basal insulin secretion at low glucose levels or the glucose-stimulated insulin release. However, islets cultured at high glucose

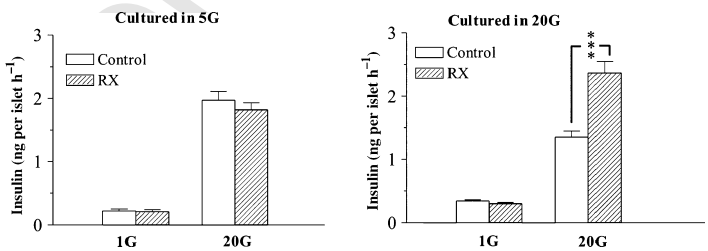

Figure 7 Effect of RX 781024 treatment on glucose-stimulated insulin secretion from islets cultured for 24 h at low and high glucose levels. Islets were cultured for 24 h at low (5 mmol L^{-1}) or high (20 mmol L^{-1}) glucose levels in the absence and presence of RX 781024 (10 μmol L^{-1}). After washing and pre-incubation (30 min), the islets were incubated at a low basal concentration of glucose (1 mmol L^{-1}) or stimulated by a high (20 mmol L^{-1}) concentration of glucose for 60 min. Mean ± SEM for eight batches of islets in each group is shown. ***P < 0.001.

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levels (20 mmol L^{-1}) in the presence of RX showed a great amplification of glucose-stimulated insulin release compared with those cultured without RX (Fig. 7). Basal insulin secretion at 1 mmol L^{-1} glucose was similar in both categories of islets.

Discussion

The present combined *in vitro* and *in vivo* study in mice shows that the imidazoline compound RX 781024 has a pronounced amplifying effect on insulin secretory processes stimulated by both glucose and the cholinergic muscarinic agonist carbachol. Data from previous *in vitro* studies with isolated rat islets have shown that RX might promote insulin release through interaction with various molecular targets (Mourtada *et al.* 1999, Efendic *et al.* 2002). In addition to its ability to inhibit the K^+_{ATP} channels with subsequent membrane depolarization and increase in $[Ca^{2+}]_i$ leading to insulin discharge, there is also evidence for the involvement of the imidazoline to further facilitate the exocytotic process through activation of the DAG–PKC system (Efanov *et al.* 2001, Efendic *et al.* 2002, Sharoyko *et al.* 2007).

Because the acute insulin-releasing effect of glucose is exerted mainly through the increase in $[Ca^{2+}]_i$ (Henquin *et al.* 2002) and that of carbachol is mainly exerted through increased sensitivity to Ca^{2+} and events related to PKC activation (Gilon & Henquin 2001, Thore *et al.* 2005), these data suggested that the facilitating and amplifying effects elicited by RX on insulin release might be explained, at least in part, by the ability of the imidazoline to suppress a release-restraining element induced by both glucose and carbachol. We have previously shown that such a common denominator might be the gaseous messenger NO. A rapid generation of NO seems to be coupled, as a negative modulator, to the acute stimulation of the insulin secretory processes induced by both glucose and carbachol (Panagiotidis *et al.* 1995, Salehi *et al.* 1996, 2008, Akesson & Lundquist 1998, 1999a,b, Henningsson *et al.* 2002). Notably, such a negative feedback by NO is not exerted on cyclic AMP–PKA-induced insulin release (Akesson & Lundquist 1999b, Salehi *et al.* 2003). In fact, NO seems to facilitate cyclic AMP-induced insulin release (Akesson & Lundquist 1999b).

The present data show that the dose–response effects of RX on insulin release from isolated mouse islets during incubation with low and high glucose levels are quite comparable with previous data in rat islets (Zaitsev *et al.* 1996). The maximal stimulating effect of RX in the presence of 12 mmol L^{-1} of glucose was achieved at 10 μmol L^{-1}, a concentration that did not affect insulin secretion at basal glucose levels but greatly amplified also the carbachol-stimulated insulin release. Moreover, the results from our acute *in vivo* experiments show that RX has an immediate amplifying effect on the insulin response following i.v. glucose or carbachol. This effect is evident within minutes and might agree with a direct inhibitory action of RX on the glucose- and carbachol-induced rapid generation of ncNOS-derived NO, which is known to suppress the insulin secretory response to both glucose and carbachol (Panagiotidis *et al.* 1995, Salehi *et al.* 1996, 2008, Akesson & Lundquist 1998, 1999a, Akesson *et al.* 1999, Henningsson *et al.* 2002). Further, the dynamic pattern of the insulin and glucose responses following glucose injection after RX pre-treatment is clearly reminiscent of a similar experiment where the mice were pre-treated with the ncNOS inhibitor N^G-nitro-L-arginine methyl ester (L-NAME) (Akesson *et al.* 1999). Similarly, previous experiments with perifused isolated islets showed that pharmacological blockade of ncNOS resulted in an immediate amplification of insulin release stimulated by glucose or carbachol (Akesson & Lundquist 1998, Akesson *et al.* 1999, Henningsson *et al.* 2002). It was also shown (Akesson & Lundquist 1998) that the blockade of ncNOS amplified insulin release induced by the phorbol ester TPA. Unlike carbachol, which stimulates both the IP_3–Ca^{2+} and the DAG–PKC messenger systems, TPA stimulates only PKC. Hence, PKC seems to be a main target for NO. This is conceivable as NO is known to inhibit many regulatory proteins through S-nitrosylation processes (Stamler *et al.* 1992, Jaffrey *et al.* 2001) and PKC is known to be equipped with critical thiol residues (Hu & el-Fakahany 1996). Our results are also in accordance with previous studies on rat islets showing that RX has a beneficial effect on the DAG–PKC system in association with an increase in arachidonic acid and its metabolites (Efanov *et al.* 2001, Efendic *et al.* 2002, Sharoyko *et al.* 2007). Such an effect might thus be explained, at least in part, through the inhibition of ncNOS-derived NO generation by RX. In this context, it should be recalled that we have shown previously that the intracellular NO donor hydroxylamine inhibits, while the ncNOS blocker L-NAME augments insulin release induced by both glucose and carbachol (Akesson & Lundquist 1998, 1999a, Akesson *et al.* 1999, Henningsson *et al.* 2002). Notably, we have also repeatedly shown (Salehi *et al.* 1998, Akesson & Lundquist 1999a, Akesson *et al.* 1999, Henningsson *et al.* 2002) that the inhibitory effects of NO are exerted mainly at distal steps in the stimulus–secretion coupling operating independent of membrane depolarization events and thus that the DAG–PKC system as well as different exocytosis-regulating proteins are likely targets for NO not only after insulin release stimulated by carbachol but also after glucose stimulation.

Hence, several lines of evidence speak in favour of ncNOS activity being an important target to suppress in

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order to achieve the amplifying action of RX on insulin release. The ensuing *in vitro* short-term experiments, where we incubated islets in the presence of high glucose levels with and without the addition of RX and then measured the activities of both ncNOS and iNOS, showed that this could well be the case. The glucose-stimulated increase in ncNOS-derived NO production was totally suppressed by RX and associated with amplification of insulin release. Moreover, iNOS activity was increased by glucose and suppressed by RX. However, our previous experiments (Henningsson *et al.* 2002) have shown that, in contrast to ncNOS activity, which raises within minutes after exposure to high glucose levels, iNOS activity does not appear until the end of an incubation period of 60 min and thus would have only a minor influence on glucose-stimulated insulin release during this short time period. Notably, electron microscopy has shown that a major part of the ncNOS enzyme is associated with the insulin secretory granules (Lajoix *et al.* 2001) and thus is conveniently situated to directly control the insulin release process. The iNOS enzyme, on the other hand, is mainly located in the cytoplasm (Knowles & Moncada 1994). However, highly elevated NO levels derived from iNOS, especially during long-term exposure to glucose and lipids (Salehi *et al.* 2001, 2008, Ekelund *et al.* 2006, Qader *et al.* 2007) or pharmacological blockade of ncNOS (Henningsson *et al.* 2000, Salehi *et al.* 2008), are able to modulate glucose-stimulated insulin release. In the present study, we found that RX abrogated the activity and expression of iNOS in short-time experiments. Moreover, culturing islets for 24 h with high glucose levels in the presence of RX abolished iNOS expression and restored the associated impairment of glucose-stimulated insulin release, which is seen after 24 h of exposure to high glucose levels, back to control levels. Notably, fluorescence intensity measurements showed that the fluorescence intensity reflecting β-cell iNOS expression was much higher after 24 h of exposure to high glucose levels than after 90 min, making a progressive NO-induced dysfunction of the β cell a likely scenario over time. In this context, it should be recalled that the regulation of NOS activities has been shown to occur at all levels from gene transcription to covalent modification and allosteric regulation of the enzyme itself (Alderton *et al.* 2001).

In conclusion, the present data bring evidence that the beneficial effects of the imidazoline RX on short-term insulin release induced by glucose and cholinergic muscarinic stimulation are coupled, at least in part, to an inhibitory action by RX on islet ncNOS-derived NO production. Moreover, RX profoundly suppressed iNOS expression after both short-time and long-time exposures to hyperglycaemic concentrations of glucose in association with an amplifying action on glucose-stimulated insulin release. The results underline the notion that imidazoline derivatives might be potential candidates for treatment of β-cell dysfunction in hyperglycaemia and type 2 diabetes and that inhibition of islet overproduction of reactive nitrogen species might be of great importance in this context.

Conflict of interest

None.

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