Regulation of Insulin Secretion in Relation to Nitric Oxide, Carbon Monoxide and Acid alpha-Glucoside Hydrolase Activities

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2005

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"[…] jo, svarade jag, då dimma är, går jag i skyn, och då dimma faller neder, strax regnar det nedanför mig […] Då jag det nekade, och han styrkt sig med ett *dictum scripturæ*, log han åt min enfaldighet, sade sig dock skola lära mig att ett slem kontinuerligen efter regn sitter på bergen, där molnet strykit […] Rådde mig dock tro folk, som förstodo sådant, och icke strax jag komme hem, skriva en disputation med allt sådant galet.

Den andra […] reprehenderade mig, att man lägger sig så mycket på detta världsliga gycklerit, och alltså tyvärr försummar det andliga och mången med sitt fikande i studier bliver fördärvad."

From Carl Linnæus' *Lappländska resan 1732*.

To Kristina, Johan and Carl

Regulation of Insulin Secretion in Relation to Nitric Oxide, Carbon Monoxide and Acid α-Glucoside Hydrolase Activities

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2005

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Printed in Sweden
Media Tryck, Lund 2005

ISBN 91-85439-97-5
ISSN 1654-8220
Lund University, Faculty of Medicine Doctoral Dissertation Series 2005:96

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LIST OF ORIGINAL PAPERS

The thesis is based on the following papers, which in the text will be referred to by their Roman numerals.

I. Salehi A, Mosén H and Lundquist I 1998 Insulin release transduction mechanism through acid glucan-1,4-α-glucosidase activation is Ca^{2+} regulated. *American Journal of Physiology* **274** E459-E468.

II. Salehi A, Henningsson R, Mosén H, Östenson C-G, Efendic S and Lundquist I 1999 Dysfunction of the islet lysosomal system conveys impairment of glucose-induced insulin release in the diabetic GK rat. *Endocrinology* **140** 3045-3053.

III. Mosén H, Salehi A and Lundquist I 2000 Nitric oxide, islet acid glucan-1,4-α-glucosidase activity and nutrient-stimulated insulin secretion. *Journal of Endocrinology* **165** 293-300.

IV. Mosén H, Salehi A, Alm P, Henningsson R, Jimenez-Feltström J, Östenson CG, Efendic S and Lundquist I 2005 Defective glucose-stimulated insulin release in the diabetic Goto-Kakizaki (GK) rat coincides with reduced activity of the islet carbon monoxide signaling pathway. *Endocrinology* **146** 1553-1558.

V. Mosén H, Östenson CG, Lundquist I, Alm P, Henningsson R, Jimenez-Feltström J, Guenifi A, Efendic S and Salehi A 2005 Impaired glucose-stimulated insulin secretion in the GK rat is associated with abnormalities in islet nitric oxide production. *Manuscript.*

VI. Mosén H, Salehi A, Henningsson R and Lundquist I 2005 Nitric oxide inhibits, and carbon monoxide activates, islet acid α-glucoside hydrolase activities in parallel with glucose-stimulated insulin secretion. *Manuscript.*

ABBREVIATIONS

AC	adenylate cyclase
ADP	adenosine 5'-diphosphate
ATP	adenosine 5'-triphosphate
BSA	bovine serum albumin
$[Ca^{2+}]_i$	intracellular calcium concentration
cAMP	adenosine 3',5'-cyclic monophosphate
cGMP	guanosine 3',5'-cyclic monophosphate
CCK	cholecystokinin
CO	carbon monoxide
DAG	diacylglycerol
DTT	D,L-dithiothreitol
EDTA	ethylenediamine tetraacetic acid
EGTA	ethylene glycol-bid(β-amino-ethyl ether) N,N,N',N'-tetraacetic acid
ER	endoplasmic reticulum
FITC	fluorescein isothiocyanate
GC	guanylate cyclase
GIP	gastric inhibitory peptide
GK	Goto-Kakizaki
GLP-1	glucagon-like peptide-1(7-36) amide
GLUT	glucose transporter
GSH	glutathione (reduced)
GSSG	glutathione (oxidized)
HEPES	*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid
HO	heme oxygenase
HO-1	inducible form of HO
HO-2	constitutive form of HO
HPLC	high-performance liquid chromatography
IBMX	3-isobutyl-1-methylxanthine
IDDM	insulin-dependent diabetes mellitus
iNOS	inducible form of nitric oxide synthase
IP_3	inositol 1,4,5-triphosphate
i.m.	intramuscular
i.v.	intravenous
K_{ATP} channel	ATP-sensitive K^+ channel
KIC	α-ketoisocaproic acid
KRB	Krebs Ringer bicarbonate buffer
LADA	late-onset autoimmune diabetes of the adult
L-NAME	N^G-nitro-L-arginine methyl ester
L-NMMA	N^G-monomethyl-L-arginine
LPS	bacterial lipopolysaccharide
MODY	maturity-onset of diabetes in the young
NADPH	nicotinamide adenine dinucleotide hydrogen phosphate
NANC	non-adrenergic, non-cholinergic
ncNOS	constitutive form of nitric oxide synthase
NIDDM	non-insulin-dependent diabetes mellitus
NMRI	naval medical research institute
NO	nitric oxide
NOS	nitric oxide synthase
PBS	phosphate-buffered saline
PKA	cAMP-dependent protein protein kinase
PKC	protein kinase C
PLC	phospholipase C
PP	pancreatic polypeptide
Rp-cAMPS	Rp-adenosine-3',5'-cyclic phosphothioate
SNP	sodium nitroprusside

INTRODUCTION

Historical background

This thesis is devoted to certain mechanisms that are involved in the regulation of insulin secretion. Insulin in itself has quite a long history, being found in mammals, as well as in reptiles, birds and amphibians, thus having an impressive phylogenetical age.[1]

We don't have very many descriptions of what might be recognized as diabetes mellitus from the times of phylogenetic adolescence, but in more modern times, more specifically in an Egyptian papyrus[2] dating some 3500 years back, a polyuric state corresponding to diabetes mellitus is described. A couple of years later, in the 2nd century AD, a man named Aretaeus of Cappadocia was the inventor of the term "diabetes" (syphon in Greek). The sweetness of the urine was for many years the main diagnostic tool, apart from symptoms like increased urinary flow. One of the greater ancients in the history of medicine is the Arab Avicenna ('Abu 'Ali al-Husin ibn 'Abdullah ibn Sina, 980-1037 AD) who among others described the sweet taste of the diabetic urine, *e.g.* in his medical encyclopaedia.

For many years diabetes mellitus remained an unknown entity, and as with medicine in general, it was recognized by the Greeks, and later by the Arabic physicians, but in northern Europe there seems to be very little to speak of in terms of descriptions of the disease. Thomas Willis (1621-1675) wrote 'Diabetes, or the Pissing Evil', and Matthew Dobson (1735-1784) made the first description of hyperglycaemia in 1776. The term 'mellitus' (honey in Greek and Latin) in this context, was first used by John Rollo (died 1809).

It was not until the late 19th century (1889) that Oskar Minkowski (1858-1931) and Josef von Mering (1849-1908) reported the quite sensational observation, that pancreatectomy in the dog caused severe diabetes. Only a few years later, in 1893, Gustave-Edoard Laguesse (1861-1927) gave birth to the term 'Islets of Langerhans' when he suggested that it might be the 'secretions' of the small 'islands' of cells, earlier (in 1869) described by the young Paul Langerhans (1847-1888), where the cause and cure of the disease might be found. The term 'insuline' (from the Latin word 'insula', *i.e.* island) was introduced by the Belgian Jean de Meyer in 1909. A few years later the term 'insulin' was independently coined by the professor of physiology in Toronto John James Richard Macleod (1876-1935).

Much effort to produce a pure pancreatic extract made from the islets of Langerhans was spent through the years, a challenging task taking the proteolytic capacity of the proteases of the exocrine pancreas into account. It was not until 1921 that insulin was discovered, and this by yet another young scientist, namely the orthopaedic surgeon Frederick Banting (1891-1941), who finally managed to convince J.J.R. Macleod to give him some space to work in. While the professor was on a fishing holiday in Scotland, Banting and the student Charles Best (1899-1978) started their work, which took about 6 months.[3]

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1 This short historical background is mainly based on Robert B. Tattersall, *Textbook of Diabetes I*, Section 1, *Diabetes in its historical and social context*, chapter 1, *The history of diabetes mellitus* (2003), s 1.1-1.22. Some biographical details have been found on the Internet at *www.whonamedit.com*.

2 Called the Ebers papyrus, named after its discoverer Georg Ebers.

3 This is described in detail in a book by Michael Bliss, *The Discovery of Insulin*, (1982).

The islets of Langerhans

The endocrine pancreas is localized as cell islets in the exocrine pancreas, separated from the exocrine part by a capsule mainly made of fibroblasts and collagen fibers. These endocrine cell groups contain four major cell types, and the size of the islets vary from a few cells to about 5000. The number of islets in a normal adult pancreas in man contains about 1 million islets, which corresponds to about 2-3% of the total mass of the pancreas. The insulin-secreting β-cells (B-cells) comprise about 60-80% of the total islet cell population, the glucagon-secreting α-cells (A-cells) about 15-20%, the δ-cells (D-cells) which produce somatostatin about 5-10%, and the PP-cells producing pancreatic polypeptide less than 1-2% [135]. More recently ghrelin-secreting cells has been identified in the islets [171]. The β-cells are mainly localized in the core of the islets and the α-cells form, toghether with the δ-cells, a mantle in the periphery of the islets. The less abundant PP-cells are mainly localized in the head of the pancreas (in the mantle of the islets), while the larger part of the α-cells are found in the tail and the body of the pancreas.

The blood supply of the islets is disproportionally large. The islets only constitute about 2-3% of the total pancreatic mass, but receive about 20% of the blood supply of the gland during resting conditions [96] and this flow increases after a bolus dose of glucose [83]. The mechanisms regulating the islet blood flow increase induced by glucose involves both nervous and metabolic mediators [30]. The increased blood flow is partly dependent on NO formation within the islets [82, 119]. The circulation of the islets is constructed in a way where the arteriols enter the islet and reaches the centre of the islet, and from the centre a fine capillary network giving rise to fenestrated venules lead out of the islets, making an extensive exchange of islet hormones possible [151]. There is a dense innervation of the islets, both by sympathetic and parasympathetic nerves, as well as other nerves, *i.e.* non-adrenergic-noncholinergic (NANC). The latter group includes nerves releasing ATP as well as nitric oxide synthase-containing nerves. Another enzyme, discussed in detail in this thesis, is heme oxygenase, which has been detected in most endocrine cells in the islets of the mouse, and was also prominently seen in pancreatic ganglionic cell bodies, often associated with the islets [109].

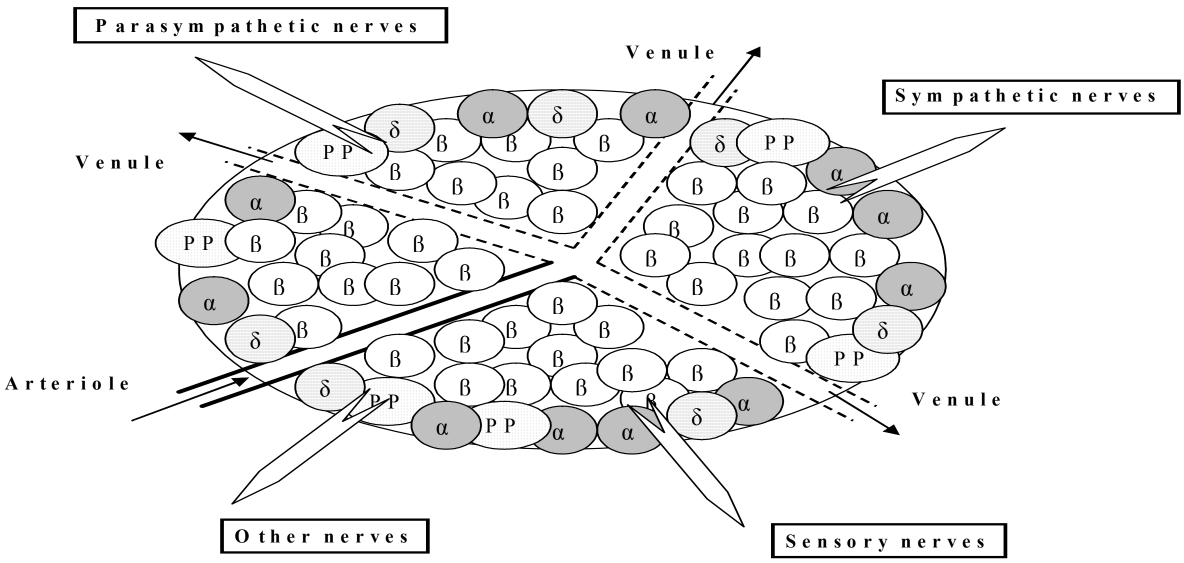

Figure 1. *Schematic illustration of the anatomy of a pancreatic islet, showing a core consisting mainly of β-cells surrounded by a mantle zone formed by a-cells, δ-cells and PP-cells. Afferent arterioles penetrate into the centre of the islet and efferent fenestrated venules lead out of the islet. Also shown are the sympathetic, parasympathetic, sensory and "other" nerves with branches terminating on the islet cells (adopted from [3]).*

Diabetes

Diabetes mellitus is a metabolic disease, caused by inherited and/or aquired factors. It is characterized by a high level of blood glucose. The disease is divided into two major forms, type 1 diabetes and type 2 diabetes.

In *type 1 diabetes* (also called insulin dependent diabetes mellitus (IDDM) or juvenile diabetes mellitus since the onset is usually below the age of 30) the β-cells are destroyed and the cause of this form is thought to be an autoimmune disease. Individuals with type 1 diabetes mellitus are in need of a life-lasting insulin treatment.

In *type 2 diabetes* (also called non insulin dependent diabetes mellitus (NIDDM) there is a relative deficiency of insulin secretion. Many times the blood insulin levels are increased in the early stages of type 2 diabetes. Individuals with type 2 diabetes usually get some kind of oral drug treatment (*e.g.* sulphonylureas), but although there is no immediate need of insulin treatment in the early phases of this form of the disease, many patients with type 2 diabetes benefit from insulin treatment and in some cases, especially in the later stages of the disease, insulin treatment is mandatory.

There are also other forms of diabetes mellitus of differing etiology, like maturity-onset diabetes of the young (MODY), late-onset autoimmune diabetes of the adult (LADA) and gestational diabetes.

Type 2 diabetes

Type 2 diabetes constitutes about 90% of all individuals with diabetes mellitus. The remaining 10% constitutes mainly of type 1 diabetic individuals, but also other forms of the disease, as already mentioned above. The pathogenesis of type 2 diabetes is genetically multifactorial, and the resulting clinical course of the disease is probably dependent on interactions between many genes interacting with different environmental factors [173]. Diabetes in itself is a disease that restricts life in many ways, but to make things even worse, the complications of the disease, mostly macro- and microvascular diseases, are many times devastating, *e.g.* increased morbidity in cardiovascular disease. Early phenomenons in type 2 diabetes is β-cell dysfuntion, insulin insensitivity and impaired glucose tolerance, partly related to obesity. Gradually the β-cell function decreases, in the end leading to a clinical hyperglycaemia.

Characteristics of insulin release

Several hormones are produced in the endocrine pancreas. Insulin and glucagon are the major islet hormones involved in the complex regulation of glucose homeostasis. In general, insulin acts as an anabolic hormone and the role of glucagon is usually the opposite. Somatostatin, produced in the δ-cells have an inhibiting effect on both insulin and glucagon secretion.

Insulin and insulin release

Insulin is a molecule consisting of two polypeptide chains, an A chain (21 aminoacid residues) and a B chain (30 aminoacid residues) connected by two linking disulphide bridges. This molecule is derived from a larger molecule, namely preproinsulin (110 aminoacid residues), which by proteolytic cleavage in the endoplasmic reticulum (ER) to proinsulin, is transported to the Golgi apparatus and there, in the secretory vesicles (now maturing), by proteolytic removal of the connecting peptide (C peptide) yields the resulting insulin molecule. About 60% of the insulin released into the portal vein is removed by the first pass metabolism in the liver.

Insulin is released in pulses in the portal vein [94, 133], and both nonadrenergic and non-cholinergic (NANC) neurons seems to be involved in the induction of this pulsatile pattern [158].

One β-cell has been shown to contain more than 10,000 secretory granules containing insulin. These granules can be divided into different pools depending on their morphological localization and how easily they can be exocytosed [26, 141].

Pools of insulin granules

- reserve pool
- readily releasable
- immediately releasable

Glucose-stimulated insulin secretion is typically described as biphasic [160]. First there is a rapid and transient phase (lasting 5-10 minutes), and then follows a more pro-longed second phase. In individuals with type 2 diabetes the first phase is usually suppressed, whereas the second phase many times is exaggerated during the initial stage of type 2 diabetes [39]. The first phase is mainly attributed to a K_{ATP} channel dependent pathway whereas the second phase is attributed to both K_{ATP} channel dependent and independent pathways acting in synergy [73].

Glucose-stimulated insulin secretion

Insulin secretion is a very complex process and it is far from completely elucidated in what ways this strict regulation of insulin release is achieved. Glucose is the main activator of insulin secretion and it enters the β-cell through a specific glucose transporter (GLUT-2 in rodents, GLUT-1 in man [134] in direct proportion to the extracellular glucose level. In the β-cell glucose is rapidly phosphorylated by glucokinase. There are two main signalling pathways involved in glucose-stimulated insulin secretion, where one pathway is relatively well described, although it is not fully understood in more detail, and the other pathway is of a more enigmatic nature:

- ➢ **K_{ATP} channel-dependent pathway** (involved in both first and second phase insulin release). Also called the *triggering pathway*.
- ➢ **K_{ATP} channel-independent pathway** (involved in second phase insulin release). Also called the *amplifying pathway*.

The classical K_{ATP} channel-dependent pathway might be described as follows [134, 141, 176]:

- Glucose enters the β-cell and the concentration of phosphorylated glucose increases in the β-cell
- ➔ glucose metabolism is increased (via the glycolytic and the mitochondrial pathways)
- ➔ increase in the ATP/ADP ratio and closure of K_{ATP} channels
- ➔ depolarization of the plasma membrane
- ➔ activation of voltage-dependent L-type Ca^{2+} channels and influx of Ca^{2+} and a 10-fold increase in $[Ca^{2+}]_i$
- ➔ finally resulting in insulin release through exocytosis of insulin containing granules

The K_{ATP} channel-independent pathway was first demonstrated in 1992 [51]. In the presence of an elevated $[Ca^{2+}]_i$ this pathway augments the glucose-stimulated stimulatory response. When studying this pathway the drug diazoxide is often used in combination with a high K^+ concentration, since diazoxide activates (opens) K_{ATP} channels and a high K^+ concentration depolarizes the plasma membrane (used in paper V). The underlying factors in the K_{ATP} channel independent pathway are poorly understood, but several factors have been suggested, *e.g.* ATP, GTP and NADPH [42, 47, 50, 79].

Glucose has also been shown to stimulate insulin secretion by a K_{ATP} channel-independent and Ca^{2+}-independent mechanism, but this pathway seems to be of minor importance [152].

Cyclic AMP and insulin secretion

Receptor-mediated activation, by *e.g.* glucagon, GLP-1 and forskolin, of the G protein which activates adenylate cyclase (AC) generates cAMP from ATP [12, 75, 95]. An increased formation of cAMP, activates in its turn protein kinase A (PKA), but also cyclic-nucleotide-gated ion channels and a family of cAMP-regulated binding proteins (implicated in incretin-potentiated insulin secretion) [46, 89, 127].

PKA stimulates exocytosis in several ways by phosphorylating different intracellular proteins and increasing the uptake of extracellular Ca^{2+}. PKA is also more directly involved in the distal events in the secretory process, *e.g.* by mobilization of insulin containing granules from the reserve pool to the readily releaseable pool [136, 141]. PKA is also involved in inhibition of cell apoptosis and inhibition of iNOS expression [87].

The role of cAMP in the regulation of glucose-stimulated insulin secretion is not fully understood and the results are sometimes contradictory, probably due to the fact that cAMP might well act through several pathways in the β-cell. There is also evidence for a subcellular compartmentation of different cAMP actions [159, 164].

Phospholipase C and insulin secretion

Activation of phospholipase C (PLC) by *e.g.* acetylcholine, by binding to a muscarinic β-cell receptor, leads to hydrolysis of phospho-inositides, and results in production of inositol-1,4,5-triphosphate (IP_3) and diacyl glycerol (DAG). These second messengers have different actions. IP_3 diffuses into the cytoplasm and promotes liberation of Ca^{2+} from Ca^{2+} storage sites, resulting in raised $[Ca^{2+}]_i$. DAG activates protein kinase C (PKC) which is involved in stimulatory mechanisms in the distal event of the secretory process in the exocytosis, by enhancing Ca^{2+} influx through voltage dependent L-type Ca^{2+} channels [177].

NO and CO as messenger molecules

In the present thesis I will restrict myself to mainly discuss the impact of less well elucidated pathways involved in insulin secretory mechanisms, *i.e.* the nitric oxide synthase-nitric oxide (NOS-NO) pathway, the heme oxygenase-carbon monoxide (HO-CO) pathway and the pathway involving activation of the acid α-glucoside hydrolases.

Conventional neurotransmittors, like noradrenaline, serotonin, dopamin and acetylcholine are enzymatically synthesized, stored in vesicles, exocytised after membrane depolarization and subsequently reaching membrane receptors, inducing one kind or the other of secondary action. More unconventional are atypical messenger molecules like the gases NO and CO. Both gases are now established as messenger molecules, though their role as messengers remains to be more extensively studied. NO was in 1987 identified as the enigmatic Endothelium Derived Relaxing Factor (EDRF) [77, 128], and a few years later it was suggested that endogenous CO might also act as a messenger molecule [114].

Both gases are somewhat bothersome to use in experimental settings, and inhalation of the gases might cause severe injury or death. Well known is the use of CO as an instrument of committing suicide. The gas NO is used as a pharmacological agent in some cases, and CO might well be used as such in the future, but the handling of both gases makes their use, in the clinic as well as in the laboratory, quite hazardous unless a strict protocol and necessary safety precautions are attended to.

NO and CO share common properties that make them unique as messenger molecules in biological systems. NO is a more reactive molecule than CO, but both have a short half life during normal conditions. This makes them into messengers with a limited range of action, and they are most probably synthesized "on demand". Both penetrate biological membranes easily since they are lipophilic. It follows, that due to their short lived nature their main targets of action are localized in the synthesizing cell or in adjacent cells. CO is much less reactive than NO and has conceivably a more extended range of action, both in time and space, than NO [17, 58].

The most important signaling mechanism for both NO and CO is thought to be the cyclic GMP system [20, 33, 113, 121, 168], and this activating action is accomplished through NO or CO binding to the heme prosthetic group of guanylate cyclase [114].

There is increasing evidence for an intricate connection between the NOS-NO-system and the HO-CO-systems in different organs and cell types [43, 53, 78], *e.g.* their role as neurotransmittors [23]. In our laboratory we have earlier presented evidence for an interaction between NO and CO in islets of Langerhans [66, 69] (see fig. 2). These results aslo suggest a protective role of the HO-CO system in the islets, counteracting the negative effect of *e.g.* LPS-induced iNOS expression and NO-production [69].

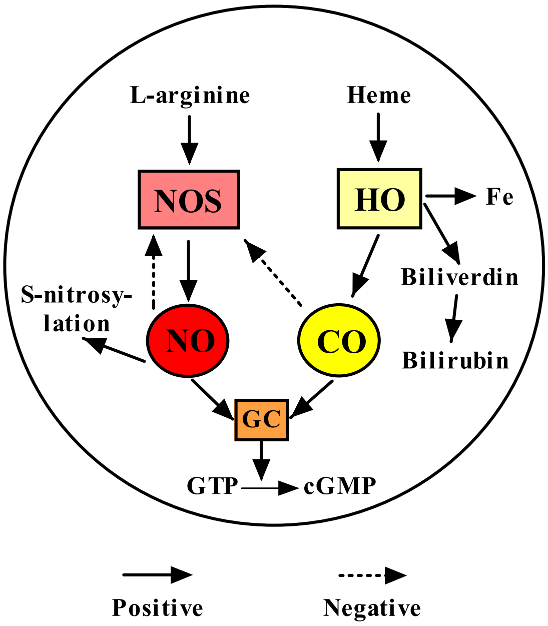

Figure 2. *Schematic illustration of the interaction between the HO-CO system and the NOS-NO system in the islets of Langerhans. Biliverdin is converted into bilirubin, a compound with antioxidant properties.*

Nitric oxide synthase and nitric oxide

Nitric oxided (NO) is produced from the amino acid L-arginine in equimolar concentrations to the amino acid L-citrulline. In the reaction producing NO the nitrogen atom of NO is derived from the guanidino group of L-arginine and the oxygen is derived from molecular O_2 [120, 122] (see fig. 3).

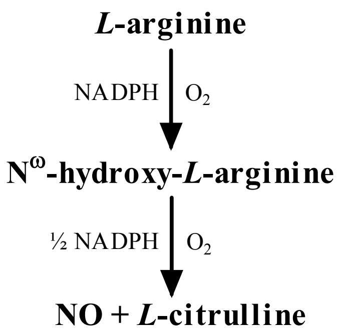

Figure 3. *Schematic illustration of the synthesis of nitric oxide and L-citrulline from L-arginine.*

All three major isoforms of NOS catalyzing the reaction shown in figure 4 have been detected in the islets of Langerhans and in the vessels supplying them [34, 38, 131, 154, 161]. More recently ncNOS has been detected in all four major cell types in the islets [11]. There are two constitutively expressed isoforms, neuronal ncNOS (NOS-I) and endothelial ecNOS (NOS-III) which produce low amounts of NO in a pulsatile manner [10, 21]. Both ncNOS and ecNOS are calmodulin dependent whereas the third isoform, *i.e.* the inducible isoform iNOS (NOS-II) is Ca^{2+}/calmodulin independent [21] since it has calmodulin tightly bound to the enzyme [32].

When iNOS is active it produces continuous large amounts of NO [21]. When NO is produced in large amounts by iNOS, it seems to play an important role in the pathogenesis of type 1 diabetes via a noxious influence on the islet β-cells [34, 37, 44, 112, 117]. Thus different cytokines have been shown to induce the expression of iNOS in islet tissue [36, 40, 45]. In contrast, ncNOS-derived NO, which is produced in much smaller amounts, seems to be able to serve as a physiological modulator of islet hormone secretion [6, 9, 15, 56, 67, 70, 71, 86, 129-131, 142, 143, 148, 154, paper III].

We have previously, and repeatedly, shown that NO evolution by islet ncNOS activity seems to serve as a negative modulator of nutrient-stimulated insulin release [67, 70, 71, 86, 129-131, 142, 143, 148, 150, paper III], although there are also reports from other research groups indicating that NO might have a different influence on insulin secretion [84, 154, 157].

There are several NOS inhibitors available, and in the papers presented in this thesis L-NAME is the inhibitor we have chosen to use. However, the effect of NOS inhibition is not completely straightforward. At high glucose the NOS inhibitor L-NMMA has been shown to increase islet NO production and inhibit insulin release when used at a low concentration (0.5 mM), while a higher concentration (5 mM) inhibited islet NOS activities and increased the insulin release [71].

Since the radical NO has a very short half life, it is a challenge to measure NO production in the islets of Langerhans. A commonly used method to estimate NO production is to determine nitrite (NO_2^-) and nitrate (NO_3^-), the end products of NO-decomposition [80, 163]. However, much NO produced intracellularly is trapped by S-nitrosylation and thus we have used a different and very sensitive method, based on HPLC-technique [29], where L-citrulline is measured. L-citrulline is

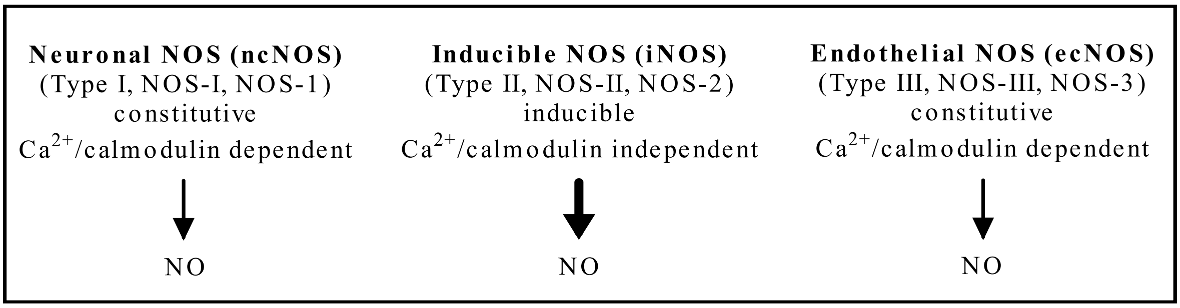

Figure 4. *Isoforms of nitric oxide synthase. Besides the fact that the figure illustrates a differing nomenclature, it also shows if the isoform is calcium dependent or not. It also illustrates (see arrows) that iNOS, when active, produces much larger quantities of NO than the constitutive forms.*

produced in equimolar amounts as NO by NOS (see fig. 3). A similar, although radioisotopic method was described by Bredt & Snyder 1989 [27].

Heme oxygenase and carbon monoxide

Carbon monoxide (CO) is mainly produced through degradation of heme groups by the microsomal enzyme heme oxygenase (HO) and the heme groups are mainly derived from hemoglobin [111, 113, 153]. In the reaction equimolar amounts of CO, biliverdin-IXa and Fe^{2+} are produced, and HO catalyses the first step in the degradation of heme (se figure 5). The degradation of heme requires the activity of NADPH-cytochrom c (P450) reductase which transfers reducing equivalents from NADPH to the heme-HO complex, resulting in a reduction of iron ($Fe^{3+} \rightarrow Fe^{2+}$) [140]. Oxidative cleavage of the α-methene bridge of heme follows, liberating CO. The biliverdin is subsequently converted into bilirubin by the cytosolic biliverdin reductase enzyme [140]. NADPH-dependent peroxidation of microsomal membrane lipids can also produce CO, but at a much lower rate [172].

There are two major isoforms of the heme oxygenase enzyme, one inducible (HO-1) and one constitutive (HO-2). A third isoform with unknown function has also been isolated from rat brain (HO-3), closely related to HO-2 but characterized as a poor heme catalyst [116]. HO-1 is also known as heat shock protein-32 (hsp-32) and is induced by *e.g.* oxidative stress, endotoxin and UV-radiation. HO-2 has recently been shown to be activated by calcium-calmodulin [22]. HO-2 has been detected in all endocrine cells in the islets of Langerhans of both rats [11, 68] and mice [66].

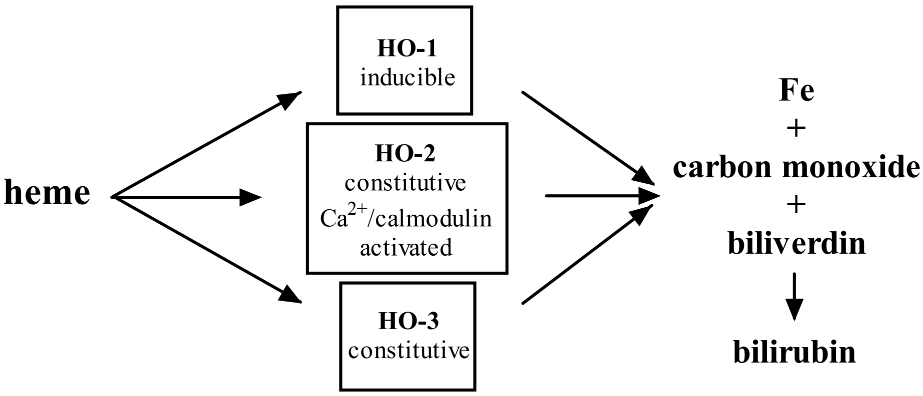

Figure 5. *Schematic illustration of the known isoforms of heme oxygenase synthase. Biliverdin is subsequently converted into bilirubin.*

Acid α-glucoside hydrolases

Introduction

In this thesis the relationship between the acid α-glucoside hydrolases and insulin release is studied. We have also tried to elucidate if there exists any connection between the HO-CO system, the NOS-NO system and the acid α-glucoside hydrolase system, in relation to insulin release. We think that there are a number of reasons to investigate the possible role of the acid α-glucoside hydrolases in the obviously very complex process of insulin granule exocytosis. Here follows a short introduction to this area of research.

Several years ago, our laboratory has reported an unexpectedly high activity of exo-amylolytic enzymes (acid glucan-1,4-α-glucosidase or acid amyloglucosidase, EC 3.2.1.3 and/ or acid α-glucosidase, EC 2.3.1.20) in the pancreatic islets of the mouse [98, 99, 101, 108]. These enzymes are associated with the acidic lysosomal/vacuolar system [101]. The main localization of this acid glucan-1,4-α-glucosidase is suggested to be the β-cell in the endocrine pancreas, since very little activity of the enzyme is found in alloxan diabetic mice [101].

Glycogen has been shown to be a normal constituent of mammalian β-cells [65, 115], and early studies, both in humans and experimental animals, have reported that diabetes might be accompanied by glycogen infiltration in the pancreatic islets [165]. The acid glucan-1,4-α-glucosidase acts preferentially on α-1,4-linked glucose polymers, such as glycogen, through consecutive removal of glucose units from the non-reducing end of the polymer. In this way the enzyme has the ability to produce non-phosphorylated glucose in the β-cell. The acid α-glucosidase acts preferentially on oligo-saccharides, but the effects of the two iso-enzymes are markedly overlapping. Hence, because of difficulties to differentiate between isoforms of these acid α-glucoside hydrolases and because their physiological effects also are markedly overlapping I prefer to refer to them collectively as *acid a-glucoside hydrolases*.

The importance of the acid α-glucoside hydrolases is mainly unknown, but it should be noted that they obviously are of great importance in glycogen metabolism, since patients suffering from deleterious forms of type II glycogenosis (Pompe's disease) have a severly deficient/absent acid glycogenolytic enzyme activity [74, 138, 139, 169], and these patients develop progressive cardiomyopathy and respiratory deficiency. It has recently been shown that patients with classical infantile Pompe disease improve when they receive enzyme replacement therapy, *i.e.* recombinant acid α-glucosidase [90], which is of specific interest also regarding the role of these enzymes in the regulation of insulin release, since exogenous (fungal) acid glucan-1,4-α-glucosidase has been shown to dose-dependently increase the insulin response to glucose and other nutrients *in vivo* and also improve the impaired glucose-stimulated insulin release seen during fasting in mice [104, 106, 110].

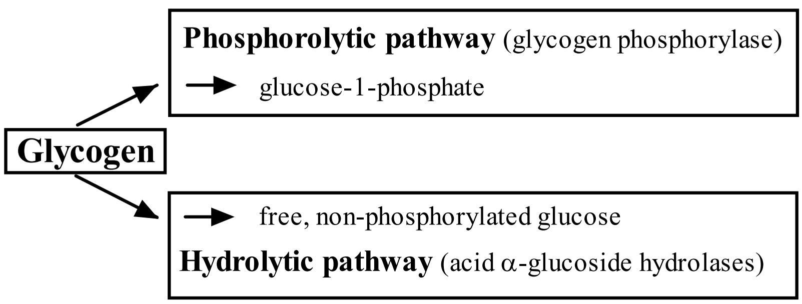

Figure 6. *The degradation of glycogen occurs in the pancreatic islets, as in most glycogen storing tissues [156], by two distinct pathways; the phosphorolytic pathway [115] and the hydrolytic pathway [98, 99, 101]. It is still not definitely proven if free non-phosphorylated glucose is involved in the regulation of insulin release.*

Acid α-glucoside hydrolases and insulin release – a brief background

Previous morphological studies have found evidence for interactions of lysosomes with secretory granules of β-cells [118, 124]. There is however, as far as I know, no convincing morphological evidence for a direct involvement of acid α-glucoside hydrolases in the insulin secretory process. It has also been shown, as is mentioned above, in our laboratory, that there is an unexpectedly high activity of the acid α-glucoside hydrolases, in the pancreatic islets in the mouse [98, 101, 108]. Our laboratory has also repeatedly presented evidence for a close link between the activity of acid glucan-1,4-α-glucosidase and nutrient-stimulated insulin secretion [98, 100-108, 144-147, 149, paper I, II and III]. Such evidence includes the correlation of high plasma insulin levels to high acid α-glucoside hydrolase activities in islets of the obese (*ob/ob*) mouse [105-107]. There is also an obvious relation between the acid α-glucoside hydrolase activities in islets of fed and fasted normal as well as obese mice and the plasma insulin levels in these animals [106, 108].

More recently it has been shown that granular acidification is involved in β-cell exocytosis [18], and in yeast it has been shown that vacuole acidification is required for the pairing of the SNARE-proteins, which take part in the exocytotic process [167]. Moreover, very recent findings show that different secretory stimuli use different Ca^{2+} organelles to elicit unique responses, and in the β-cell glucose was found to mobilize Ca^{2+} from a lysosome-related organelle, while acetylcholine (which has has no effect on the acid α-glucoside hydrolases) [145] only used the endoplasmic reticulum [174].

These data speak much in favor of the existence and potential importance of Ca^{2+}-dependent enzymes, active in acidic milieus, as are the acid α-glucoside hydrolases. These enzymes seem to be involved in the exocytotic regulation of the β-cell. This involvement has previously been hypothesized, by Lundquist and Salehi, to take place at a distal step in the secretory process [110, paper II] and in association with exocytosis at the entry of Ca^{2+} in the region of the β-cell with the highest density of secretory granules [24].

Among the more interesting findings in our laboratory [110, 144-146, 148, 149, paper I and II], regarding the role of the acid α-glucoside hydrolases, are the results from experiments using different selective inhibitors of the α-glucoside hydrolases, (*e.g.* the deoxynojirimycin derivatives miglitol and emiglitate, the indazol-derivative castanospermine and the pseudo-tetrasaccharide acarbose) both *in vitro* and *in vivo*. These chemically very different inhibitors display a direct relationship between the inhibition of the activities of the acid α-glucoside hydrolases and the inhibition of glucose-stimulated insulin release. In this context, it is also well worth mentioning that while acarbose has profound, and parallel, effects on both the activities of the acid α-glucoside hydrolases and glucose-stimulated insulin release, the acarbose analogue maltotetrose is completely devoid of any effect, neither on the acid α-glucoside hydrolase activities nor on glucose-stimulated insulin release [149].

It is also important to note that pretreatment with fungal acid glucan-1,4-α-glucosidase ("enzyme replacement") not only markedly enhances the *in vivo* insulin secretory response to glucose [100], but also to ketoisocaproic acid (KIC) [110], while receptor-activated insulin response through CCK-8 [110], carbachol [145] or β-adrenergic stimulation [4] are having no effect when cholecystokinin-8 (CCK-8) is injected [110]. This is in accordance with the finding that selective inhibition of the acid α-glucoside hydrolases very markedly suppresses the insulin secretory response to KIC, but has no effect on insulin release stimulated by CCK-8, carbachol, isoprenaline or IBMX [110, 146].

To explain the correlation between the activity of these acid α-glucoside hydrolases and insulin release, it has been hypothesized that these acid hydrolases might attack pools of vacuolar glycogen, liberating free glucose which in turn might serve as an insulin secretory signal in itself and/or affect key membrane glycoproteins containing α-glucosides (primarily α-1,4-glucoside residues) in membranes taking part in the exocytotic process of the β-cell.

The GK rat – a model of type 2 diabetes

Since the Goto-Kakizaki (GK) rat has been used as an animal model of type 2 diabetes in three of the papers in this thesis (paper II, IV, V), some of the characteristic features of the GK animal model are summarized below. The GK rat model was developed in 1972, by selective breeding of normal Wistar rats with the highest blood glucose values [54]. The GK rats that we have used in our studies come from the Stockholm colony, which was started in the late 1980's [126].

- a spontaneous mildly diabetic animal
- lean all through life
- is non insulin dependent all through life
- has fasting blood levels ranging between 8-12 mM
- developes a mild insulin resistance (apparently due to hyperglycaemia)
- displays a markedly impaired glucose-stimulated insulin secretion (both in the K_{ATP}-dependent and K_{ATP}-independent pathways)
- reportedly often responds in an exaggerated manner to non-metabolised stimulators of insulin secretion

AIMS

General aims

In the studies presented in this thesis the regulation of glucose-stimulated insulin release is in the focus. The general aim has been to further study less well-known regulatory systems in the β-cell, emphasizing on the acid α-glucoside hydrolases and their role in the complex exocytotic process. Beside the lysosomal/vacuolar system two other systems have been studied, on one hand the NOS-NO system and on the other hand the HO-CO system. It has been of special interest to try to elucidate if there exists any interconnections between the latter two systems and the lysosomal/vacuolar system in relation to the regulation of glucose-stimulated insulin release.

To add another dimension to the studies, and to make them more relevant from a clinical viewpoint, we have chosen to perform several of the studies in an animal model with a spontaneous mild diabetes, the Goto-Kakizaki (GK) rat. In this way we have tried to further clarify the potential abnormalities in this type of diabetic animal model regarding the lysosomal/vacuolar system as well as the NOS-NO system and the HO-CO system, in relation to glucose-stimulated insulin release. This is of special interest since the GK rat is known to have a greatly impaired insulin response to glucose.

Specific aims

- Study the Ca^{2+}-dependency of the acid α-glucoside hydrolases and to characterize the potential involvement of these enzymes in Ca^{2+}-glucose-stimulated insulin release (paper I).
- Study the importance of the acid α-glucoside hydrolases in glucose-stimulated insulin release in the GK rat (paper II).
- Study the NOS-NO-dependency of the acid α-glucoside hydrolases in glucose-stimulated insulin release (paper III, VI).
- Study the potential involvement of the HO-CO system, in regard to glucose-stimulated insulin release in the GK rat (IV).
- Study the potential involvement of the NOS-NO system, in regard to glucose-stimulated insulin release in the GK rat (paper V).
- Study potential interactions between the NOS-NO system and the HO-CO system and especially their effects on the activities of the acid α-glucoside hydrolases in relation to glucose-stimulated insulin release (paper VI).

MATERIALS AND METHODS

Animals

Female mice of the Naval Medical Research Institute (NMRI) strain, weighing 25-30g, were used in the studies in papers I, III and VI. Age- and sex-matched GK rats of the Stockholm colony and Wistar controls (B&K Universal, Sollentuna, Sweden) were used in papers II, IV and V. A standard pellet diet (B&K, Sollentuna, Sweden) and tap water available *ad libitum*, were used throughout the different studies. The animal experiments were approved by local animal welfare committees (Lund and Stockholm, Sweden), and were in accordance with the international standard recommended by NIH.

Experimental methodology and procedures

Isolation of islets

Isolation of pancreatic islets from mice and rats was accomplished by retrograde injection of a collagenase solution via the bile-pancreatic duct [55]. The animals were killed by elongation of the neck and were immediately injected with collagenase. The islets were after isolation hand-picked under a stereomicroscope at room temperature.

Islet batch incubation studies

Freshly isolated islets were preincubated for 30 min at 37°C in Krebs Ringer bicarbonate buffer (KRB), pH 7.4, supplemented with 10 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES), 0.1% bovine serum albumin, and 1 mM glucose. Each incubation vial contained 10 islets in 1.0 ml (or 30-40 islets in 1.5 ml) buffer solution and was gassed with 95% O_2/5% CO_2 to obtain constant pH and oxygenation. After preincubation for 30 min, the buffer was changed to a medium containing different concentrations of glucose as well as the different test agents, and the islets were then incubated for 60 or 120 min. All incubations were performed at 37°C in an incubation box (30 cycles/min). Immediately after incubation, an aliquot of the medium was removed and frozen for the subsequent radioimmunoassay of insulin [59]. It should be noted that in the experiments in paper I, in the experiments with high concentrations of Ca^{2+}, phosphate and sulfate in the KRB-HEPES buffer were replaced with equmolar amounts of chloride [61].

Analysis of lysosomal enzyme activities

Islet preparations: Immediately after incubation, and the removal of an aliquot for insulin determination, the islets were thoroughly washed in glucose-free KRB buffer and collected in 200 µl ice-cold acetate-EDTA buffer (1.1 mM EDTA and 5 mM sodium acetate pH 5.0) and thereafter stored at –20°C. After sonification on ice, islet homogenates were analyzed for lysosomal enzyme activities. In the experiments in which the direct influence of different Ca^{2+} concentrations added to islet homogenates on the lysosomal enzyme activities (paper I), the islets were washed in a glucose- and Ca^{2+}-free KRB buffer and collected and stored in 5 mM acetate in the absence of EDTA.
Incubation of islet homogenates: In some studies the enzyme activities were measured after incubation of islet homogenates (see paper III and VI) where aliquots of islet homogenates were either incubated with test substances (*e.g.* sodium nitroprusside [SNP]) or directly gassed with helium, followed by NO or CO until saturation.
Enzyme activity determination: The procedures for determination of acid phosphatase (pH 4.5), acid α-glucosidase (pH 4.0/5.0), N-acetyl-β-D-glucosaminidase (pH 5.0) and neutral α-glucosidase (pH 6.5) with methylumbelliferyl-coupled substrates, as previously described [106]. Islet glucan-1,4-α-glucosidase activity with glycogen as substrate was determined at pH 4.0 [99, 106]. Protein was determined according to the method of Lowry *et al.* [97] or Bradford [25].

Islet perifusion experiments

In the islet perifusion experiments (paper I) 150-200 islets were first incubated for 90 min in 800 µl of KRB medium supplemented with 10 mM HEPES, 0.1% bovine serum albumin

(BSA), 20 mM glucose and 50 µl $^{45}CaCl_2$ (50-100 µCi), which was added from a stock solution with a specific activity of 10-40 mCi/mg Ca^{2+}. The islets were then washed three times with nonradioactive medium, divided into two or three groups with 75-100 islets per group, and transferred to perifusions columns. The islets were thereby sandwiched between two layers of gel (Bio-gel P-4, 200-400 mesh; Bio-Rad Laboratory, Richmond, CA, USA) and perifused at a rate of 0.1 ml/min with the KRB buffer supplemented with 1 mM glucose. Test substances were introduced according to the protocols. A Ca^{2+}-deficient medium was obtained by omitting calcium chloride and adding 0.5 mM EGTA. The radioactivity lost by the islets was measured in effluent fractions collected every 2 min (50 µl of the sample were added to 5 ml of scintillation fluid) and counted in a scintillation counter (Packard Instrument, Downers Grove, IL, USA). The fractiononal efflux rate was calculated for each period (radioactivity lost by islets during the time interval/radioactivity present in the islets during the same time interval), and the mean value calculated for *minute 40* and *42* was then normalized to 100%. Insulin was determined with a radioimmunoassay [59].

HPLC determination of islet NO production

Freshly isolated pancreatic islets were either incubated as described above, or washed and collected in ice-cold buffer (200 islets in 840 µl buffer) containing 20 mM HEPES, 0.5 mM EDTA, and 1 mM D,L-dithiothreitol (DTT), pH 7.2, and immediately frozen at –20°C. On the day of assay, the islets were sonicated on ice, and the buffer solution containing the islet homogenate was reconstituted to contain, in addtion to the above mentioned compounds, also 0.2 mM L-arginine, 0.45 mM $CaCl_2$, 2 mM NADPH, and 25 U calmodulin in a total volume of 1 ml. To determine iNOS activity both Ca^{2+} and calmodulin were omitted. This buffer solution was essentially the same as previously described for assay of NOS in brain tissue using radiolabelled L-arginine [27]. The crude homogenate was then incubated at 37°C under constant air bubbling, 1.0 ml/min, for 180 min. Aliquots of the incubated homogenate (200 µl) were then passed through an 1 ml Amprep CBA cation-exchange column for high-performance liquid chromatography (HPLC) analysis of the L-citrulline formed according to Carlberg [29, 71, 148]. Since L-citrulline is is created in equimolar concentrations to NO, and L-citrulline is stable whereas NO is not, L-citrulline is the preferred parameter when measuring NO production.

Gas chromatographic analysis of islet CO production

CO production was determined with a sensitive gas chromatographic method essentially as previously described [31, 66, 68]. Freshly isolated islets were either incubated as described above, or washed and collected in ice-cold phosphate buffer (0.1 M, pH 7.4; approximately 300 islets in 200 µl buffer), and thereafter immediately frozen at –20°C. On the day of assay the islets were sonicated on ice, and methemalbumin (30 µl), β-NADPH (100 µl; 4 mg dissolved in 1 ml phosphate buffer [0.1 M]) and hemoglobin (2 mg) were added together with phosphate buffer up to a final volume of 1 ml. The methemalbumin solution was prepared by dissolving 25 mg hemin, 82.5 mg NaCl and 12.1 mg Tris base in 5 ml 0.1 M NaOH, followed by the addition of 5 ml albumin solution (20 g/l) and 5 ml distilled water. The homogenate was then incubated at 37°C under protection from light. Aliquots (330 µl) were taken after 6 min of incubation, which was terminated by placing the tubes on ice. The samples were then injected into reaction tubes containing ferricyanide-citric acid (100 µl). Nitrogen was used as a carrier gas, as well as to purge the reaction vessels for 4 minutes before the samples were injected into them. After a reaction time of 4 minutes, the liberated CO was brought to a nickel catalyst, mixed with H_2, and then brought further as methane to the detector. 99.9% CO was used as standard. The amount of CO produced was calculated from the area under the curve.

Western blot analysis

Approximately 150 freshly isolated islets were collected in Hanks' buffer (100 µl) and sonicated on ice (3 x 10 s). Homogenate samples, representing 10 μg of the islet protein, were then run on 10% SDS-polyacrylamide gels. After electrophoresis, proteins were transferred to nitrocellulose membranes by electrotransfer (10-15 V, 60 min) (semi-dry transfer cell, B10-RAD, Richmond, CA). The membranes were blocked in 9 mM Tris-HCl (pH 7.4), containing 5% non-fat milk powder, for 40 min at 37°C. Immunoblotting with rabbit anti-mouse HO-1 (OSA 100) (1:500) and HO-2 (OSA 200) (1:1,000) or rabbit anti-mouse ncNOS (N-7155) and iNOS (N-7782) (1:2,000; Sigma, St Louis, MO) was performed for 16 h at room temperature. The membrane was washed twice and then incubated with alkaline-phosphatase conjugated goat anti-rabbit IgG (1:10,000) (Sigma) for 90 min. Antibody binding to HO-2 and HO-1 or ncNOS and iNOS was detected using 0.25 mM CDP-Star™ (Tropix, Bedford, MA) for 5 min at room temperature. The chemiluminiscence signal was visualized by exposing the membranes to Dupont Cronex® X-ray films for 1-5 min. The intensities of the bands were, when applicable, quantified by densitometry (Bio-Rad GS-710 Densito-meter).

Immunocytochemistry

The animals were anaesthesized with ketamine (100 mg/kg *i.m.*) and xylazin (15 mg/kg *i.m.*), and perfused transcardially through the ascending aorta, first with 100 ml of ice-cold calcium-free KRB buffer (containing 0.5 g/l sodium nitrite and 10,000 IU/l of heparin), and then with 300 ml of an ice-cold, freshly prepared solution of 4% formaldehyde in phosphate buffered saline (PBS, 0.1 M, pH 7.4). The pancreatic glands were then rapidly dissected out and divided into pieces, which were fixed in the same fixative for four hours, followed by rinsing in ice-cold 15% sucrose in PBS (three rinses during 48 hours). The tissue specimens were frozen in isopentane at -40°C and then stored at -70°C. Cryostat sections, cut at a thickness of 8 μm, were incubated overnight (HO-2) or 2 days (ncNOS) in the presence of HO-2 or ncNOS rabbit antisera. For the demonstration of ncNOS, sections were preincubated in PBS with 0.2% Triton X-100 for about 2 hrs After rinsing the sections were incubated for 90 min with fluorescein isothiocyanate- (FITC) or Texas Red-conjugated donkey anti-rabbit immuno-globulins (IgG), rinsed and mounted. The antiserum was diluted with phosphate-buffered saline (PBS, 0.1 M, pH 7.4). Epi-illumination and appropriate filter settings for Texas Red- and FITC-immunofluorescence were used in the microscopical examination of the sections.

Isolated perfused pancreas

Rats were anaesthetized with sodium thiopental (100 mg/kg, *i.p.*) and each pancreas was dissected free from adjacent tissues and removed to a perfusion chamber as previously described [126]. The perfusion medium was directed into the isolated pancreas through a cannula in the aorta by a non-recycling perfusion system with a flow rate of 2.8 ml/min. The basal perfusion medium consisted of KRB buffer, pH 7.4, gassed with 95% O_2/5% CO_2 supplemented with 10 mM HEPES, 20 g/l bovine plasma albumin and 5.5 mM glucose. Following an equilibration period with a glucose-free medium, 5 mM L-NAME was added in the presence of 3.3 mM glucose. After 30 min the glucose concentration in the medium was raised to 16.7 mM for another 20 min. Integrated insulin responses were calculated as areas under the curve, using the hormone concentration at start of the test period as basal value.

In vivo experiments

Young adult GK and Wistar rats, 1-2 months of age, were injected *i.v.* with either glucose (11.1 mmol/kg) (paper II) or L-arginine (3.6 mmol/kg) (paper V), and blood sampling was performed as previously described [137]. The volume load was 5 μl/g rat. Concentrations of insulin and glucose in plasma were determined by the methods of Heding [59] and Bruss and Black [28], respectively. In paper II one group of GK rats were injected with phlorizin and another group was injected with solvent (propylene glycol). Phlorizin (0.4 g/kg BWxday), made up as a 20% solution in propylene glycol, or propylene glycol alone was administered as a *s.c.* injection every morning and afternoon for 9 days. Wistar control rats receiving solvent were included. Protein was determined according to the method of Lowry *et al.* [97] or Bradford [25].

The concentrations of insulin in plasma were determined by radioimmunoassay [59].

Glucagon was also determined by radio-immunoassay [5, 132].

Statistics

Probability levels of random differences were determined by Student's unpaired *t*-test with Welch correction when appropriate, or where applicable, analysis of variance followed by Tukey-Kramer's or Newman-Keuls' multiple comparisons test.

RESULTS AND DISCUSSION

Calcium-dependency of acid α-glucoside hydrolases (I)

Many cellular events are in some way dependent on Ca^{2+}. Insulin release is only one of many cellular processes where the distrubution and fluxes of Ca^{2+} are significant. It was for this reason that we performed the first study in this thesis, where we studied the Ca^{2+}-dependency of the acid α-glucoside hydrolases. These results have recently become even more interesting, since evidence has been found for an organelle selection which determines agonist-specific Ca^{2+} signals in pancreatic β-cells [174].

Normal and high Ca^{2+} at basal glucose

To study the activity of the acid α-glucoside hydrolases at a low, substimulatory concentration (1 mM) of glucose, and possible effects of different extracellular Ca^{2+} concentrations, we performed incubations of islets in the presence of normal extracellular Ca^{2+} (2.5 mM) and at a high maximal extracellular Ca^{2+} (see fig. 7). Since Hellman had earlier shown [61] that at substimulatory levels of glucose, Ca^{2+} was able to increase insulin release from isolated islets, up to a Ca^{2+} concentration of 30 mM, we chose 30 mM as the maximal Ca^{2+} concentration. In accordance with Hellman's earlier data we found that insulin release was increased at high extracellular Ca^{2+}. In parallel we detected an increase in the acid α-glucoside hydrolase activities, while other lysosomal enzyme activities were unaffected.

These effects on insulin release and enzyme activities are most likely a result of an increased $[Ca^{2+}]_i$ since it has been shown that a high extracellular Ca^{2+} can induce a rise in $[Ca^{2+}]_i$, primarily due to Ca^{2+} influx through dihydropyridine- and voltage-insensitive non-selective cation channels [155].

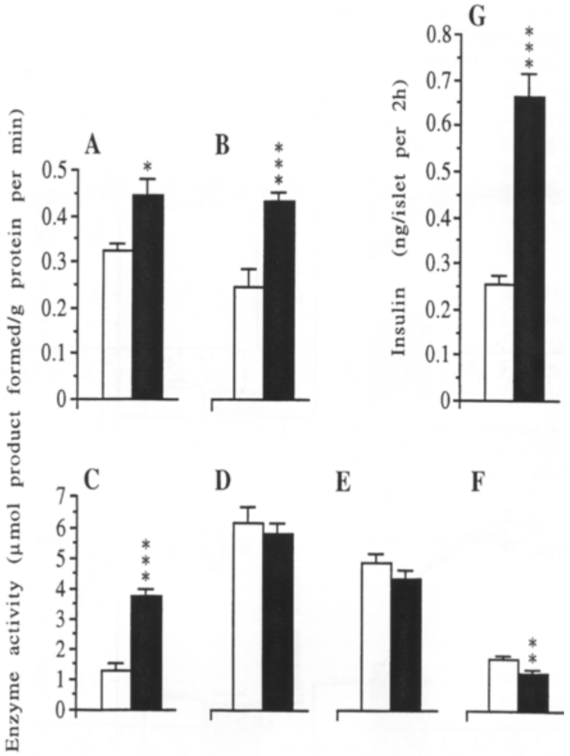

Figure 7. *Effect of normal extracellular (2.5 mM, open bars) and at very high (30 mM, solid bars) Ca^{2+}, on islet enzyme activities as well as insulin secretion, at 1 mM glucose; A) acid a-glucosidase pH 4.0, B) acid a-glucosidase pH 5.0, C) acid glucan-1,4-a-glucosidase, D) acid phosphatase, E) N-acetyl-β-D-glucosaminidase, F) neutral a-glucosidase, G) insulin release.*

Dose-response effect of Ca^{2+} and nifedipine in islet homogenates

Since we found that a high extracellular Ca^{2+} increased the acid α-glucoside hydrolase activities in intact islets at basal glucose (see above), it was of interest to see if Ca^{2+} could exert any direct effect on the acid α-glucoside hydrolase enzymes in islet homogenates. The experiments were conducted both in the presence and the absence of calmodulin, and different Ca^{2+} concentrations had no apparent influence on the enzyme activities within known intracellular fluctuations of the cation.

These results suggested that Ca^{2+} has no direct effect on the islet acid α-glucoside hydrolases at physiological intracellular Ca^{2+} concentrations [62, 134], although it cannot be completely ruled out that very high local Ca^{2+} concentrations might be found at the sub-cellular compartment/organelle level as part of normal β-cell physiology.

Influence of nifedipine in relation to insulin release

We also studied the effect of nifedipine in islet incubations as well as islet perifusion experiments. We found, as expected, that nifedipine markedly suppressed glucose-stimulated insulin release. Unexpectedly, we detected a marked amplification of the activities of the acid α-glucoside hydrolases at basal glucose. In the presence of high glucose, which in itself markedly enhanced the acid α-glucoside hydrolase activities, nifedipine had no further effect on their activities.

Since both high extracellular Ca^{2+} and nifedipine induced an increased activity of the acid α-glucoside hydrolases in intact islets we searched for an effect of nifedipine on intracellular Ca^{2+} that was independent of its Ca^{2+} channel-blocking effect. First we found that at substimulatory (1 mM) glucose and normal Ca^{2+}, nifedipine produced a marked decrease of $^{45}Ca^{2+}$ efflux, although at this basal glucose level the nifedipine-sensitive Ca^{2+} channels are already closed. To further study this we performed the same experiment in a Ca^{2+}-deficient medium at basal glucose where nifedipine had the same strong inhibitory effect even in the presence of the intracellular Ca^{2+} mobilizer carbachol. The conclusion we drew from this was that at least under our experimental conditions, nifedipine in addition to its blocking effect on voltage-dependent L-type Ca^{2+} channels also inhibits the outflow of Ca^{2+} and/or causes a redistribution of intracellular Ca^{2+}, leading to accumulation of Ca^{2+} in acid α-glucoside hydrolase-containing organelles in the lysosomal/vacuolar system. Another finding which also speaks in favor of the redistribution-interpretation, rather than a sole effect of an inhibited $^{45}Ca^{2+}$ efflux, is the fact that the initial acute increase in $^{45}Ca^{2+}$ efflux in the biphasic response to carbachol stimulation was converted into a marked initial decrease followed by a modest and highly suppressed second phase, a pattern reminiscent of the effect of glucose stimulation in the presence of extracellular Ca^{2+} [49].

Effect of high Ca^{2+} in the absence and presence of emiglitate

To further study the relationship between insulin release and the acid α-glucoside hydrolases in relation to Ca^{2+} we performed a series of experiments at 20 mM glucose where we used a normal or a high (maximal) concentration of Ca^{2+} (10 mM). Greater concentrations of Ca^{2+} at high glucose are inhibitory to insulin release [60]. We also tested the effect of the selective α-glucoside hydrolase inhibitor emiglitate and performed the islet incubations at high glucose.

We found that the acid α-glucoside hydrolase activities as well as the glucose-stimulated insulin release were increased by high Ca^{2+}. In contrast classical lysosomal enzyme activities, such as acid phosphatase and N-acetyl-β-D-glucosaminidase were unaffected. When emiglitate was added the amplifying effect of Ca^{2+} was virtually abolished and even greatly suppressed below the control level, both with regard to the acid α-glucoside hydrolases as well as the parallel effect on insulin release.

This strong relationship between the activity of Ca^{2+}, the acid α-glucoside hydrolases and glucose-stimulated insulin release is not only striking, but also speaks much in favor of the hypothesis that an important Ca^{2+} effect in the stimulus-secretion coupling of glucose-stimulated insulin release is elicited closely proximal to the action of the acid α-glucoside hydrolases (but not exerted as a direct effect on the enzymes).

Conclusions – paper I

The primary conclusion from the present studies is that the activities of the acid α-glucoside hydrolases are dependent on Ca^{2+}, and that Ca^{2+}-induced changes in the activity of these enzymes were intimately coupled to similar changes in Ca^{2+}-induced insulin release. The effect of Ca^{2+} was not elicited on the enzyme itself, but presumably activated either acid α-glucoside hydrolase-containing organelles or closely interconnected messengers. This hypothesis is, as is mentioned above, further encouraged by very recent finding showing that different secretory stimuli use different Ca^{2+} organells to elicit unique responses [174].

Islet acid α-glucoside hydrolase activities and insulin release in the GK rat (paper II)

Since we had earlier found much evidence for an involvement of the acid α-glucoside hydrolases in the regulation of glucose-stimulated insulin release, we sought an animal model where we could study the role of the lysosomal/vacuolar system in animals with a spontaneous form of diabetes. We chose to perform this study using the GK rat, an animal model with spontaneous mild diabetes and highly impaired insulin response to glucose [52, 126].

Lysosomal enzyme activities in islets of Langerhans and liver

When we compared the lysosomal activities in islets and liver tissue, we found profound differences. In freshly isolated islets from GK and Wistar control rats the activities of the acid α-glucoside hydrolases were about 10-fold higher than the same activities in liver tissue. On the other hand other classical lysosomal enzyme activities (*e.g.* acid phosphatase) were of the same magnitude in both islets and liver tissue. The much higher activities of the acid α-glucoside hydrolases in islets than in liver tissue provide an intriguing aspect of cell physiology. Why is there such a difference? We found it conceivable that the acid α-glucoside hydrolases were involved in the regulation of insulin secretion, which might explain their prominent presence in islets, compared to the liver, another carbohydrate regulating organ.

When we compared the acid α-glucoside hydrolase activities in GK and Wistar control rats we found certain differences worth mentioning. In the GK islets the activities of these enzymes were significantly higher, while the classical lysosomal enzyme activities were lower than in Wistar controls. The latter fact suggests that at least there is no degenerative/ catabolic processes active in these young (6-8 weeks old) GK rat islets, since the classical lysosomal enzymes are known to increase during degeneration/catabolism [107]. The islet insulin content was normal in the GK rat islets, consistent with earlier data showing no difference in morphological appearance in young GK rat islets compared to Wistar control islets [57].

Effect of phlorizin treatment

To further study the characteristics of the GK rat we treated them with phlorizin for 9 days. Other control GK rats and Wistar rats received solvent. Phlorizin is known to normalize elevated plasma glucose levels by inhibiting glucose transport through the renal tubuli, and the plasma levels of glucose were, as expected, fairly well normalized in our study. It has earlier been shown that glucose utilization is 2-3-fold increased in GK rat islets [76, 125, 126], and that exposure of isolated islets to high glucose *in vitro* augments the activity of the acid α-glucoside hydrolases [144, 146]. Hence, it seems likely that the hyperglycemia itself and/or the increased rate of glycolysis are conceivable mechanisms contributing to the enhanced levels of the acid α-glucoside hydrolase activities in GK islets.

Interestingly, although the plasma glucose levels were decreased in the phlorizin-treated GK rats, no significant differences were seen in the lysosomal enzyme activities compared to solvent-treated GK control rats. This is also in agreement with our finding in short-time *in vitro* experiments, that incubation of isolated GK islets for 2.5 hrs in low (3.3 mM) glucose did not restore the enhanced activity of the acid α-glucoside hydrolases to normal levels. These results are thus in accordance with our earlier observations that old, previously hyperglycemic, *ob/ob* mice that spontaneously returned to close to normal plasma glucose levels still displayed markedly elevated levels of islet acid α-glucoside hydrolase activities [107]. However, in contrast to the GK rat, the elevated enzyme activity still correlated with the markedly increased plasma insulin levels [107], suggesting a proper function of the islet lysosomal/vacuolar system in the *ob/ob* mouse, whereas in the GK rat there is an apparent dysfuntion of this system.

Selective α-glucosidase inhibition

In another series of experiments we performed a dose-response study using the selective α-glucosidase inhibitor acarbose in islet homogenates. By using homogenates we could provide a direct path for acarbose to the target enzyme, making it possible to elucidate whether acarbose was able to influence the activities of the enzymes in GK rats compared to the effect of acarbose in Wistar control islets. We found no difference between GK and Wistar rats in the ability of acarbose to dose-dependently inhibit the α-glucoside hydrolases in islet homogenates. In contrast to these findings, we found that there was a profound difference between GK rats and the Wistar control rats when we performed incubations with intact islets (see fig. 8). The differences were small at low glucose, but at high glucose the differences were dramatic. The pattern is striking, with a markedly impaired glucose-stimulated insulin release in the GK islets with the pseudo-tetrasaccharide acarbose not being able to exert any effect on the acid α-glucoside hydrolase activities. In contrast, a nice parallellity was evident in Wistar rat islets between the activities of the acid α-glucoside hydrolases and glucose-stimulated insulin secretion, where both insulin release and the acid α-glucoside hydrolase activities were markedly inhibited by acarbose.

These findings show that in GK rats there is a profound dysfunction in the lysosomal/vacuolar system, since acarbose was unable to have any effect on the α-glucoside hydrolase activities in intact islets, while acarbose had a perfectly normal inhibitory effect on the enzyme activities in islet homogenates from GK rats. It seems plausible to assume that there exists some kind of deficiency in the lysosomal/vacuolar system in the intact GK islets that prevents acarbose from being endocytosed and thereby being hindered from entering the system and having its effect.

Adenylate cyclase activation and insulin release

Forskolin activates adenylate cyclase, and in the present study we confirmed earlier observations [2] that forskolin greatly potentiates glucose-stimulated insulin release, both in GK and Wistar control rats. In GK rats the insulin response to glucose is, as mentioned earlier, markedly reduced. This reduction was completely ameliorated by forskolin, suggesting that the actual exocytotic machinery in GK rat β-cells is fully functional, as long as it is properly activated.

Interestingly, we have previously shown in isolated mouse islets that blockade of the acid α-glucoside hydrolase activities results in an increased insulin response to forskolin, suggesting the cyclic AMP system as an important compensatory pathway when the glucose-acid-α-glucose hydrolase pathway is suppressed [148].

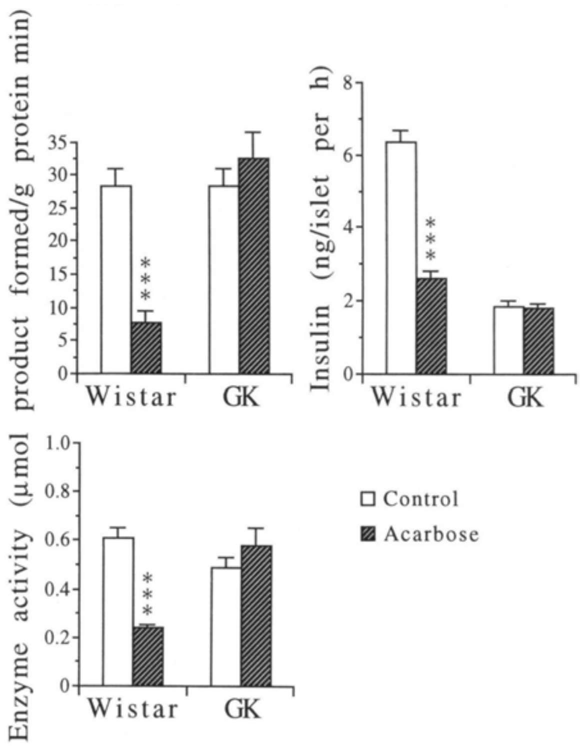

Figure 8. *Effect of selective a-glucoside hydrolase inhibition (10 mM acarbose) in Wistar and GK islets incubated at 16.7 mM glucose. Activities of acid glucan-1,4-a-glucosidase (top left) and acid a-glucosidase (bottom left) are shown, as well as insulin release.*

Conclusions – paper II

The present findings suggest that the GK rat has a dysfunctional lysosomal/vacuolar system in the islets of Langerhans. The dysfunction brings about profound changes in the normal physiology of the islets of Langerhans, *e.g.* decreased activities of classical lysosomal enzymes like acid phosphatase and N-acetyl-β-D-glucosaminidase and increased activities of the acid α-glucoside hydrolases. The increase in activity of the acid α-glucoside hydrolases does not bring about an increased insulin response to glucose in the GK rat, and the reason for this is conceivable related to the inability of the lysosomal/vacuolar system to respond to selective inhibition of the acid α-glucoside hydrolases in intact islets. Since the enzymes themselves respond normally to inhibition there is probably a malfunction in the uptake of the inhibitor acarbose. This defective lysosomal/vacuolar system seems to explain, at least partly, the impaired insulin secretory respons to glucose in the GK rat.

Glucose-stimulated insulin release in relation to islet NOS/NO (paper III)

NO might have multiple effects on the stimulus-secretion coupling of the β-cell, and several possible targets have been suggested, where NO seems to be involved in a suppression of the stimulus-secretion coupling of nutrient-stimulated insulin release, *e.g.* opening of K_{ATP} channels [15], suppression of phosphofructokinase activity [166], binding to iron-sulfur enzymes like aconitase [35, 170] or S-nitrosylation of various plasma membrane or cytosolic regulator proteins containing critical thiol groups [6, 70, 81, 129-131, 148]. In this context it is important to note that constitutive NOS has been regarded as a cytosolic enzyme in most tissues [91] although more recent findings [92] suggest that a significant part of ncNOS in rat islet β-cells is localized to insulin secretory granules.

Since we have for long argued that the acid α-glucoside hydrolases are involved in the complex regulation of insulin release, we have performed a number of studies (among those paper III, V and VI) to better characterize the nature of this involvement. Isoforms of acid α-glucoside hydrolases are known to contain several cysteine residues [19] and the very reactive radical NO might conceivably interact with those through nitrosylation of important thiol groups leading to a changed function (inactivation). In paper III we tried to investigate the influence of NO on glucose-stimulated insulin release in relation to the islet acid α-glucoside hydrolase activities.

Effect of exogenous NO in islet homogenates

To study the direct effect of NO on the acid α-glucoside hydrolase activities, as well as other lysosomal enzyme activities, we performed a series of experiments where mouse islet homogenates were incubated in the absence or presence of exogenous NO or with the intracellular NO donor sodium nitroprusside (SNP) in the incubation medium.

We detected a markedly suppressive effect of NO on the acid α-glucoside hydrolase activities (~ 60%), as well as a less marked suppressive effect on other classical lysosomal enzyme activites (~ 30%), *e.g.* acid phosphatase and N-acetyl-β-D-glucosaminidase. SNP which is known to deliver NO in a spontaneous way [48] also suppressed the acid α-glucoside hydrolase activities, although to a lesser degree than exogenous NO gas.

The effect of exogenous NO (saturated solutions) in this study was very convincingly consistent with the results from the results retrieved in paper VI, where we also used exogenous NO, as well as exogenous CO gas in the same type of experimental settings.

Effect of exogenous NO and hydroxylamine

Apparently NO acts a negative modulator of the acid α-glucoside hydrolases when it has direct access to the enzymes (see above), but it was of greater interest to see what effects might be seen in intact islets, where the conditions are more physiological. To investigate the effect of NO on insulin release and enzyme activities in intact islets, we used a similar approach as when we studied the direct effect of NO in islet homogenates; *i.e.* we used exogenous NO and a NO donor.

When we selected a suitable NO donor we chose to use hydroxylamine, a compound which in contrast to most other NO donors, such as SNP [48], delivers its NO by the intracellular route after having been metabolized by catalase [41]. The intracellular route of NO production in this context is important, because several previous studies have shown that a deranged balance of the intracellular reduced/oxidized thiol groups (especially the GSH/GSSG system) will induce an impairment of glucose-stimulated insulin release [14, 64]. In contrast, agents affecting thiol groups facing the outside of the plasma membrane might stimulate insulin release [8, 13, 63], and thus different NO donors delivering NO directly into the extracellular milieu, such as SNP, will not mimic intracellular NO production in an appropriate way. Hence, this makes hydroxylamine more attractive as an NO donor in experiments with intact islets.

In this study we found that hydroxylamine inhibited the acid α-glucoside hydrolase activities in parallel with a suppression of glucose-stimulated insulin release. The findings regarding NO's suppression of acid α-glucoside hydrolase activities accompanied by a similar parallel inhibition of glucose-stimulated insulin release is in accordance with our previous findings [110, 144-146] suggesting an intact activity of these enzymes being one of several important links in the insulin secretory process induced by glucose.

Effect of NOS-inhibition on nutrient-stimulated insulin release

The intracellular NO donor hydroxylamine inhibited the acid α-glucoside hydrolase activities in parallel with a suppression of glucose-stimulated insulin release from isolated islets. To further investigate the involvement of NO, we performed experiments with the NOS-inhibitor L-NAME. To study the effect of L-NAME on the activities of the acid α-glucoside hydrolases in relation to the effect on insulin release, we used 5 mM L-NAME, a concentration which has been shown to suppress the endogenous evolution of ncNOS-derived NO [6, 71].

Again, we found a striking correlation between the activities of the acid α-glucoside hydrolases and insulin release (see fig. 9), both in glucose-stimulated insulin release and L-arginine-stimulated insulin release. When we used 10 mM L-arginine to stimulate insulin release at 7 mM glucose the effect of L-NAME on the acid α-glucoside hydrolase activities was somewhat less pronounced than with glucose. The slight difference in effect on enzyme activites of L-arginine compared with glucose, might be explained by the fact that the insulin releasing effect of L-arginine is not only nutrient-stimulated but is due in major part to its cationic property [70, 72].

L-NAME amplified the acid α-glucoside hydrolase activities in parallel with an increased insulin release stimulated both by glucose and L-arginine. No effect of L-NAME was seen either at 1 mM or 7 mM glucose, which is consistent with previous data showing that there is is only a low endogenous NO production from islets incubated at low glucose [6, 66]. These findings are also consistent with our findings in the Wistar rat, which is discussed later in this section, dealing with the experiments in the mildly diabetic GK rat.

This striking correlation between the activities of the acid α-glucoside hydrolases and nutrient-stimulated insulin release suggests that the acid α-glucoside hydrolases might be one of several possible targets behind the inhibitory action of NO on nutrient-stimulated insulin release. In contrast to the above mentioned possible targets for NO (*e.g.* K_{ATP} channels, phosphofructokinase and aconitase), the acid α-glucoside hydrolases are located in the lysosomal/vacuolar system of the β-cell [101]. NO penetrates easily membranes and hence might very well exert its actions also in this system.

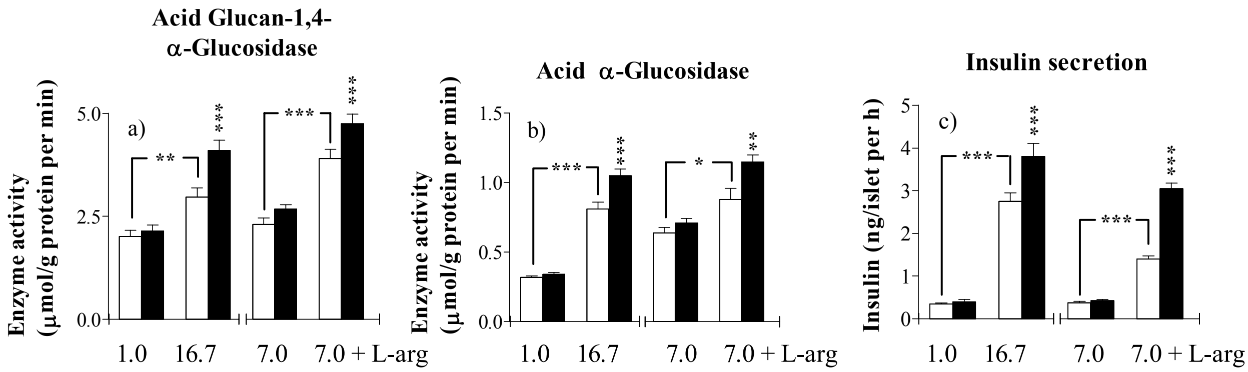

Figure 9. *Effect of NOS inhibition with L-NAME on islet activities of the acid a-glucoside hydrolases and on insulin release, at low and high glucose and in the presence of L-arginine. The islets were incubated in the absence (open columns) or presence (black columns) of 5 mM L-NAME at 1 or 16.7 mM glucose (the four columns to the left) and 7 mM glucose ±10 mM L-arginine (the four columns to the right).*

Conclusions – paper III

In the present study we show that NO inhibits the activities of the acid α-glucoside hydrolases and glucose-stimulated insulin release in parallel. Inhibition of NOS enzymes results in an amplification of both L-arginine- and glucose-stimulated insulin release as well as an amplification of the activities of the acid α-glucoside hydrolases. These results suggest that the inhibitory effect of NO on glucose- as well as L-arginine-stimulated insulin release is, at least in part, exerted via inactivation of the acid α-glucoside hydrolases.

Glucose-stimulated insulin release in relation to islet HO/CO in the GK rat (paper IV)

We have earlier shown in our laboratory (Henningsson 1999, 2001) that the HO-CO system is implicated in the regulation of insulin release, and that the NOS-NO system and the HO-CO system have different roles in this regulation, see fig. 2. The HO-CO system seems to have a protective role in the islets of Langerhans, counteracting negative effects of the NOS-NO system [69, 175]. In the present study we wanted to study the HO-CO system in the diabetic GK rat, to see if there were any differences in the nature and function of this system in relation to glucose-stimulated insulin release.

Heme oxygenase and CO production in GK islets

Immunocytochemistry showed a diffuse cytoplasmic HO-2 immunoreactivity in most endocrine cells in GK as well as Wistar control islets, and no apparent difference was seen between GK and Wistar islets in regard to number of immunolabelled cells or inten-sity of fluorescence. In contrast, we could detect a clear reduction of HO-2 expression in the GK islets compared to controls, evident in Western blots of HO-2. The GK islets also expressed HO-1, the inducible form of heme oxygenase, while no such expression was detected in control islets. We also measured CO production in freshly isolated islets ("ex vivo") and could reveal a prominent reduction of CO production (~ 50%) in GK islets compared to Wistar control islets (see fig. 10).

A marked dysfunction of CO production in GK islets was further found after incubation at high glucose, where we found that the glucose-stimulated CO production and the associated insulin release was considerably reduced, as compared to Wistar control islets.

The presence of HO-1 in the GK islets is of great interest, since HO-1 is known to be expressed in response to various noxious stimuli, such as endotoxin, heavy metals and oxidative stress [69, 113, 175]. It has been assumed that HO-1 activity protects the cells through metabolizing heme to bilirubin, which is known to have strong antioxidant properties [113]. HO-1 has also been shown to be expressed in obese hyperglycemic (*ob/ob*) mice [109], in partially pancreatectomized, hyperglycemic mic rats [93] as well as in normal rat islets cultured in high glucose [88]. The HO-1 expression, probably induced in the β-cells of the islets, seems to be a response to prevent glucotoxicity.

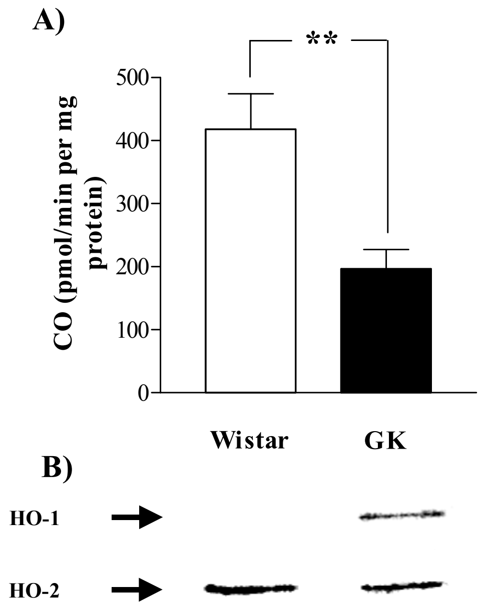

Figure 10. *HO activity measured as CO production and Western blots of HO-2 and HO-1 in freshly isolated islets from GK rats and Wistar controls. A) CO production in isolated islets. B) Representative Western blot showing protein expression in islets.*

Hemin-stimulation in GK islets

We have earlier shown that the HO substrate hemin is a potent stimulator of islet CO production [66, 68], and that hemin dose-dependently can potentiate glucose-stimulated insulin release. Glucose is in itself a potent stimulator of CO production [66], which has led to the suggestion that it might act as a positive modulator of glucose-stimulated insulin release.

We now studied the effect of hemin as well as exogenous CO gas on basal and glucose-stimulated insulin release, which was of specific interest since we had observed a dysfunction of the GK islet CO production, see above. We found that hemin and CO gas both potentiate glucose-stimulated insulin release in a similar manner, both in GK and Wistar control islets. The amplification of the insulin response (Δ) to glucose was not significantly different in GK *vs* control islets.

The results from these studies in the GK rat, suggest that the working capacity of the HO enzymes and the transduction targets of the CO molecule in the stimulus-secretion coupling are unimpaired in GK islets compared to Wistar control islets. Our results rather speak in favor of a major defect in glucose-stimulation of the HO-CO pathway in the diabetic islets.

Conclusions – paper IV

The present study provides evidence for a reduced activity of the islet HO-CO system, where the total CO production is suppressed in the GK rat, while at the same time the islets in the GK rat express the inducible form of HO, which might be interpreted as a consequence of the hyperglycemia in the GK rats. The impairment of the glucose-HO-CO signalling pathway is also seen in the decreased glucose-stimulated CO-production in parallel with a decreased glucose-stimulated insulin release. A possible involvement of HO-1-derived CO in the insulin secretory process is still unclear.

Glucose-stimulated insulin release in the GK rat in relation to islet NOS/ NO (paper V)

To further study the GK rat and its potential regulatory discrepancies regarding insulin release, we now performed a study where we focused on NO and its involvement in the regulation of glucose-stimulated insulin release, using a number of different approaches, including immunocytochemistry as well as batch incubations and results from experiments in the perfused pancreas.

Nitric oxide synthase in freshly isolated GK islets

Immunocytochemistry showed a diffuse cytoplasmic ncNOS immunoreactivity in most endocrine cells in GK as well as Wistar control islets. The cells generally appeared larger and swollen in GK islets compared to control islets, but no overt difference between GK and control islets in intensity of immunofluorescence or number of immunolabelled cells could be seen.

In freshly isolated islets Western blot revealed a clear expression of iNOS protein in GK islets, while no iNOS expression could be detected in Wistar control islets. The activity of ncNOS was modestly reduced in GK islets *vs* control islets. This reduction of ncNOS activity could be explained by the observation that the GK rats displayed a marked hyperglucagonemia *in vivo*. We have recently shown that cAMP generating agents, *e.g.* glucagon and glucagon-like peptide 1 (7-36) amide (GLP-1) are potent inhibitors of islet NOS activities [86, 87, 142, 143], and this hyperglucagonemia could, at least partly, explain also the hyperglycemia seen in the GK rats. In this context it should be mentioned that the islet cyclic AMP system is highly upregulated in GK rats [1], which again might depend, at least partly, on the hyperglucagonemia seen in these rats.

NOS activities and insulin release at low glucose

In contrast to freshly isolated GK islets, incubated GK islets at low glucose displayed an enhanced NO production compared to Wistar control islets (see fig. 11). The GK islets displayed markedly higher NOS activities, both NO derived from an increased ncNOS activity as well as from the appearance of a highly significant iNOS activity. The Wistar control islets, in contrast, displayed a similar NO production derived from ncNOS activity at low glucose, as was seen in freshly isolated islets. A neglible iNOS activity was also seen in control islets at low glucose. Previous data from our laboratory have repeatedly shown a low ncNOS activity and a non-detectable/neglible iNOS activity after incubation at low glucose in normal healthy rat and mouse islets [69, 87, 142, 143]. Why the GK rat displays this abnormal increase of iNOS activities at low glucose is unclear, but conceivably both glucagon and other unknown neural/hormonal factor/s restrain the activity of iNOS *in vivo*.

In the perfused pancreas we detected higher levels of insulin in GK than Wistar control pancreata at low glucose, when the NOS-inhibitor L-NAME was present. This is also consistent with a presence of an abnormal increase in basal NOS activities and NO production in the GK rat, that might act as a negative modulator of basal insulin release. There was no difference in basal insulin release between depolarized GK and Wistar islets with L-NAME added to the medium, which probably is explained by an elevated influx of Ca^{2+} in depolarized β-cells, where the increase in $[Ca^{2+}]_i$ might stimulate the Ca^{2+}/calmodulin dependent ncNOS activity [91] to approximately the same levels of NO in both types of islets. In this context it should be mentioned that the intracellular NO donor hydroxylamine has been shown to activate K_{ATP} channels, in mouse islets [15]. However, earlier data from our laboratory using K^+ diazoxide-treated mouse islets speak in favor of the major inhibiting effect of endogenously produced NO being exerted at more distal events in the stimulus-secretion coupling [7, 70, 71]. The cyclic AMP stimulator forskolin greatly potentiated the insulin response in depolarized islets in the presence of L-NAME, which again suggests that a low NO production and a marked stimulation by the cyclic AMP system is favourable in amplifying insulin release.

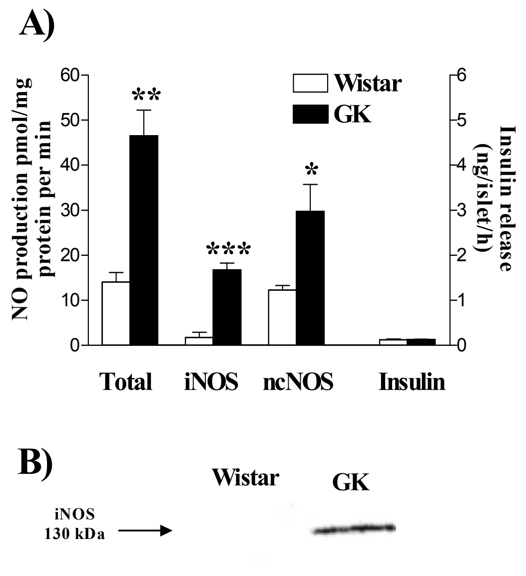

Figure 11. A) *NO production and insulin secretion in Wistar and GK islets incubated at low (3.3 mM) glucose.B) Western blot of iNOS in Wistar and GK islets incubated at low glucose.*

It remains to be clarified whether the predisposition of GK islets to readily display iNOS expression and activity, both at low and high glucose, might have any harmful effects in the long run, since an elevated iNOS activity in the β-cells is known to have a toxic influence [37, 45], even in the absence of an accompanying insulitis as described for transgenic mice overexpressing iNOS in their β-cells [162].

NOS activities and insulin release at high glucose

Glucose is a strong stimulator of islet NO production derived both from ncNOS and iNOS activation in normal mice [71] and rats [87, 143]. NO derived from ncNOS has been shown to increase within minutes following glucose stimulation [71, 157], while iNOS expression and activity is first evident after approximately 1 hour of hyperglycemic glucose levels, both *in vitro* and *in vivo* [71, 87, 143].

In the present study we wanted to further investigate the characteristics of the GK rat in glucose-stimulated insulin release. We saw the expected pattern in Wistar control islets incubated at high glucose, with an increase of both ncNOS and iNOS activities. The GK islets in contrast, showed a different pattern where the iNOS activity was highly increased, while the ncNOS activity which was already raised at low glucose did not display a further increase. NOS inhibition by L-NAME at high glucose was associated with an increased insulin release in the perfused pancreas from the GK rat, although the amplification of the insulin secretory response in the presence of L-NAME was more pronounced in the Wistar controls.

Similarly, experiments with islet incubations at high glucose in the presence of L-NAME revealed that the absolute amounts of insulin secreted after glucose stimulation were lower in the GK islets than in the control islets. However, the relative increase of the insulin response in GK islets was greater than in the control islets, suggesting that also iNOS-derived NO might be implicated in the inhibitory action of NO on glucose-stimulated insulin release, as previously has been shown in our laboratory [86, 87, 142, 143].

Glucose-stimulated insulin release dynamics and NOS inhibition

In the perfused pancreas the glucose-stimulated first phase insulin release was of similar magnitude in GK and Wistar pancreata when recorded in the presence of L-NAME, while the second phase was much more pronounced in control pancreata, suggesting that NOS inhibition by itself was not sufficient to completely restore the impaired insulin response to glucose in the GK rat. In contrast the first phase of insulin release was of similar magnitude in GK and Wistar controls in the presence of L-NAME, which is in accordance with our findings that the ncNOS activity was of the same magnitude in islets incubated at high glucose, since ncNOS, as mentioned above, is increased acutely after glucose-stimulation, while iNOS activity is delayed in appearing. This is also consistent with previous data [67, 71] showing that L-NAME is a more efficient inhibitor of ncNOS activity than of iNOS activity in islets exposed to high glucose, and also showing that high amounts of iNOS-derived NO in certain situations apparently can restrain glucose-stimulated insulin release.

It should also be emphasized that NOS inhibition with L-NAME almost abrogated the negative peak (nadir) separating first and second phase of glucose-stimulated insulin release, suggesting that NO derived from the rapid activation of islet ncNOS by glucose is of importance as a negative feedback inhibitor of the early insulin response. A similar release pattern was seen in isolated perifused islets from Sprague-Dawley rats [71] as well as our previous observations of a marked inhibition of islet NO production exerted by L-NAME already at approximately 2-3 min after an *i.v.* injection of the NOS inhibitor [6].

L-arginine-stimulated insulin release in vivo and in vitro

We have previously shown that concentrations of L-arginine that stimulate insulin release also stimulate islet NO production [70]. Interestingly however, glucose stimulates both ncNOS and iNOS, while L-arginine exerts a major effect on ncNOS only [85].

We now show, both *in vivo* and *in vitro*, that the L-arginine-stimulated insulin response was decreased in GK rats compared to Wistar control rats and addition of the NOS-inhibitor L-NAME potentiated the insulin response to L-arginine both in in GK and Wistar rats. These results are consistent with a predominant effect of L-NAME on the regulatory role of ncNOS-derived NO on the early insulin response elicited by L-arginine as well as glucose. The ncNOS-derived NO is probably only one of several factors behind the acute defective insulin response to these secretagogues in the young GK rat, since NOS inhibition by itself is not sufficient to restore the insulin response to control levels.

Conclusions – paper V

In the present study NO was again found to exert a negative influence on glucose-stimulated insulin release, and inhibition of NO production amplified the release of insulin. In the GK rat immunoblotting revealed iNOS expression and islets of the GK rat displayed a marked iNOS activity when incubated both at low and high glucose. The results suggest that NO is a negative feedback inhibitor of glucose-stimulated insulin release in the rat and that an enhanced iNOS activity rather than an impaired ncNOS activity in the GK rat seems to contribute to the defective insulin response to glucose in the young GK rat. The propensity of the GK rat to display iNOS expression and activity in the islets of Langerhans might be deleterious for the β-cell over time.

Islet acid α-glucoside hydrolases and glucose-stimulated insulin release in relation to NO and CO (paper VI)

In this study we tried to gather the findings and insights from our previous studies, and by a combination of experiments, mainly in batch incubations of intact islets, try to further elucidate the nature of the regulatory role that the acid α-glucoside hydrolases have in the β-cell, especially in relation to the novel messenger molecules NO and CO.

Effect of exogenous NO in islet homogenates and intact islets

The effect of exogenous NO (saturated solutions) was in this study consistent with similar results presented in paper III,, where we also detected a markedly suppressive effect of NO on the acid α-glucoside hydro-lase activities (~ 60%), as well as a less marked suppressive effect on other classical lysosomal enzyme activites (~ 30%), *e.g.* acid phosphatase and N-acetyl-β-D-glucosaminidase.

In paper VI exogenous NO displays a pronounced suppressive effect on the acid α-glucoside hydrolase activities as well as on glucose-stimulated insulin release in intact islets. In these experiments the suppressive effect of NO was, in contrast to the effect of hydroxylamine (paper III), slightly apparent at basal glucose where insulin release and the acid α-glucoside hydrolase activities were decreased in parallel.

Effect of exogenous CO and hemin in islet homogenates and intact islets

The direct effect of exogenous CO on the α-glucoside hydrolases was studied by adding CO to islet homogenate incubations. The effect was stimulatory , not only to the acid α-glucoside hydrolases but also to other lysosomal enzyme activities which all were increased by 150-250%.

It is of course of greater interest to study the effect of CO on intact islets. Hence, we performed experiments where exogenous CO or the HO substrate hemin was added to islet incubations at low and high glucose. Both CO and hemin induced an amplification of glucose-stimulated insulin release and a parallel increase in the activities of the acid α-glucoside hydrolases. At basal glucose CO stimulated the enzymes but had no effect on the insulin release at this low substimulatory glucose concentration (1 mM).

We have earlier shown that the intracellular messenger Ca^{2+} activates the lysosomal/vacuolar insulin secretory pathway (*e.g.* paper I), and our results show that CO too might act as an intracellular activator of the same pathway.

In these experiments we also found that CO hade a general stimulatory effect on the enzyme activities, both directly and in islet incubations, not only stimulating the acid α-glucoside hydrolases but also classical lysosomal enzymes such as acid phosphatase and N-acetyl-β-D-glucosaminidase. These findings agree with what we have found in the GK rat model (paper IV), where we detected a greatly reduced CO production in isolated islets, and this might be associated with an impaired interaction between different organelle constituents within the islet vacuolar system (paper II).

Effect of selective inhibition of soluble guanylate cylase at high glucose

It has earlier been shown in our laboratory that hemin stimulates glucose-induced insulin release, while the HO inhibitor Zn-protoporphyrin acts suppressively [68]. The hemin-enhanced glucose-stimulated insulin release can also be markedly reduced by the selective inhibitor of soluble guanylate cyclase ODQ [66] which suggests that the major effect of CO on insulin release is exerted through the guanylate cyclase/cyclic GMP system.

To investigate the involvement of the guanylate cyclase/cyclic GMP system further, we added ODQ to the incubation media at high glucose (20 mM). ODQ was able to completely abrogate the stimulatory effect of hemin on both insulin release and the acid α-glucoside hydrolase activities. In the absence of hemin a very slight decrease in the activity of acid α-glucosidase, but not of acid glucan-1,4-α-glucosidase, as well as a slight decrease in insulin release was evident. Since hemin also stimulated the activity of the lysosomal enzymes acid phosphatase and N-acetyl-β-D-glucosaminidase, it seems quite conceivable that the HO-CO-cyclic GMP system not only amplifies glucose-stimulated insulin release but also has a general stimulating effect on the vacuolar system/lysosomal enzyme activities.

Interaction of the HO-CO signalling pathway with PKA, PKC and guanylate cyclase in glucose-stimulated insulin release

The apparent involvement of the guanylate cyclase/cyclic GMP system at very high glucose (20 mM) raised the question whether also the cyclic AMP system and/or phospholipase C might be involved in the HO-CO signalling pathway. These experiments were conducted at a lower hyperglycaemic glucose level (12 mM), and we found as expected that guanylate cyclase inhibition by ODQ abrogated the stimulatory effect of CO on glucose-stimulated insulin release, but we also found that selective inhibition of cyclic AMP by Rp-cAMPS brought about the same effect, while the phospholipase inhibitor bisindolylmaleimide had no apparent effect. The marked inhibitory effect by Rp-cAMPS might indicate that a very high CO production has a great impact not only on cyclic GMP but also on the cyclic AMP pathway(s) for insulin release in a complex compensatory interaction.

We also performed experiments where exogenous CO was added to islets incubated at high glucose and the results further implicated an involvement of the cyclic AMP system, since CO amplified the glucose-stimulated increase in islet cyclic AMP content as well as in islet cyclic GMP content. The effect of exogenous NO was also studied (se above) and the results suggest that CO, at least at high glucose, has a greater impact than NO on the cyclic GMP system, which is at variance with data from *e.g.* cerebellar granule cell cultures where NO has been shown to be the major modulator of cyclic GMP compared to the role of CO [78]. Our results in the present study might be explained by the fact that we used a maximal concentration of NO gas which at high concentrations might exert a negative feedback on the NOS enzymes [16]. Endogenous NO production in most instances is likely to stimulate cyclic GMP production in many cell types, including islet endocrine cells [40, 66, 67, 69, 91, 142, 143].

The present results suggest that the CO-induced amplification of glucose-stimulated insulin release is elicited both through the cyclic GMP and the cyclic AMP pathways, and that an important part of the cyclic GMP effect is transduced through the activation of the acid α-glucoside hydrolases and the lysosomal/vacuoular system, although an additional direct action of CO on this system cannot be excluded. The secretory pathways induced through direct activation of the cyclic AMP or phospholipase C systems seem to operate independently of the acid α-glucoside hydrolases [110, 144, 145, 146, paper I]. Indeed, we have earlier shown that selective α-glucoside hydrolase inhibition of glucose-stimulated insulin release by emiglitate can be compensated for by stimulation of the cyclic AMP pathway through the adenylate cyclase activator forskolin [148]. Similarly, a dysfunction of glucose stimulation of the lysosomal/ vacuolar system in the islets of the GK rat (paper II) is associated with a compensatory increase in the cyclic AMP pathway.

Effect of hemin at high glucose on islet NOS activities

The interaction between NO and CO on the different NO- and CO-synthesizing enzymes is unclear and presently not predictable [58]. We have previously observed that exogenous CO greatly suppresses islet NO production, and this effect was not afflicted by ODQ and thus appeared to be operating independent of the cyclic GMP system [69]. In the present study we added hemin to the incubation media, and found that the inhibition of the islet NO production is mainly exerted on iNOS-derived NO. Since there is a concomitant increase in glucose-stimulated insulin release these data also indicate that iNOS-derived NO might restrain the release process as previously suggested [86, 87, 142, 143].

It is important to note that freshly isolated islets, from both mice and rats, display a more than 5 to 10-fold higher production of CO than of NO [66-69, 71, paper IV], a difference that might partly compensate for the low levels of the antioxidant enzymes superoxide dismutase, catalase and glutathione peroxidase in the β-cell [123].

Effect of selective inhibition of the acid α-glucoside hydrolases in the absence and presence of CO

In the present study we also added the selective α-glucoside hydrolase inhibitor emiglitate to islet incubations and could show that emiglitate greatly suppressed the glucose-stimulated insulin release in parallel with an inhibitory effect on the activities of the acid α-glucoside hydrolases. When exogenous CO was added to the incubations glucose-stimulated insulin release was amplified as expected, but this amplification was completely abrogated by the addition of emiglitate.

The suppressive effect of emiglitate and ODQ on CO-stimulated amplification of glucose-stimulated insulin release shows the relative importance of the cyclic GMP-acid α-glucoside hydrolase pathway in relation to the cyclic AMP pathway in this context. However, as shown by the present results as well as by previous data [66, 110] emiglitate greatly reduced the insulin response to glucose in the absence of exogenously added CO, while ODQ had only a minor inhibiting effect. Hence, most likely emiglitate inhibited not only the cyclic GMP signalling pathway but also other transduction pathways related to the acid α-glucoside hydrolases in glucose-stimulated insulin release, and it should be recalled that emiglitate exerts its inhibitory effect on glucose-induced insulin release at a distal step in the stimulus-secretion coupling, since it also inhibits insulin release stimulated by nutrients directly entering the mitochondrial metabolism, *e.g.* leucine and KIC [110] and it does not influence glucose oxidation [149].

In contrast, a possible defect in endogenously produced CO is most likely associated with an early step in glucose-stimulated insulin release, since the glucose-stimulated induction of the HO-CO signalling pathway is defective in the islets of the diabetic GK rat, and these islets respond to exogenous CO with a normal amplification of glucose-stimulated insulin release (paper IV).

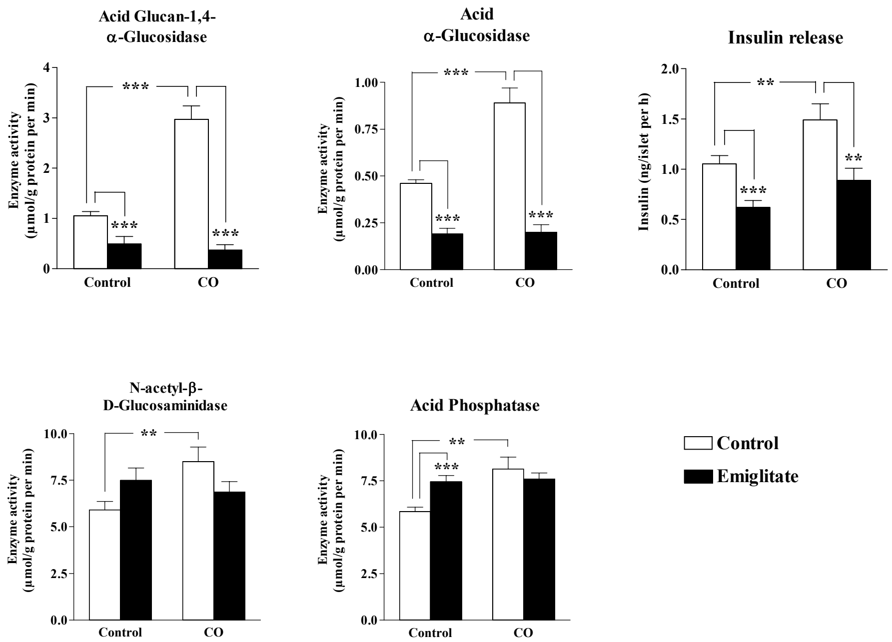

Figure 12. Effect of the selective α-glucoside hydrolase inhibitor emiglitate (black columns) on glucose-stimulated insulin release and islets lysosomal enzyme activities at 12 mM glucose, in the absence and presence of exogenous CO.

Conclusions – paper VI

In this study we showed that NO inhibits and CO amplifies glucose-stimulated insulin release and the activities of the acid α-glucoside hydrolase activities in parallel. Moreover, the HO substrate hemin markedly enhanced glucose-stimulated insulin release and the activities of the acid α-glucoside hydrolases in parallel. Guanylate cyclase inhibition had a suppressive influence on the effects of hemin. Exogenous CO was shown to raise the islet content of both cGMP and cAMP in parallel with a marked amplification of glucose-stimulated insulin release, while exogenous NO had the opposite effect on insulin release and cAMP, but did not affect cGMP. Selective inhibition of the acid α-glucoside hydrolases by the selective inhibitor emiglitate could counteract the stimulatory effect of CO and glucose on both insulin release as well as on the activities of the acid α-glucoside hydrolases.

The results suggest that NO and CO, which both are produced in significant amounts in the islets of Langerhans, have interacting regulatory roles on glucose-stimulated insulin release, and that this regulation is, at least in part, transduced through the activity of cyclic GMP and the lysosomal/vacuolar system and the associated acid α-glucoside hydrolases, but most probably also through a direct effect on the cyclic AMP system.

SUMMARY AND GENERAL CONCLUSIONS

Glucose-stimulated insulin release – its regulation by the acid α-glucoside hydrolases, NO and CO

Acid α-glucoside hydrolases

In this thesis the activities of acid α-glucoside hydrolases are discussed in relation to insulin secretion. There are two isoforms called acid glucan-1,4-α-glucosidase and acid α-glucosidase. The former preferentially cleaves α-1,4-linkages in glycogen, while the latter preferentially acts on oligosaccharides, but it should be kept in mind that their activities are overlapping. The enzymes are located in the lysosomal/vacuolar compartment of cells, and their direct effects are therefore probably limited to this acidic mileu, but their secondary effects are not necessarily restricted to an acidic environment.

In paper I the Ca^{2+}-dependency of the acid α-glucoside hydrolases are studied in some detail, and the conclusions that might be drawn is that the activity of the enzymes is dependent on Ca^{2+} and that the effects that Ca^{2+}-glucose-induced changes seem to be coupled to Ca^{2+}-glucose-stimulated insulin release. The effect of Ca^{2+} is not exerted directly on the enzyme molecules, but is probably brought about by activation of the acidic organelles where the acid α-glucoside hydrolases are located.

The NOS-NO system and the HO-CO system

The activity of the acid α-glucoside hydrolases is also dependent on the NOS-NO system, where we in the present studies have shown that NO inhibits the acid α-glucoside hydrolases and glucose-stimulated insulin release in parallel. NOS inhibition results in increased acid α-glucoside hydrolase activities and a parallel amplification of both L-arginine- and glucose-stimulated insulin release. NO was also shown to decrease both the islet content of cAMP and glucose-stimulated insulin release.

I have also presented evidence for a stimulatory role for the HO-CO system, in contrast to NO, on the activities of the acid α-glucoside hydrolases and on glucose-stimulated insulin release in parallel. Inhibition of guanylate cyclase could partly suppress the effects of hemin-stimulation and CO increased the islet content of both cGMP and cAMP and increased glucose-stimulated insulin release in parallel.

Studies in the mildly diabetic GK rat

In the studies of the GK rat I present evidence for several, partly interacting, abnormalities that might contribute to the impaired response to glucose stimulation seen in this animal model of mild spontaneous diabetes.

We show that the GK rat has a dysfunctional lysosomal/vacuolar system in the islets of Langerhans where the acid α-glucoside hydrolases display a normal catalytic activity in islet homogenates, but their action is restrained by a malfunction in the lysosomal/ vacuolar compartment which seems to prevent the acid α-glucoside hydrolase signalling pathway from functioning normally. Hence, this might explain, at least partly, the marked impairment of glucose-stimulated insulin release in this rat model.

The islets of the GK rat express the inducible form of NOS, iNOS, and the islets display a marked iNOS activity when incubated at low glucose, which is in contrast to normal control rats, although iNOS expression and activity is seen as a normal response to incubation at high glucose. Inhibition of NO production on the other hand, resulted in an amplification of glucose-stimulated insulin release. The results suggest that an enhanced iNOS activity rather than an impaired ncNOS activity seems to contribute to the defective insulin response to glucose in the GK rat. The iNOS expression and activity might in the long run conceivably be harmful for the β-cells, since NO has been shown to be involved in the diabetogenic process in β-cells of type 1 diabetes.

The islet HO-CO system displayed a reduced activity in the GK rat, where total CO production was suppressed in islets isolated "ex vivo". The GK islets were shown to express inducible HO (HO-1), which might be in response to hyperglycemia. The GK islets also displayed a decreased glucose-stimulated CO production. A possible role of HO-1 in the insulin secretory process is unclear.

Concluding remarks

In this thesis evidence is presented for two evolutionary very old messenger molecules, CO and NO, to have a profound regulatory influence on glucose-stimulated insulin release and that the glycogenolytic acid α-glucoside hydrolases, associated to the lysosomal/vacuolar compartment of the β-cell, seem to be deeply involved in the regulation of glucose-stimulated insulin release, and in the action of both CO and NO.

The results presented in this thesis suggest that NO and CO, which both are produced in significant amounts in the islets of Langerhans, have interacting roles on glucose-stimulated insulin release, and that this regulation is, at least partly, transduced through the activity of cGMP and the lysosomal/vacuolar system and the associated acid α-glucoside hydrolases, but also through a direct effect on the cAMP system. NO has an inhibitory role and CO has a stimulatory role in the very complex process of regulation of insulin release. Schematic overviews of the findings presented in this thesis are shown in fig. 13, both in healthy animals and in diabetic GK rats.

In the mildly diabetic GK rat I have presented evidence for abnormalities in all three enzyme systems studies, and these findings might hopefully contribute to the understanding of the impaired insulin response to glucose stimulation seen in type 2 diabetes.

In the future

Future studies might establish the actual importance *in vivo* in mice and rats, as well as humans, of the acid α-glucoside hydrolases. It should be kept in mind that, with regard to glucose-stimulated insulin release, there are recent findings that point out the importance of organelle specific signalling pathways through acidic organelles in the β-cell, and there are also data showing that acidification of secretory granules is of importance for the exocytotic process, and an acidic milieu is just the right place for enzymes like the acid α-glucoside hydrolases.

In the future it should be of interest to establish the exact location of the acid α-glucoside hydrolases on a subcellular level, and to study the actual physiological processes at this level, *e.g.* production of non-phosphorylated glucose and/or the effects of these enzymes on membrane glycoproteins and membrane merging in relation to events in the exocytotic process. It might also be helpful to pursue studies in acid α-glucoside hydrolase knock-out animal models.

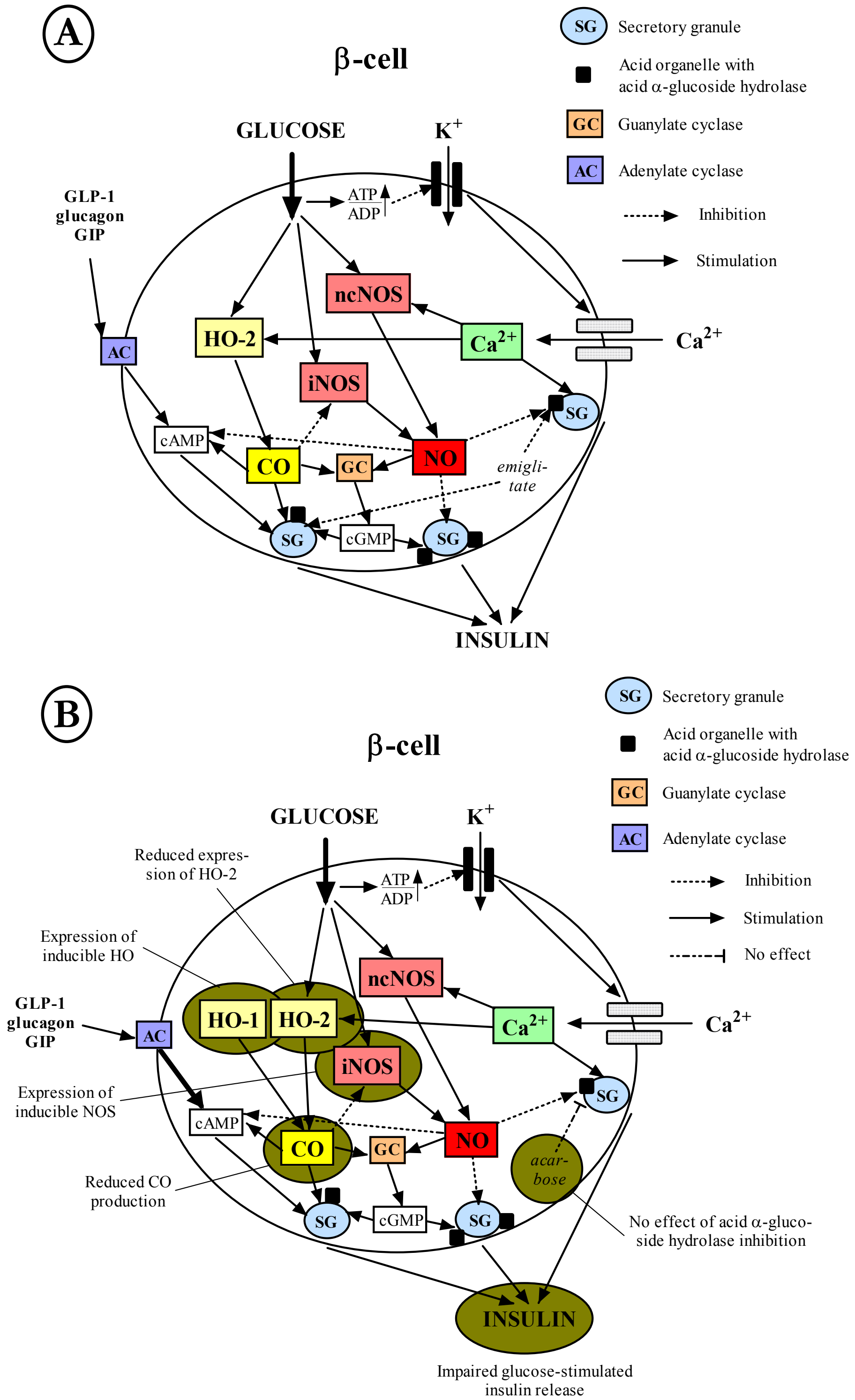

Figure 13. *Simplified scheme of the data obtained in the present thesis, illustrating the putative interaction of the NOS-NO, HO-CO and acid a-glucoside hydrolase-containing organelles in glucose-stimulated insulin release in normal animals (A) and in the mildly diabetic GK rat (B). Differences compared to normal healthy animals are highlighted. In this thesis we show that in healthy animals: the acid a-glucoside hydrolases are Ca^{2+}-dependent and coupled to Ca^{2+}-glucose-stimulated insulin release; NO inhibits, and CO stimulates, the acid a-glucoside hydrolases and glucose-stimulated insulin release in parallel; NOS inhibition has the opposite effect to NO; guanylate cyclase inhibition has a suppressive effect on hemin-glucose-stimulated insulin release, and exogenous CO raised both cGMP and cAMP levels as well as resulted in an amplified glucose-stimulated insulin release, while NO had the opposite effect on cAMP levels; selective inhibition of acid a-glucoside hydrolase activities counteracts the effect of CO and glucose on insulin release as well as on the activites of the acid a-glucoside hydrolases; inhibition of the acid a-glucoside hydrolases also suppresses the effect of high Ca^{2+} on glucose-stimulated insulin release.*

POPULÄRVETENSKAPLIG SAMMANFATTNING PÅ SVENSKA

Allmän introduktion

Diabetes (sockersjuka) är en folksjukdom som inte minst drabbar oss i Sverige. Det finns olika former av sjukdomen, men de har gemensamt att kroppen inte kan kontrollera halterna av glukos i blodet (blodsockret). Det finns två stora grupper av diabetes, typ 1 och typ 2, där man i den första typen ofta insjuknar tidigt i livet och alltid är beroende av hormonersättning med insulin, medan den senare formen ofta debuterar senare i livet och åtminstone till en början inte nödvändigtvis kräver insulinbehandling. Det är nu drygt 80 år sedan man lyckades identifiera hormonet insulin, och på så sätt fann ett sätt att rädda alla dem som tidigare dog tidigt i livet då de drabbats av diabetes typ 1. Under denna tid har man ägnat mycket tid och inte minst pengar åt att försöka klargöra vad som reglerar insulinets frisättning i kroppen. Det har visat sig vara ett ytterst komplicerat arbete, då denna för kroppen livsviktiga hormonfrisättning, har ett otal olika reglermekanismer för att säkerställa att blodsockernivåerna ligger rätt. Insulin frisätts från β-celler i små öar av hormonproducerande celler som ligger utspridda i bukspottkörteln, och en anledning till att det är svårt att studera insulinfrisättning är att just denna miljö är svår att imitera i försök i provrör. I mina studier har jag främst använt mig av isolerade cellöar (Langerhanska öar).

Bakgrund och målbeskrivning

I den här avhandlingens arbeten har främst glukosstimulerad insulinfrisättning studerats eftersom glukoshalten i blodet är den främsta reglermekanismen för insulinfrisättning. Det finns dock andra ämnen (bl.a. aminosyror som L-arginin och leucin samt vissa fettsyror) som också kan stimulera till frisättning av insulin.

Tyngdpunkten i avhandlingen ligger på studier av olika enzymsystem som kan vara involverade i regleringen av insulinfrisättningen. Ett av de enzymsystem som vi fokuserade på är lokaliserat i det ”lysosomala/vakuolära systemet” som är ett system av rörliga ”blåsor” eller ”rum” i cellen, som också innefattar de insulininnehållande granula och som kännetecknas av en hög surhetsgrad (lågt pH). Detta har gjorts delvis i förhållande till två andra enzymsystem som också finns i β-cellerna, nämligen hämoxygenas-kolmonoxid-systemet (HO-CO-systemet) och kväveoxidsyntas-kväveoxid-systemet (NOS-NO-systemet). Reglermekanismer för frisättning av insulin i relation till ovannämnda enzymsystem har dels studerats i normala möss och råttor såväl som i en råttmodell av spontan mild typ 2 diabetes, kallad Goto-Kakizaki (GK).

De sura α-glukosidhydrolaserna finns i de Langerhanska öarna och i β-cellerna i två former som båda har till uppgift att bilda sockermolekyler från större molekyler, dels surt glukan-1,4-α-glukosidas (som föredrar att dela på stora glykogenmolekyler) och dels surt α-glukosidas (som föredrar små kolhydrater, sk oligosackarider), men de två enzymerna har överlappande effekter och är båda lokaliserade i lysosomala/vakuolära organeller i cellerna. Det viktiga med dessa enzymer är att de kan producera intracellulärt glukos och/eller förändra membranstrukturer som innehåller glukosmolekyler, och därvid stimulera insulinfrisättningen.

Gaserna CO och NO är väldigt reaktiva och mycket benägna att reagera med omgivningen. Båda gaserna är farliga att andas in i större mängder. CO finns t.ex. i bilavgaser, och har som självmordsredskap tyvärr nyttjats av många personer med självmordstankar och tillgång till en bil, vilket inte minst skildrats i olika varianter på samma tema i oräkneliga film- och teveproduktioner. Det har dock visat sig att dessa för kroppen dödliga gaser, i små mängder och lokalt på cellnivå, har viktiga uppgifter. Inte minst har detta visats för NO som vidgar kärl, något som används dagligen av hjärtsjuka patienter som har sitt nitroglycerinpreparat i fickan.

Målet med studierna är att klargöra vilken roll de nämnda enzymsystemen har för regleringen av glukosstimulerad insulinfrisättning, dels normalt och dels i den diabetiska GK-råttan.

Resultat

I den första artikeln framkommer att de sura α-glukosidhydrolaserna är beroende av kalcium (Ca^{2+}), och vi finner ytterligare belägg för att de har en roll i regleringen av glukosstimulerad insulinfrisättning. Man vet sedan länge att Ca^{2+} är inblandat i flera led av insulinfrisättningsprocessen. Vi fann att Ca^{2+} inte stimulerar själva enzymmolekylerna utan istället sannolikt aktiverar de organeller som innehåller enzymet.

I den andra artikeln studeras den diabetiska GK-råttan med de sura α-glukosid-hydrolaserna i fokus. Här visas att en orsak till GK-råttans nedsatta insulinsvar troligen kan knytas till en felaktig funktion i det lysosomala/vakuolära systemet då aktiviteten av klassiska lysosomala enzymer som surt fosfatas är sänkt medan de sura α-glukosid-hydrolasernas aktivitet är förhöjd. Dessutom visas att i dessa diabetiska råttor ses ingen effekt av selektiv hämning av aktiviteterna hos de sura α-glukosidhydrolaserna, vilket normalt åtföljs av ett nedsatt insulinsvar på glukos. Denna brist på effekt beror förmodligen på att hämmaren av någon anledningen inte kommer åt de sura α-glukosid-hydrolaserna i GK-råttan, således ytterligare ett tecken på en felaktig funktion i det lysosomala/vakuolära systemet.

I den tredje artikeln studeras de sura α-glukosidhydrolaserna och glukosstimulerad insulinfrisättning i förhållande till NOS-NO-systemet. Försöken görs dels med en NO-givare i form av hydroxylamin som avger NO inne i β-cellen, och dels genom att hämma själva produktionen av NO genom att specifikt blockera NOS. Jag finner där att NOS-NO-systemets effekter är slående parallella på de sura α-glukosidhydrolaserna och på insulinfrisättningen. Slutsatsen som kan dras är att NO:s hämmande effekt på de sura α-glukosidhydrolaserna är en bidragande orsak till NO:s hämmande inverkan på insulinfrisättningen.

I den fjärde artikeln vänds åter blickarna till GK-råttan, men nu studeras HO-CO-systemet i denna diabetiska djurmodell. GK-råttorna visar sig uttrycka (producera) den normalt förekommande HO-formen (HO-2) i mindre utsträckning än normalt, medan GK-råttans öar, till skillnad från i normala friska djur, uttrycker en inducerbar form av HO, något som tidigare visats vara fallet vid olika för celler skadliga omständigheter. HO-1 har där antagits vara skyddande för cellerna då en av slutprodukterna av enzymets aktivitet är bilirubin, vilket är en betydande antioxidant. Samtidigt fann jag i denna studie att den glukosstimulerade CO-produktionen, liksom insulinfrisättningen, var minskad i GK-råttan. Av detta drog jag den slutsatsen att ett nedsatt svar på glukosstimulering i HO-CO-systemet kan vara ännu en orsak till GK-råttans dåligt fungerande insulinsvar på glukosstimulering.

I den femte artikeln studeras så NOS-NO-systemet i GK-råttan. Vi finner där att de Langerhanska öarna i GK-råttan uttrycker den inducerbara formen av NOS (iNOS) och att den totala NO-produktionen i inkuberade öar är förhöjd hos GK-råttan. Då NO har en hämmande effekt på insulinfrisättningen kan även detta vara en bidragande orsak till GK-råttans dåliga insulinsvar på glukosstimulering, och i längden kan denna NO-produktion vara skadlig för β-cellerna och leda till för cellen skadliga processer och i slutändan celldöd. Återigen visar vi i olika försök att NO:s effekt på insulinfrisättning huvudsakligen är hämmande.

I den sista artikeln studeras så glukosstimulerad insulinfrisättning sett ur ett holistiskt perspektiv där alla tre ovannämnda enzymsystem studerats ur olika synvinklar. Vid en jämförelse mellan CO- och NO-gas visar jag att NO hämmar och CO stimulerar de sura α-glukosidhydrolaserna parallellt med samma effekter på insulinfrisättningen. Vid hämning av ett cellsignalsystem kallat cykliskt GMP-systemet ses viss hämning av både aktiviteten hos sura α-glukosidhydrolaserna och av glukosstimulerad insulinfrisättning, vilket tyder på att cykliskt GMP-systemet åtminstone delvis är involverat i signalöverföringen. I övrigt visas också att CO hämmar NO-produktionen i öarna och har en allmänt stimulerande effekt på det lysosomala/vakuolära systemet.

Sammanfattning

I avhandlingen framläggs resultat som visar att NO:s hämmande effekter och CO:s positiva effekter på aktiviteten hos de sura α-glukosidhydrolaserna är parallella med deras respektive inverkan på glukosstimulerad insulinfrisättning. Selektiv hämning av de sura α-glukosidhydrolaserna leder dessutom till hämning av glukosstimulerad insulinfrisättning. Sammantaget kan man konstatera att resultaten ger ytterligare tyngd åt tidigare data som visat på ett samband mellan de sura α-glukosidhydrolaserna och glukosstimulerad insulinfrisättning, och resultaten är väl i linje med senare års studier från andra forskargrupper, där man bland annat funnit belägg för att en surgörning (acidifiering) av sekretoriska granula (i vilka insulinet lagras och frisätts) föregår själva frisättningsprocessen ut ur β-cellerna. I studier av den diabetiska GK-råttan ses flera, sannolikt delvis av varandra beroende, avvikande fynd vad gäller de tre studerade enzymsystemen. Resultaten visar på en koppling mellan dessa avvikelser och den spontandiabetiska GK-råttans dåligt fungerande insulinsvar på glukosstimulering vilket således antyder att liknande defekter kan bidra till utvecklingen av typ 2 diabetes hos människor.

ACKNOWLEDGEMENTS

It is a pleasure to finally finish the doctoral studies that I commenced many years ago. My studies have not been of the straight-forward kind, since I have oscillated from doctoral studies, to medical school, to clinical work, and to finish it off, taking care of my two lovely sons, and of course trying to get some time to spend with my wife.

Over the years I have had the oppurtunity to get to know many individuals, and this is my oppurtunity to mention one or two of them. I started out with a memory of questionable logic, and I can only say that it has not improved over the years. This might explain if I have forgotten to mention someone I should have remembered.

First of all I want to give my supervisors **Albert Salehi** and **Ingmar Lundquist** the credit they so very much deserve. They have actually managed to follow me through all those years of now-and-then studies, studies that sometimes were very focused and sometimes not so focused. They have provided me with all the support I have needed, and shared their extensive knowledge. It has been a pleasure being tutored by the two of them.

When I try to dig deeply in memory I seem to remember the place I began my days as a PhD student. A building reminiscent of a medival fortress, like Glimmingehus but in a more cosmopolitical environment. It was always a special, and to a guy like me an enriching, experience to pass through the entrance hallway of the old **Department of Pharmacology**, hearing the massive door close behind me. Now the days of medical glory that actually had a small place right there, are gone and soon almost forgotten.

In the beginning I was lured into the doctoral swamp of studies by a course mate and friend of mine, **Georgios Panagiotidis**. I am really glad I got to know you. You also taught me many valuable lessons in the laboratory, for which I am grateful.

Another collegue that I have spent many hours talking to is **Ragnar Henningsson**, who I got to know as a sharp mind in a boyish body. I always envied you your stamina and speed, but I don't think I would ever manage to get up in the morning to go for a run by the sea. We were quite even on the badminton court, which was great fun.

I must not forget to mention **Björn Åkesson**, who also taught me one or two things, and I will always remember when you danced "double Jitterbug", which I had never seen done before, or even heard of.

Naturally I also want to give loads of thanks to **Britt-Marie Nilsson**, for never-ending patience when it comes to all those assays, as well as friendly and uplifting morning talks. There is a girl with a steady hand using the pipette, as well as with the gun perhaps? In the beginning, there was also **Elsy Ling** to help out with all the samples I managed to gather. Many thanks to you both.

I also want to thank my last, but not at all least, acquaintance at the lab, **Javier Jimenez Feltström** who is a really great guy. You have added many laughs to just as many lousy, grey and rainy winter days. You were quite nice in other seasons as well, of course.

On other floors in the old building I mentioned above, I had the pleusure of getting to know **Rolf Håkanson** and his research group, including **Per Norlén**, **Maria Björkqvist** and **Erik Lindström**, as well as the always trustworthy and helpful **Eva Björkbom**. I also want to mention **Anna Themner-Persson** and **Britt Carlsson**, always with a bright word or so to share. And many thanks to **Janeric Kronroth** for your relentless help with the technical stuff.

I also want to mention **Ding Xi-Qin** for nice chats in the basement and elsewhere, and for teaching me some Chinese, of which I today don't remember much.

In the later years I have had the pleasure to get to know **Peter Ericsson, Charlotta de la Cour**, **Andreas Lindqvist** and **Maria Bernsand**.

After we moved from the fortress on Sölvegatan to BMC, I had the oppurtunity to meet **Patrik Rorsman** and members of his research group, it was really nice meeting you all.

It would perhaps be unfair not to mention one of the more consuming passions of my life. I suppose that if I had not got to know you, now long gone and stone dead people, I might have had more time to spend on my doctoral studies. But then again, I am not so sure that I would have liked the world of insulin regulation as much if I had not had something completely different to turn to, when I got tired of islets of Langerhans. So anyhow, I must thank you for distracting me. My dear fellows (and relatives): **Lars** in Torpa (who shot his first wife in 1616 – by mistake he told the court, and then got her cousin pregnant a few months later), **Nils Haraldsson** in Torpa (not the same Torpa), **Lars Eriksson** in Norra Karleby, and all the rest of you. Not only men by the way, lets not forget the fascinating woman (not a relative of mine as far as I know) **Kerstin** in Vist and many more enchanting individuals. I hope I will get to know you better, now that I have more time for you.

Now, lets get back to the living.

My loving mother **Eva** has always supported me in my small ventures, for which I am very greatful. And I am glad you have got **Ove**, a really fine man.

If it had not been for my father **Harald**, nowadays almost a Norwegian, I would of course not be here, and surely not spending my days in a hospital. Obviously, I have you to thank for much.

Let me send my best wishes to grandma **Greta** as well. I spent some of my most memorable moments of youth in your and grandpa **Bengt**'s company, for which I am very greatful. You taught me the importance of collecting knowledge, and how fun it really is to learn new things.

My sister **Anna** and brother **Anders** have followed me through life. It is really a privilige to have you around.

I am also most greatful to my father and mother in law, **Bo** and **Barbro**, who have been so very kind helping out with the children in the latest months, when I have tried to get some fragmentary reading and writing done.

Lastly I want to thank my beloved **Kristina**, and my two sons, **Johan** and **Carl**. I am so very lucky to have you with me by my side, though of lately I have had reason to be greatful when you have stayed away from my side, in this way supporting me admirably in my final doctoral work at the computer. I will always love you!

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