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The Free Fatty Acid Receptor GPR40 – expression and role in islet hormone secretion

Erik Flodgren

Doctoral Thesis

LUND UNIVERSITY
Faculty of Medicine

With due permission from the Faculty of Medicine, Lund University, the public defense of this thesis, for the degree of Doctor of Philosophy in Medicine, will take place in Segerfalksalen, Wallenberg Neuroscience Center, Lund, Sweden on Friday, March 23, 2007 at 9 a.m.

Faculty opponent: Professor Valdemar Grill, The Norwegian University of Science and Technology in Trondheim, Norway

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The Free Fatty Acid GPR40 - expression and role in islet hormone secretion

Abstract

Type 2 diabetes (T2D) is a serious condition of growing proportions. Developing via an increasing imbalance between insulin sensitivity in the peripheral tissues and insulin release from pancreatic beta-cells, it ultimately renders the individual incapable of regulating the blood glucose concentration, e.g. after a meal. The increased prevalence of T2D is associated with an increase in the prevalence of obesity, with obesity being the single largest risk factor for the development of T2D. This work describes a molecule in the border zone between T2D and obesity. Our initial characterization of GPR40 identified it as a receptor for medium- to long-chain free fatty acids (FFAs). With a marked expression in pancreatic beta-cell lines, we expected GPR40 to be involved in FFA-mediated augmentation of insulin release. This was confirmed when we examined the dose-response relationship between FFA stimulation of GPR40 and both intracellular second messengers in a beta-cell line and insulin release from isolated pancreatic islets. A similarly increased glucagon secretion from alpha-cells was demonstrated after we established that these cells also express GPR40. Antisense knock-down of GPR40 abolished the effect of FFA stimulation on hormone secretion from both cell types. In the final part of this work, FFAs that activate GPR40 were shown to negatively regulate its mRNA expression, indicating a mechanism of protection from detrimental effects of sustained GPR40 stimulation. FFAs mediate effects on both alpha- and beta-cells that are potentially harmful in the development of T2D and it is possible that at least part of those occur via GPR40.

Key words: GPR40, free fatty acid, insulin, glucagon, diabetes

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Äntligen!

Gert Fylking

The cover illustration displays a pancreatic β-cell stained for GPR40 expression. From paper II © Springer Science

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ABBREVIATIONS

2BrP 2-bromo palmitate
Ab Antibody
AC Adenylyl cyclase
Ac-CoA Acetyl-Coenzyme A
ADP Adenosine diphosphate
ATGL Adipose triglyceride lipase
ATP Adenosine triphosphate
$[Ca^{2+}]_i$ Intracellular Ca^{2+} concentration
C12 Lauric acid
C6 Capric acid
cAMP Cyclic adenosine monophosphate
cDNA Complementary deoxyribonucleic acid
CPT-1 Carnitine palmityol transferase 1
DAG Diacyl glycerol
DNA Deoxyribonucleic acid
eEF1α Eukaryotic transcription elongation factor 1α
ER Endoplasmatic reticulum
FFA Free fatty acid
G-6-P Glucose-6-phosphate
GAPDH Glyceraldehyde phosphate dehydrogenase
GDP Guanosine diphosphate
GH Growth hormone
GK Glucokinase
GLP-1 Glucagon-like peptide 1
GLUT Glucose transporter
GPCR G protein-coupled receptor
GSIS Glucose stimulated insulin secretion
GTP Guanosine triphosphate
HFD High fat diet
hGPR40 Human GPR40
HSL Hormone sensitive lipase
IP_3 Inositol-1,4,5-triphosphate
K_{ATP} ATP-sensitive K^+(-channel)
LA Linoleic acid
LC-CoA Long-chain acyl-Coenzyme A
LTCC L-type Ca^{2+}-channel
MAPK Mitogen activated protein kinase
mRNA Messenger ribonucliec acid
NEFA Non-esterified fatty acid
OA Oleic acid
ORF Open reading frame
PA Palmitic acid
PC Prohormone convertase
PCR Polymerase chain reaction
PhA Phosphatidic acid
PIP_2 Phosphatidylinositol-4,5-bisphosphate
PKA Protein kinase A
PKC Protein kinase C
PLC Phospholipase C
PPAR Peroxisome proliferator-activated receptor
PTX Pertussis toxin
RNA Ribonucleic acid
RNAi Ribonucleic acid interference
RRP Readily releasable pool
RT Reverse transcription
siRNA Small interfering ribonucleic acid
T2D Type 2 diabetes
TCA Tricarboxylic acid
TG Triglyceride
TM Transmembrane domain
TZD Thiazolidinedione
UCP-2 Uncoupling protein 2

LIST OF PUBLICATIONS

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

I. **A human cell surface receptor activated by free fatty acids and thiazolidinedione drugs**
Knut Kotarsky, Niclas E. Nilsson, Erik Flodgren, Christer Owman, and Björn Olde
Biochemical and Biophysical Research Communications. 2003 Feb 7;301(2):406-410

II. **Free fatty acid receptor 1 (FFA_1R/GPR40) and its involvement in fatty-acid-stimulated insulin secretion**
Albert Salehi[†], Erik Flodgren[†], Niclas E. Nilsson, Javier Jimenez-Feltström, Jun-ichi Miyazaki, Christer Owman, and Björn Olde
Cell and Tissue Research. 2005 Nov;322(2):207-215

III. **GPR40 is expressed in glucagon producing cells and affects glucagon secretion**
Erik Flodgren, Björn Olde, Sandra Meidute-Abaraviciene, Maria Sörhede Winzell, Bo Ahrén, and Albert Salehi
Biochemical and Biophysical Research Communications. 2007 Mar 2;354(1):240-245

IV. **GPR40 in mouse islets is transcriptionally down-regulated by agonistic free fatty acids**
Erik Flodgren, Bo Ahrén, and Maria Sörhede Winzell
manuscript in preparation

[†] These authors contributed equally to this work.

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INTRODUCTION

This thesis describes work with a newly discovered cellular receptor called GPR40. It is involved in the vital regulation of blood sugar, or glucose homeostasis. The following pages will give an overview of this particular type of receptor and some key concepts of glucose homeostasis.

G protein-coupled receptors (GPCRs)

Every living cell needs to be able to interact with its surroundings, whether it is part of a multicellular organism or constitutes the organism itself. A single-cellular organism, *e.g.* a bacterium, needs to be able to sense the presence of essential nutrients or toxic substances and, if motile, initiate movement accordingly. Many cells in multi-cellular organisms like animals and plants also have the ability to detect external stimuli, like light, taste, and temperature sensations, but to benefit from the specialization possible in a multi-cellular organization, they also need to be able communicate this information to each other in order to act in concert. Regardless of the source of the stimulus, these signals are detected by the use of receptors. They help relay the information from the outside, so the cell can take appropriate action.

There is a seemingly infinite number of receptors and they can be classified in many different ways depending on factors like function, molecular structure or the nature of the activating agent, the receptor ligand (which can, as a matter of fact, also be an inactivating agent). A complete overview of all known receptors is beyond the scope of this work that will instead focus on one member of a certain class of receptors, the G protein-coupled receptors or GPCRs. Anchored in the cell membrane, GPCRs, like many other types of receptors, enable their ligands to cause effects inside cells without actually having to cross the cell membrane into the interior of the cell. With several hundred members, the GPCR superfamily is the largest class of cell surface receptors [1] and one of the largest protein families in the human genome [2]. In fact, estimates have reported GPCRs to make up as

much as 2% of the total human genome [3]. As a consequence of their widespread use in the body, the activation of different GPCRs ultimately leads to end results as diverse as *e.g.* muscular contraction, sensation of smell, or the activation of immune cells. Another fact pointing to the importance of GPCRs is that they have been conserved throughout evolution. GPCRs are found in early organisms such as yeast and slime molds, in plants, in the fruit fly, as well as in vertebrates [4].

Perhaps even more astonishing is the versatility of this class of molecules, with regards to its ligands. The first, and so far only GPCR to be crystallized (enabling deduction of its molecular structure) was the bovine rhodopsin receptor [5]. It is expressed in the retina and is activated by photons, which trigger the perception of light via a complex network of signals. At the other extreme of the ligand size spectrum are complex macro-molecules such as hormones and chemokines.

Being involved in such a variety of physiological phenomena, GPCRs are also of great interest to the pharmaceutical industry. It has been estimated that with worldwide sales netting over $50 billion annually, over 50% of all drug targets involve GPCRs [6] – directly or indirectly – and address cardiovascular, respiratory, neurological, and gastrointestinal conditions as well as allergies, tumors, and other debilitating illnesses [7]. With many GPCRs still orphans, it is conceivable that the therapeutic importance of this receptor class will continue to grow even larger as new (and old) receptors are associated with new aspects of physiology.

Molecular structure

A striking feature of GPCRs is their molecular structure. Their amino acid sequences all have seven stretches of around 25 amino acids with hydrophobic properties separated by segments of a more hydrophilic nature (Figure 1) [7]. The cell membrane, separating the interior of the cell from the outside, is a hydrophobic lipid bilayer with an aqueous milieu on either side, so GPCR proteins will insert themselves into the membrane with the hydrophobic domains forming seven transmembrane alpha-helices connected by hydrophilic extra- and intracellular loop domains (Figure 2). For this reason, GPCRs are often referred to as seven

```
              TM1
  1  atggacctgccccgcagctctccttcggcctctatgtggccgcctttgcgctgggcttcccgctcaacgtcctg
  1  M  D  L  P  P  Q  L  S  F  G  L  Y  V  A  A  F  A  L  G  F  P  L  N  V  L

                                                       TM2
 76  gccatccgaggcgcgacggcccacgcccggctccgtctcaccctagcctggtctacgccctgaacctgggctgc
 26  A  I  R  G  A  T  A  H  A  R  L  R  L  T  P  S  L  V  Y  A  L  N  L  G  C

151  tccgacctgctgctgacagtctctctgcccctgaaggcggtggaggcgctagcctccggggcctggcctctgccg
 51  S  D  L  L  L  T  V  S  L  P  L  K  A  V  E  A  L  A  S  G  A  W  P  L  P

              TM3
226  gcctcgctgtgccccgtcttcgcggtggcccacttcttcccactctatgccggcgggggcttcctggccgccctg
 76  A  S  L  C  P  V  F  A  V  A  H  F  F  P  L  Y  A  G  G  G  F  L  A  A  L

                                                                      TM4
301  agtgcaggccgctacctgggagcagccttccccttgggctaccaagccttccggaggccgtgctattcctggggg
101  S  A  G  R  Y  L  G  A  A  F  P  L  G  Y  Q  A  F  R  R  P  C  Y  S  W  G

376  gtgtgcgcggccatctgggccctcgtcctgtgtcacctgggtctggtctttgggttggaggctccaggaggctgg
126  V  C  A  A  I  W  A  L  V  L  C  H  L  G  L  V  F  G  L  E  A  P  G  G  W

451  ctggaccacagcaacacctccctgggcatcaacacaccggtcaacggctctccggtctgcctggaggcctgggac
151  L  D  H  S  N  T  S  L  G  I  N  T  P  V  N  G  S  P  V  C  L  E  A  W  D

                                        TM5
526  ccggcctctgccggcccggcccgcttcagcctctctctcctgctctttttctgcccttggccatcacagccttc
176  P  A  S  A  G  P  A  R  F  S  L  S  L  L  L  F  F  L  P  L  A  I  T  A  F

                                                                         TM6
601  tgctacgtgggctgcctccgggcactggcccgctccggcctgacgcacaggcggaagctgcgggccgcctgggtg
201  C  Y  V  G  C  L  R  A  L  A  R  S  G  L  T  H  R  R  K  L  R  A  A  W  V

676  gccggcggggccctcctcacgctgctgctctgcgtaggaccctacaacgcctccaacgtggccagcttcctgtac
226  A  G  G  A  L  L  T  L  L  L  C  V  G  P  Y  N  A  S  N  V  A  S  F  L  Y

                                 TM7
751  cccaatctaggaggctcctggcggaagctggggctcatcacgggtgcctggagtgtggtgcttaatccgctggtg
251  P  N  L  G  G  S  W  R  K  L  G  L  I  T  G  A  W  S  V  V  L  N  P  L  V

826  accggttacttgggaaggggtcctggcctgaagacagtgtgtgcggcaagaacgcaagggggcaagtcccagaagtaa
276  T  G  Y  L  G  R  G  P  G  L  K  T  V  C  A  A  R  T  Q  G  G  K  S  Q  K  -
```

Figure 1. DNA (lower case) and amino acid (UPPER CASE) sequences of human GPR40 (GenBank accession no. AF024687). TM1-7 denotes transmembrane domains 1-7 as predicted by TMHMM 2.0 (http://www.cbs.dtu.dk/services/TMHMM/), highlighted in grey.

transmembrane domain, or 7TM, receptors. This structural feature was exploited in the first paper of this work and has been used to postulate the number of GPCRs encoded in the human genome. By finding stretches of DNA fitting the template of seven potential alpha helices interspersed by hydrophilic domains in genome sequences, the number of GPCRs have been predicted and recent estimates land at over 800 [1,3]. Such hypothetical GPCRs are often referred to as “orphan GPCRs” in that they have no known ligands or functions. There are several approaches to finding the ligands to these orphans, one of which was used in our first study and will be discussed later.

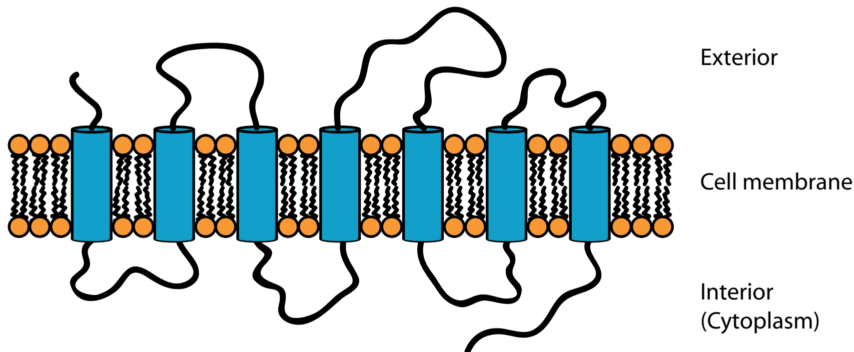

Figure 2. Schematic 2D representation of a GPCR, with hydrophilic extra- and intracellular domains and seven hydrophobic transmembrane domains (in blue). In reality a GPCR is not stretched out like this. Instead, it forms a barrel-like structure, like in Figure 3.

Signalling

The receptor protein spans the cell membrane and serves to relay information from the extracellular environment, by the binding of its ligand(s), to the inside of the cell to invoke an appropriate response. Anchored in the cell membrane, GPCRs spontaneously oscillate between active and inactive conformations. Receptor ligands will stabilize it in either an active or inactive conformation and were originally classified as either agonists or antagonists. An agonist is an agent that increases the signalling from the receptor, via the G protein and the downstream pathways, whereas an antagonist has the opposite effect. The realization that receptors are not just "on" or "off", but can in fact be stabilized in many different conformations in-between, has lead to a refinement of this classification to include more precise designations, *e.g.* partial agonists, inverse agonists, and neutral antagonists.

As the name implies, a common feature of this class of receptors is that they are coupled to G proteins. The G protein, found inside the cell, consists of three different subunits; α, β, and γ. In its resting state the G protein α-subunit is bound to a guanosine diphosphate (GDP) (① in Figure 3), but upon agonist binding (②) and receptor activation this GDP is exchanged for a guanosine triphosphate (GTP) (③

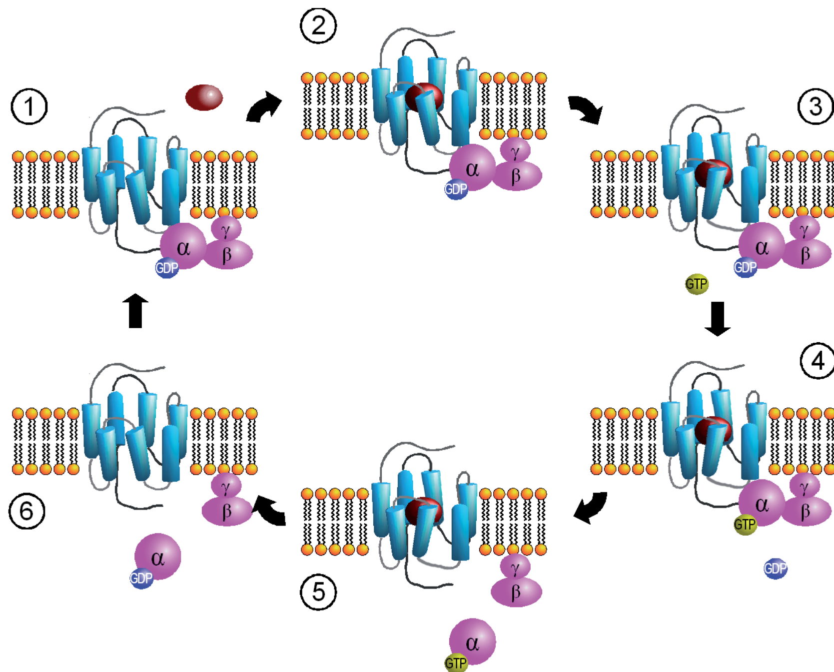

Figure 3. The cycle of activation of G proteins. The numbers ① through ⑥ are referred to in the text. From http://en.wikipedia.org/wiki/G-protein. Reprinted with permission granted from the author, Dr. Sven Jähnichen.

and ④). This forces a dissociation of the G protein into separate α- and βγ-moieties (⑤) and these two molecules will then in turn induce changes in other enzyme effector molecules, propagating the signal down different intracellular signalling pathways. Which pathway is induced depends on the classes of the α- and βγ-subunits involved. The signalling event is turned off when the intrinsic phosphatase activity of the α-subunit dephosphorylates the GTP to GDP (⑥). The trimeric G protein then reassembles and is ready for another round of activation (①) [1]. As a testimony to the importance of these mechanisms, the 1994 Nobel Prize in Physiology or Medicine was awarded to Alfred Gilman and Martin Rodbell "for their discovery of G proteins and the role of these proteins in signal transduction in cells".

The α-, β-, and γ-subunits have 16 (more if splice variants are included), 5, and 13 known isoforms, respectively [7]. The α-subunits can be divided into four categories; $G\alpha_{q/11}$, $G\alpha_s$, $G\alpha_{i/o}$, and $G\alpha_{12/13}$. $G\alpha_{q/11}$ subunits have the ability to phosphorylate and activate phospholipase C-β (PLC-β). The role of this enzyme is to catalyze the cleavage of membrane-bound phosphatidylinositol-4,5-bisphosphate (PIP_2) into the second messengers, inositol-1,4,5-triphosphate (IP_3) and diacylglycerol (DAG). IP_3 then binds to its receptors on the endoplasmatic reticulum (ER) and mediates the release of Ca^{2+}, which can lead to a number of effects depending on the cell type. DAG, on the other hand, activates protein kinase C (PKC), which in turn can phosphorylate a number of downstream effectors. $G\alpha_s$ proteins activate the adenylyl cyclase (AC) enzyme, with the ability to form cyclic adenosine monophosphate (cAMP). The production of cAMP leads to the activation of protein kinase A (PKA) and changes in gene transcription. In contrast, the $G\alpha_i$ family instead inhibits the activity of AC. The smallest of the families, the $G\alpha_{12/13}$ activate c-Jun N-terminal kinase, which phosphorylates the transcription factor c-Jun, and phospholipase D, which can produce phosphatidic acid (PhA) from the cleavage of membrane lipids [8]. The function of the Gβγ-subunit was previously unclear, but it has now been shown to bind and activate a multitude of effectors, including ion channels, phospholipases, and AC (if $G\alpha_s$ is concomitantly active) [9].

The combinatorial possibilities enable an exquisite specialization and it is also clear that a GPCR is not restricted to interacting with a single G protein, but can instead couple to different G protein heterotrimers, allowing signalling via different pathways by a single receptor. There is also evidence for G protein independent signalling (making the well-established term GPCR somewhat outdated), where the receptor couples directly to downstream effector molecules. Making the picture even more complex is the fact that two or more receptors can aggregate to form homo- or heteromers [10], and these aggregates may even recruit other membrane proteins. The signalling characteristics of a receptor can also be modulated by different intracellular modifying proteins.

Glucose homeostasis

Glucose metabolism is under strict control as its dysregulation can have dire consequences and it is imperative that glycemia is kept within a narrow range, normally in the range of 4-7 mM. When it rises (*e.g.* after a meal), mechanisms are triggered to bring it back down, and conversely, if it falls (*e.g.* during fasting or physical exercise), other regulatory actions are invoked to elevate it. Prolonged periods of elevated blood sugar can result in hypertension, retinopathy, nephropathy, atherosclerosis, and cardiovascular disease [11]. Hypoglycemia, on the other hand, can lead to neural damage and ultimately death [12]. The brain and neurons are dependent on glucose, because they cannot easily oxidize fat or amino acids for use as fuel.

The corner stones of glucose metabolism are the two hormones, insulin and glucagon. They are produced in and secreted by the β- and α-cells found in the islets of Langerhans, respectively. Making up the endocrine part of the pancreas, the about 1 million islets (in a healthy adult human pancreas) are found throughout the pancreas and account for about 2% of the organ. The β- and α-cells that will be the focus of this presentation are the two most predominant cell types in the islet, with the β-cell constituting about 60-80% of the total number, followed by the α-cell at 15-20% [13]. The α-cells are typically located in the periphery of the islet, surrounding the core of mainly β-cells. Other cells found in the islet are δ-cells, PP-cells, and ghrelin-producing cells [14]. The islets are thoroughly vascularized and the secretion of different polypeptides from the islet cells is triggered by factors in the blood, including hormones and nutrients. Furthermore, a rich innervation of the islets by both sympathetic and parasympathetic nerve fibers also regulates endocrine pancreatic secretion.

Insulin at high plasma glucose

Insulin is a protein consisting of two polypeptide chains, A and B, with interlinking disulfide bonds. It is produced in pancreatic β-cells by the cleavage of its precursor, proinsulin, to yield the two polypeptide chains, removing the interspersing

C-peptide. After this post-translational modification, the insulin molecule is stored in specialized secretory granules granules in the cell, ready to be secreted in response to elevated plasma glucose levels, *e.g.* after a meal. The β-cell senses an elevation of glucose via the membrane GLUT2-transporter. This transporter enables the facilitated diffusion of glucose down its concentration gradient and into the β-cell. GLUT2 is not easily saturated and ensures a continuous transport of glucose into the β-cell, even at high plasma concentrations of glucose. Inside the β-cell, glucose is rapidly phosphorylated to glucose-6-phosphate (G-6-P) primarily by the enzyme glucokinase (GK), suggested critical for β-cell glucose sensing almost 40 years ago [15]. There is also a second enzyme able to phosphorylate glucose, hexokinase I. The activity of hexokinase I, expressed in all mammalian tissues, is negatively regulated by G-6-P, however, and therefore plays a limited role in the release of insulin. GK has a low affinity for glucose, making it active only at plasma glucose concentrations above 5 mM [16]. This works as a safeguard to ascertain that insulin is secreted only when it is needed, namely when the blood glucose levels are high. The action of GK keeps the intracellular glucose concentration low, enabling a steady influx via GLUT2 while the plasma glucose concentration remains elevated. The G-6-P undergoes glycolysis to yield pyruvate, followed by conversion to acetyl-CoA (Ac-CoA) in the mitochondria and entry into the tricarboxylic acid (TCA) cycle. The net result of the TCA cycle and the ensuing oxidative phosphorylation is an elevation of intracellular adenosine triphosphate (ATP) at the expense of adenosine diphosphate (ADP). This leads to closure of ATP-sensitive K^+-channels (K_{ATP}-channels) in the cell membrane, which in turn leads to a depolarisation of the electrically excitable β-cell and the opening of membrane voltage-gated L-type Ca^{2+}-channels (LTCC). The Ca^{2+} influx via the LTCC leads to the increase of the intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) necessary for exocytotic release of insulin from the intracellular storage granules. This process is referred to as glucose-stimulated insulin secretion (GSIS) or the triggering pathway of insulin secretion [17].

Modulating the triggering pathway, there is also an amplifying pathway, the mechanisms of which are less well established. It was first identified when it was found that glucose was still able to augment insulin secretion even when the

triggering pathway was invoked pharmacologically, bypassing the membrane K_{ATP}- and LTCC-channels [18,19]. While it did not itself cause an elevation of the $[Ca^{2+}]_i$, the amplifying pathway was found to be dependent on the increased $[Ca^{2+}]_i$ resulting from the triggering pathway. Such a hierarchal system again ensures that the amplifying pathway does not cause insulin release unless the plasma glucose concentration is high enough to activate the triggering pathway, another safeguard against hyperinsulinemic hypoglycemia. This is especially important in view of that the amplifying pathway in isolated human islets is active already at 1 mM glucose [17]. Besides glucose (possibly after being metabolized to produce ATP), several agents have been suggested as amplifiers, including cAMP, glutamate, and free fatty acids (FFAs). Interestingly, FFAs have pleiotropic effects on insulin secretion. In the acute setting, they augment GSIS [20-22]. However, chronic exposure of rats to elevated levels of FFAs instead leads to inhibition of GSIS [23], an effect that is seen also in isolated islets [24].

Insulin release in response to high glucose levels is biphasic, with a rapid first phase resulting in a peak of plasma insulin many times above the normal concentration just 5-10 minutes after the rise in plasma glucose concentration. The decline is similarly rapid and followed by the second phase starting after 15-20 minutes. This phase is more sustained and plasma insulin levels remain high for up to a few hours until the blood sugar is brought down to normal levels. The biphasic nature of the secretion is explained by the occurrence of two distinct pools of insulin granules. The readily releasable pool (RRP) of granules resides docked on the inner face of the plasma membrane, ready to be rapidly released in response to an elevation of $[Ca^{2+}]_i$, as elicited by the triggering pathway [25]. The RRP accounts for just a small fraction of the total insulin content and after release it is replenished from the much larger reserve pool. The insulin released from the reserve pool makes up the second phase of insulin secretion and has been reported to be dependent on the amplifying pathway [26].

Insulin stimulates uptake of glucose into the liver by different mechanisms. By increasing GK activity, insulin makes sure that glucose taken up via GLUT2, which is expressed also in hepatocytes [27] and allows efficient glucose uptake. Glucose is

then readily converted to G-6-P, preventing it from leaving the hepatocyte. Insulin also activates a number of enzymes involved in glycogenesis, the process of glycogen formation from G-6-P. Conversely, enzymes involved in the opposite process, glycogenolysis, are instead inhibited by insulin. When glucose uptake is higher than what can be stored as glycogen, hepatocytes instead convert the excess glucose to FFAs (via conversion to puryvate and Ac-CoA) for transport to and deposition in adipose tissue. Between meals, when the blood glucose and insulin concentrations drop, glycogenolysis is activated, converting the stored glycogen back to glucose for release into the blood. In this fashion, the liver acts as a glucose buffer to decrease the fluctuations of blood sugar between meals. Its importance is made clear in patients with severe liver disease, who often exhibit a perturbed glucose homeostasis [28].

After release into the blood stream, insulin causes rapid uptake and metabolism of glucose in most tissues of the body, but besides the liver, the most important insulin sensitive tissues are skeletal muscle and adipose tissue. In these tissues, insulin signalling leads to the translocation of GLUT4 transporters from intracellular stores to the plasma membrane. Unlike GLUT2, which is constitutively expressed underlining its importance in glucose sensing, GLUT4 is dependent on insulin for its cell surface exposure. In skeletal muscle, glucose is taken up after insulin stimulation and again stored as glycgogen. This glycogen is later used for energy consumption by the muscle itself, as myocytes lack the ability to release glucose into the blood. In adipose tissue, some of the glucose taken up in response to insulin is converted to FFAs, but the majority is instead converted to glycerol. This glycerol forms the backbone of the triglycerides (TGs) resulting from combination of glycerol with the FFAs primarily synthesized in the liver. The adipose tissue stores the triglycerides to be used as fuel when the plasma glucose concentration is low. When this happens, the TGs are broken down by the enzymes adipose triglyceride lipase (ATGL) [29] and hormone-sensitive lipase (HSL) (which is inactivated by insulin and therefore only active when the plasma glucose concentration is low) [30] and the FFAs are again released into the blood stream to be transported to other tissues for oxidation to produce energy for cellular processes.

Glucagon at low plasma glucose

Glucagon is a polypeptide processed from proglucagon. Proglucagon is produced in a number of tissues and tissue-specific proteolytic cleavage yields different hormones involved in glucose homeostasis. In islet α-cells proglucagon is converted to glucagon, but intestinal L-cells instead produce the incretin, glucagon-like peptid 1 (GLP-1) [31]. The glucagon receptor is a GPCR expressed in multiple tissues [32] and its natural ligand, glucagon, is the counter regulatory hormone of insulin and in many aspects they are thus each other's opposite. Glucagon is secreted when blood glucose levels are low, *e.g.* between meals or during exercise, and its primary function is to promote hepatic glucose output. The molecular mechanisms behind the release of glucagon are much less well studied and understood than those involved in insulin release, but a number of ion channels appear to be involved in addition to the K_{ATP}- and L-type Ca^{2+}-channels proven crucial in β-cells [33]. While insulin promotes glycogenesis in favor of taking up and storing an abundance of blood glucose, glucagon inhibits this and instead promotes glycogenolysis to produce glucose from the stored hepatic glycogen to improve glycemia. When liver glycogen stores are starting to become depleted, glucagon also has the ability to enhance gluconeogenesis in the liver to further increase the availability of glucose by conversion of amino acids or the glycerol backbone of TGs.

Insulin is the only hormone with the direct effect of lowering plasma glucose, but glucagon is only one of several hormones behind the opposite effect. Adrenaline, corisol, and growth hormone (GH) also promote increased glycemia. Adrenaline is released in response to acute severe hypoglycemia to increase hepatic glucose output, whereas cortisol and GH are released over longer periods of hypoglycemia and promote fat oxidation over glucose utilization [34].

Diabetes mellitus

There are two main types of diabetes mellitus; type 1 and type 2. Type 1 diabetes is an autoimmune disease leading to the destruction of the pancreatic β-cells. Despite an enormous amount of effort, the cause of type 2 diabetes has not been

established, but it is associated with a declined release and action of insulin. In the healthy individual, there is a balance between the amount of insulin secreted from the β-cells and the amount needed for the peripheral tissues to keep a normal blood glucose concentration. If the tissues become less sensitive to the action of insulin, then the β-cells have to adapt by increasing the amount of secreted insulin to maintain normoglycemia. By increasing the number of β-cells or their secretory capacity, the pancreas can often handle this increased need for insulin, resulting in hyperinsulinemia. However, if the insulin resistance progresses further, the β-cells may become incapable of compensatory insulin hypersecretion and this may eventually cause β-cell exhaustion and ultimately β-cell death in susceptible individuals [35]. There are many, possibly additive, explanations to insulin resistance and β-cell dysfunction, two of which are lipotoxicity and glucotoxicity, as discussed below. People with type 2 diabetes often display a perturbed first phase of insulin secretion.

A prominent risk factor for developing type 2 diabetes (referred to from here on as T2D) is abdominal obesity, more so than overall obesity [36]. The tight link between the two conditions even sparked the term 'diabesity'. Along with a steadily increasing prevalence of obesity, the recent years have seen a dramatic increase in the number of cases of T2D worldwide and this development is projected to continue. Data from the International Diabetes Federation estimate that there are 246 million adult diabetics worldwide, and project that this will increase to 380 million by 2025 [37]. This corresponds to a rise in prevalence from 5.9% to 7.1%. The limited increase in the number of type 1 diabetics is, by far, over shadowed by the increase in T2D [38]. A common explanation for the rising numbers is a high caloric intake combined with an increasingly sedentary lifestyle [38]. However, not everybody who is obese develops T2D. Other proposed contributors include a genetic susceptibility and early nutritional factors [39] (even as early as in the uterus [40]).

Lipotoxicity

Obesity is often a consequence of caloric intake in excess of energy expenditure. Normally, surplus energy is stored as glycogen, primarily in the liver and in skeletal muscle, and as TGs in the adipose tissue. When these depots cannot store any more, however, and there are still circulating carbohydrates and fat available, TGs are ectopically stored in non-adipose tissues, interfering with normal tissue function in what is referred to as lipotoxicity.

In muscle biopsies, elevated plasma FFAs have been shown to interfere with insulin action by disrupting downstream signalling events of the insulin receptor [41], leading to impaired translocation of GLUT4 to the cell membrane and a decreased ability to take up glucose. This results in insulin resistance and is one of the strongest predictors of diabetes [42].

β-cells are also affected by chronically elevated levels of FFAs. Several effects have been reported, including a down-regulation of insulin synthesis [23,24,43], lower GLUT2 translocation [44], dysfunctional glucose metabolism [24,45,46], and increased apoptosis [47,48]. Almost all reports also include a significantly blunted GSIS.

Supporting lipotoxic effects on the liver, it has been shown that elevated FFAs can stimulate both gluconeogenesis [49] and glycogenolysis [50] and that the catalytic subunit of glucose-6-phosphatase, the enzyme with the opposite effect of GK and catalyst of the rate limiting step of glucose output from the liver [51], is positively regulated by FFAs [52]. Both of these processes are inhibited by the action of insulin, but just like in the myocytes FFAs interfere with the signalling downstream of the hepatic insulin receptor [53].

Leptin is a key hormone in lipid metabolism. Secreted by adipocytes, it signals to the brain that TG supplies are plentiful. In animal models, leptin administration results in a lowered food intake. Leptin also controls lipid partitioning, ensuring that lipids are stored as TGs in adipose tissue and keeping non-adipose tissue TGs to

a minimum, enough to cover housekeeping needs, like the production of structural lipids. In non-adipocytes, leptin causes up-regulation of enzymes promoting β-oxidation and dissipation of the resulting energy in the form of heat [54]. Kept on a high-fat diet leading to obesity, mice were shown to develop peripheral resistance to leptin [55]. The obese mice displayed significantly higher levels of leptin than their lean controls, mirroring the situation in humans [56]. Several mouse and rat models of obesity stem from disruption of the leptin gene or its receptor [57], making the animals hyperphagic. Disrupted leptin signalling in obese individuals is a potential contributor to dyslipidemia and lipotoxicity.

Glucotoxicity

Under normal conditions, glycemia is kept under strict control. However, if the primary glucose lowering tissues, as outlined above, become increasingly insulin resistant, the β-cells may or may not be able to compensate by hypersecretion. If they do not, the result is chronic hyperglyemia, which has adverse effects on the β-cell, called glucotoxicity. Effects of prolonged hyperglycemia include diminished transcription of genes, *e.g.* insulin [58], by a diminished binding of important β-cell transcription factors [59]. Chronic elevated glucose levels have also been shown to induce oxidative stress eventually ending up in β-cell death by apoptosis [60]. Sustained influx of Ca^{2+} due to prolonged hyperglycemia may also lead to apoptosis via Ca^{2+}-dependent endonucleases [61]. Experimental data support that hyperlipidemia may not be detrimental to glucose metabolism *per se*, as long as it is not accompanied by hyperglycemia [62], a potential explanation as to why not all obese persons develop diabetes.

GPR40 AND THE EFFECTS OF FFAs

Modulatory effects of FFAs on insulin secretion have been recognized for a long time. The presence of acutely elevated levels of FFAs was shown to augment insulin release both *in vivo* in dogs [63,64] and in man [65] as well as from isolated rat pancreas *in vitro* [66]. Not only do they potentiate GSIS secretion, FFAs are a requirement, as evidenced by the fact that rat islets devoid of lipids due to induced hyperleptinemia failed to secrete insulin in response to glucose perfusion. The unresponsiveness was corrected by co-perfusion with FFAs [67]. A similar effect was seen in fasted rats [68] and humans [69], where a pharmacological depletion of the elevated plasma FFAs following prolonged fasting by nicotinic acid rendered the individuals unresponsive to glucose stimulation. Again, the addition of exogenous FFAs completely normalized the deficiency.

The malonyl-CoA hypothesis

How FFAs acutely enhance the glucose induced secretion of insulin has not been satisfactorily established. One hypothesis is that an intracellular build-up of long-chain acyl-Coenzyme A (LC-CoA) in the cell cytoplasm is the reason [70], and that the LC-CoA is either converted to complex lipids, *e.g.* TG, PhA, and DAG, that in turn stimulate insulin secretion, modulate enzyme activity [71], or interact directly with the secretory mechanisms [72]. FFAs are a primary fuel for the pancreatic islet [73] and when taken up by β-cells, they are converted to LC-CoA and transported into the mitochondria via carnitine palmitoyl transferase 1 (CPT-1) for β-oxidation to Ac-CoA which then enters the TCA cycle leading to the production of ATP. However, at elevated plasma glucose concentrations, glucose is taken up and metabolized by the triggering pathway, as already described. One of the intermediates in the mitochondrial TCA cycle is citrate. Citrate can leave the mitochondria and, in the cytoplasm, be converted to malonly-CoA, in a two-step process via Ac-CoA. Malonly-CoA has been shown to inhibit CPT-1 activity, preventing the mitochondrial uptake (and oxidation) of LC-CoA, leading to increased cytoplasmic levels of LC-CoA. In effect, malonyl-CoA works as a "signal

of plenty", switching cellular energy production from using fat to using glucose, when glucose is in good supply.

There is plenty of experimental evidence for different steps of the "malonyl-CoA hypothesis" and the importance of partitioning fat away from oxidation into esterification, but other observations oppose it. Supporting the hypothesis, the inhibitor of CTP-1, 2-bromo palmitate (2BrP), has been explained to boost GSIS in HIT-cells by blocking CPT-1 more effectively than the build-up of malonyl-CoA. This would make more LC-CoA available in the cytoplasm to boost GSIS. [74,75]. Yet, others report 2BrP not to be augmenting, but instead attenuating GSIS, even after addition of exogenous FFAs [22,76,77]. It was also shown that palmityol-CoA did not mimick the effects of palmitate on exocytosis and Ca^{2+} currents when applied intracellularly in islet β-cells [78].

The initial characterization of GPR40

In a project screening orphan GPCRs with homologies to receptors for leukotriene B_4 (a derivative of the cell membrane lipid, arachidonic acid) previously identified in our lab, a HFF11 reporter cell line expressing the human GPR40 receptor (hGPR40) gave a robust and reproducible signal when challenged with linoleic acid (LA) ([79], **paper I**). Interestingly, LA was found not to be the only ligand for hGPR40. Instead an array of FFAs was found to activate the receptor, ranging in chain length from 10 to 18 carbon atoms and comprising saturated as well as both mono- and polyunsaturated FFAs, including the two other predominant FFAs in human serum [80], palmitic acid (PA) and oleic acid (OA). Such promiscuity is uncommon for GPCRs, but could be seen as an indication of the relevance of the receptor. The wide range of ligands was corroborated in studies from two other labs [81,82], and although the extremes of the FFA ligand spectrum showed some discrepancies between the different reports, as did the relative orders of potency between the individual FFAs, GPR40 was conformly identified as a receptor for medium- to long-chain FFAs with EC_{50} values in the micromolar range.

An important step in the de-orphanization of a GPCR is establishing its pattern of expression, as this may give further hints to the functional relevance. We found the strongest expression of GPR40 in pancreatic β-cells, represented by the mouse insulinoma cell line, MIN6 [83]. Other tissues showing expression of hGPR40 were the liver, heart, and in skeletal muscle. β-cellular expression was the unifying find in all three studies, while Briscoe *et al.* reported a high expression also in the brain [81]. Previous reports of FFA augmentation of GSIS by mechanisms not fully understood made the association with β-cells particularly intriguing.

All the three groups agreed that activation of the receptor leads to an elevation of $[Ca^{2+}]_i$. This is the typical response from stimulation of GPCRs coupling to the $G\alpha_q$-class of G proteins. In view of a tentative involvement of GPR40 in β-cell insulin release, the ability to cause $[Ca^{2+}]_i$ elevation seems logical. Using pertussis toxin (PTX), we found that hGPR40 also appears to couple partially to G proteins of the $G\alpha_{i/o}$-class in HeLa cells. PTX causes ADP-ribosylation of $G\alpha_{i/o}$ α-sununits, uncoupling the receptor from its effectors [84]. Itoh *et al.* also discovered a PTX-sensitive $G\alpha_{i/o}$-coupling for hGPR40, but not the mouse homolog, expressed in CHO cells [82]. In contrast, the report from Briscoe *et al.*, hGPR40-insensitivity to PTX lead to the conclusion that $G\alpha_{i/o}$ is not involved.

Itoh *et al.* also demonstrated GPR40-mediated augmentation of GSIS in MIN6 cells using several of the different FFA agonists. The specificity was tested by RNAi-mediated receptor knockdown and clearly showed that this process is highly dependent on GPR40. Furthermore, they found that GPR40 activation leads to mitogen activated protein kinase (MAPK) activation. As expected, this had no influence on insulin secretion. Instead MAPK pathways have been implicated in the regulation of cell proliferation and differentiation [85].

An interesting observation we did was that not only FFAs activated GPR40 in HFF11 cells. Another ligand is MEDICA16, an experimental hypolipidemic drug acting on lipid metabolism in the liver of rats. More conspicuously, GPR40 was also activated by the thiazolidinedione (TZD) type drugs, rosiglitazone and MCC-555 (later named netoglitazone). TZDs are well-known as ligands for the

peroxisomal proliferator-activator receptor γ (PPARγ) expressed in adipose tissue and, to a lesser degree in skeletal muscle. Several TZD compounds are or have been used clinically to increase insulin sensitivity in muscle and adipose tissue and to decrease hepatic gluconeogenesis. Located in the cell nucleus and not in the plasma membrane, the PPARs function as transcription factors that upon binding of a ligand mediate changes in gene transcription by binding to certain regions of DNA after dimerizing with the nuclear retinoid-X-receptors. FFAs and other lipid molecules are natural ligands for the different PPARs [86] and genes reported to be regulated by TZDs include genes involved in lipid metabolism, such as fatty acid CoA synthase, HSL, and glycerol phosphate dehydrogenase [87]. However, many studies have also demonstrated PPAR agonists to have non-genomic effects, like signalling through MAPK pathways [88], and affecting mitochondrial metabolism [89]. Primarily used in the treatment of T2D, some TZDs also display anti-inflammatory [90] or anti-tumorigenic [91,92] effects. Rosiglitazone has also been shown to have a negative impact on osteoblasts, via the PPARγ2 isoform [93]. While all three different subtypes (α, γ, and δ) of the PPARs are activated by long chain FFAs, only the synthetic agonists of PPARγ (*e.g.* TZDs), but not PPARα (*e.g.* clofibrate), act as GPR40 ligands. The activation of GPR40 by TZDs was later repeated by others [94,95].

Bridging cell lines and islets

With the discovery of GPR40 as a receptor for FFAs and the established effects of FFAs in the augmentation of GSIS, we wanted to study the importance of GPR40 for this process more closely. As described earlier, a key step of the malonyl-CoA hypothesis is the cytosolic build-up of LC-CoA following blocking of CPT-1 by malonyl-CoA [75]. Given both the uncertainty about the effect of 2BrP and the close structural resemblance to the GRP40 agonist, palmitate, we hypothesized that 2BrP may also have an effect on signalling through GPR40.

The coupling of GPR40 to $G\alpha_q$ was confirmed in a series of experiments measuring IP_3, a product of PLC-β-mediated hydrolysis of PIP_2 ([96], **paper II**). Both in human embryonic kidney (HEK293) cells transiently transfected with

GPR40 and in endogenously expressing MIN6 cells, the addition of LA caused a marked increase in the generation of IP_3, and in both systems 2BrP proved to be a potent antagonist of this effect. Interestingly, in the recombinant HEK293 system, 2BrP alone showed a weak agonism, whereas in the MIN6 cells 2BrP instead appeared to be an inverse agonist, resulting in a lowering of the basal level of IP_3-production. A possible explanation for this phenomenon may be a difference in receptor densities between the two systems and the reported effects of altering the stoichiometry between a receptor and its downstream signalling mechanisms on receptor pharmacology [97]. A difference in receptor density is also supported by the difference in magnitude of the response to LA stimulation.

Moving from clonal cells to isolated islets of Langerhans, we demonstrated the same effect of 2BrP effectively antagonizing stimulation with LA, as measured by a diminished LA-augmention of GSIS. This indicated that the previously demonstrated coupling between GPR40 signalling and insulin release is not limited to the MIN6 cell line [82], but is also operational in islets. The specificity of the islet β-cell response to LA was examined using antisense knockdown of GPR40 expression. Applying morpholino oligonucleotides to block the translation of GPR40 mRNA in islets, we were able to abolish the surface expression of GPR40 as well as the effect of LA stimulation of GSIS. Furthermore, this confirmed that the GPR40 knockdown previously demonstrated by RNAi in the clonal MIN6 cells is also achievable in intact islets for the study of GPR40-mediated effects in this primary tissue.

Modulation of GPR40 expression in vivo

Important *in vivo* observations about the effects of GPR40 stimulation were provided by Steneberg *et al.* in a study with genetically modified mice [98]. Apart from slightly lowered fasted serum insulin levels, GPR40 knockout ($GPR40^{-/-}$) mice showed much the same characteristics as their wildtype ($GPR40^{+/+}$) controls; they were viable and fertile, with a normal weight gain and glucose tolerance. As expected from the *in vitro* results in cell lines and isolated islets [82,96], however, islets isolated from $GPR40^{-/-}$ mice did not exhibit an augmentation of GSIS (neither

at low nor at high glucose concentrations) after acute FFA exposure. Moreover, the attenuation of FFA-mediated increase of GSIS previously reported after chronic FFA stimulation [24] and also seen in the control GPR40$^{+/+}$ islets, did not manifest itself in the GPR40$^{-/-}$ islets incubated with PA for 48 hours. This suggests GPR40 involvement in both acute and chronic effects of FFAs on insulin release.

The effects of chronic stimulation of GPR40 were then studied further in two different experimental systems. In contrast to GPR40$^{+/+}$ controls, the GPR40$^{-/-}$ mice were protected from the deleterious effects of a high fat diet (HFD), including hyperglycemia, hyperlipidemia, glucose intolerance, hepatic steatosis, and hypertriglyceridemia, despite a similar weight gain. No dramatic changes in gene expression were seen when comparing the two mouse strains on HFD or normal diet, except the lack of HFD-induced up-regulation of PPARγ expression in the GPR40$^{-/-}$ animals. Conversely, mice with a β-cell specific transgenic overexpression of GPR40, mimicking sustained receptor activation, spontaneously developed glucose intolerance and a perturbed GSIS. They also displayed a disrupted islet organization, with infiltrating α-cells, similar to what has been seen in other diabetic models [99]. PPARα mRNA expression was significantly up-regulated is islets, as were the PPARα target genes, CPT-1 and uncoupling protein 2 (UCP-2), both previously shown to be up-regulated after chronic FFA elevation [100,101] and implicated in the development of T2D [102,103]. Both GLUT-2 and prohormone convertase 1/3 (PC1/3; an enzyme involved in converting proinsulin to insulin) were down-regulated on a protein level, indicative of perturbed glucose sensing and insulin processing. Lastly, overexpression of GPR40 did not lead to increased caspase activity, further supporting loss of β-cell function, not β-cell mass, as the plausible explanation for the diabetic phenotype.

Genetic variation in the *gpr40* gene in humans has also been investigated, but the results are conflicting. In healthy Japanese men, the Arg211His polymorphism was linked with changes in serum insulin levels, insulin resistance, and β-cell function [104]. On the contrary, there was no link between this polymorphism (or an Asp175Asn mutation) and insulin release or T2D in Danish Caucasians [105].

Dissection of GPR40 signalling

Several studies have suggested that GPR40 activation by FFAs leads to signalling via coupling to $G\alpha_q$, mainly by measuring elevations of $[Ca^{2+}]_i$. Under some circumstances such an elevation may be a downstream effect of $G\alpha_{i/o}$-signalling as well, as some G protein βγ-subunits have been shown to activate certain PLC isozymes, although much less effectively than G_q α-subunits [106]. As discussed earlier, this pathway is sensitive to PTX inhibition. Further evidence for the $G\alpha_q$ coupling was provided by two reports demonstrating similarly decreased $[Ca^{2+}]_i$ mobilization after pharmacological interference at different stages of the $G\alpha_q$ signalling pathway, both in clonal [107] and in primary [108] rat β-cells. Only one of the studies explicitly examined (and ruled out) $G\alpha_{i/o}$-involvement [107]. Interestingly, both reports suggested that uptake of extracellular Ca^{2+} through LTCC is a requirement for GPR40 signalling, supporting an observation that the stimulatory effect of PA involves LTCC [78]. Shapiro *et al.* also showed that keeping the K_{ATP}-channels open with diazoxide prevents GPR40 from raising $[Ca^{2+}]_i$ [107]. This is in accordance with the previously reported requirement of stimulatory glucose concentrations for FFAs to affect insulin secretion [22]. By the use of RNAi, both groups confirmed that GPR40 is critical for the insulinotropic response to PA and OA.

Repolarization of the β-cell membrane after insulin release has been shown to occur via a voltage-gated outward K^+ current [109]. Feng *et al.* demonstrated that LA stimulation of β-cells resulted in a delay of the repolarization process by reducing the K^+ current [110]. This effect was established to be GPR40 dependent by siRNA mediated receptor knockdown and it was also shown to operate via activation of the cAMP-PKA pathway. Activation of this pathway is attributed to signalling via the $G\alpha_s$-class of G proteins [1,8], resulting in activation of AC (the opposite effect of $G\alpha_{i/o}$-signalling). A prolonged membrane depolarization would lead an increased influx of Ca^{2+} ions through LTCC and an augmentation of GSIS, providing yet another potential mechanism linking FFAs and insulin release via GPR40.

GPR40 and glucagon secretion

While the involvement of FFAs in insulin secretion is well established, relatively little is known about their effects on the secretion of glucagon. Early findings stated that FFAs lowered glucagon secretion [111], but there are several more recent observations, done on isolated mouse [112,113] and rat [114] islets, pointing to FFAs instead having a glucagonotropic effect. It is also a paradoxical fact that many people suffering from T2D, a condition associated with elevated levels of plasma FFAs [115], are hyperglucagonemic despite a prevailing hyperglycemia. We therefore decided to investigate the possibility of GPR40 involvement in glucagon secretion. When studied using confocal microscopy after staining for GPR40 and glucagon, isolated mouse islets showed indications of GPR40 expression in α-cells and we also found that clonal In-R1-G9 glucagonoma cells expressed GPR40 mRNA ([116], **paper III**). Previous attempts at identifying GPR40 expression in α-cells have been unsuccessful, possibly due to experimental factors such as sample preparation or origin. Stimulation of the glucagonoma cells with LA displayed striking parallels with previous results in β-cells, such as a dose-dependent increase in PI-hydrolysis and hormone exocytosis. Increased sensitivity to LA stimulation was conferred by transient overexpression of GPR40, strengthening the implication of GPR40 in α-cell exocytosis. Glucagon secretion from isolated mouse islets was also stimulated by LA challenge, at both low and intermediate glucose concentrations, while a morpholino-induced knockdown of GPR40 expression resulted in a loss of the ability of LA to stimulate glucagon exocytosis.

It may seem counterintuitive that the same stimulus would stimulate the secretion of counterregulatory hormones, but unlike their effect on glucagon secretion, the augmentation of insulin secretion by FFAs requires stimulatory glucose concentrations [22]. Furthermore, secreted insulin inhibits the secretion of glucagon in a paracrine fashion, preventing it from further elevating the plasma glucose concentration. In the pancreas, this is facilitated by islet microcirculation. The capillaries enter into the β-cell rich core of the islet where secreted insulin will counteract the exocytosis of glucagon from the α-cells later encountered in the islet mantle zone, before emptying into venules and leaving the pancreas [117]. In T2D,

however, hypoinsulinemia due to β-cell dysfunction relinquishes the attenuation of glucagon secretion and elevated plasma FFAs can instead exert a glucagonotropic action. This, together with an increased hepatic glucose output due to loss of insulin tonus and a lowered glucose clearance by skeletal muscle due to lipotoxicity-induced insulin resistance, contributes to diabetic hyperglycemia.

Also, it should be noted that in isolated islets the directionality of intra-islet blood flow is by-passed, allowing secretion of glucagon even without concomitant hypoinsulinemia, as in **paper III**.

Transcriptional regulation of GPR40

There are many ways for cells to regulate the signalling through GPCRs. Acute regulation includes intracellular modifications of the receptor protein itself and changes in receptor number, via *e.g.* internalization or recruitment from intracellular stores. Changes over longer time-scales often include changes in gene expression. After observing a decrease in GPR40 mRNA in the islets of mice kept on a HFD for several months, we wanted to study the effects of elevated FFA levels on the expression of GPR40 mRNA ([118], **paper IV**). Culturing isolated islets for 18h in the presence of high concentrations of FFAs, we found receptor mRNA levels to be negatively regulated by FFAs within the established chain-length range of GPR40 ligands. The six-carbon FFA, caproic acid (C6), is not within that range [79,82,119] and had no effect GPR40 mRNA expression, demonstrating that the mRNA down-regulation was not an unspecific FFA-mediated effect. PPARs, when activated by FFAs, function as transcription factors modulating gene expression and it would seem logical that the negative effect on GPR40 transcripts could be mediated by PPAR activation. However, the fact that the twelve-carbon lauric acid (C12), which activates GPR40 but not the PPARs, also resulted in down-regulation of GPR40 mRNA, speaks against this possibility. As expected, cells incubated in the presence of high concentrations of LA showed a perturbed LA-augmentation of GSIS at a permissive glucose concentration, whereas incubation with the GPR40 non-agonist, C6, had no detrimental effect on the ability of LA to increase GSIS, as compared to control.

Fasting mice overnight elicited a trend of elevated plasma FFAs, along with a decrease in GPR40 mRNA levels. Other influencing factors can not be ruled out, as both body weight and plasma glucose changed more dramatically than plasma FFA, but lowering GPR40 expression seems a physiologically adequate response in a fasting individual, so as to not have increased levels of FFAs augment insulin secretion and further the hypoglycemia.

GPR40 and proliferation

GPR40 is not only involved in secretion of hormones. Reports have shown that unsaturated long chain FFAs have a proliferative effect on breast cancer cells *in vitro*, whereas saturated long chain FFAs instead induce apoptosis. There is also epidemiological evidence of a connection between dietary fat and the risk of breast cancer. Interestingly, GPR40 mRNA was found in a breast cancer cell line and the unsaturated OA and LA, but not the saturated PA and stearic acid, were demonstrated to induce rapid $[Ca^{2+}]_i$ mobilization in a partly PTX-sensitive manner [120]. Using RNAi and transient transfections, Hardy *et al.* established that the proliferative response of different breast cancer cell lines to OA stimulation is mediated via GPR40 [121]. In a thorough examination of the signalling pathways with pharmacological inhibitors, they found the proliferative effect of OA to be dependent on a number of different effectors, many of which are known to be involved in regulation of proliferation.

Synthesis of GPR40 ligands

Efforts have also been made to produce synthetic ligands specifically targeting GPR40. In a screening of a chemical library, Garrido *et al.* found a small-molecule compound that was able to elevate $[Ca^{2+}]_i$ in transfected HEK293 cells [122]. It had a slightly higher GPR40-activating potency than fatty acids and an efficacy comparable to that of LA. By modifying the molecule they were able to study the structure-activity relationships on GPR40 and came up with a variety of agonist compounds that activated GPR40 in the low nanomolar range. The pharmacology of one of these was examined in a follow-up study, along with a small-molecule

antagonist [123]. Interestingly, the agonist also activated GPR120, another recently de-orphanized GPCR for long-chain FFAs [124], although to a much lesser extent. GPR120 is expressed in STC-1 cells, a murine entroendocrine cell line shown also to express GPR40 [125] and its activation leads to the secretion of GLP-1. The agonist dose-dependently augmented GSIS from MIN6 cells, but not from rat or mouse islets. The antagonist inhibited the response to both the synthetic agonist and to a number of GPR40-activating FFAs in a dose-dependent manner. It was without effect on the activation of GPR120, showing at least some degree of specificity. Coming from a large pharmaceutical company, it would be naïve not to think that these studies were done with drug development in mind, but they raise the interesting option of using receptor-specific ligands to pinpoint the role of GPR40 in different situations, without confounding factors like metabolic pathways running in parallel or the concomitant activation of other receptors.

GPR40 activation by endogenous ligands

One objection to the physiological relevance of GPR40 to insulin secretion has been the discrepancy between the reported EC_{50} values of receptor activation and the unbound FFA concentration in the plasma [126] that is likely to be in the low nanomolar range. Several investigators have also shown that the concentration of unbound, as opposed to albumin coupled, FFAs is of key importance to their stimulating effect [22,82,127]. However, as also pointed out [126], nothing is known about the local concentration of FFAs in the pancreatic islet, which is known to express lipases [128,129]. A very recent report demonstrated by guanidine nucleotide exchange that $G\alpha_q$ coupled to GPR40 is activated by endogenous ligands, resulting in a high basal activity and necessitating the addition of high concentrations of exogenous FFAs in order to affect signalling [95].

AIMS OF THE STUDY

* **To characterize the orphan GPCR GPR40.**

* **To study the role of GPR40 in insulin secretion from islets and the effect of 2BrP on GPR40.**

* **To examine the involvement of GPR40 in glucagon secretion.**

* **To investigate the effect of long-term elevated FFA concentrations on GPR40 transcription.**

METHODOLOGY

This section outlines the main methodologies used. More detailed descriptions can be found in papers I-IV.

Model systems

In these studies, numerous cell lines have been employed, including CHO (**paper I**), HeLa (**I** and **II**), HEK293 (**II** and **III**), In-R1-G9 (**III**), INS-1E (**I**), MIN6 (**I**), and its sub clone, MIN6c4 (**II**). The first three have been used in transfection experiments examining the effects of addition of GPR40 expression, as they are devoid of endogenous expression of GPR40. The remaining four are pancreatic hormone secreting cell lines, used because they express GPR40 endogenously and can therefore serve as model systems more closely resembling a natural situation. All clonal cells were cultured using standard cell media with supplements as indicated in the respective papers.

Unlike some tissues, isolated islets are reasonably easily cultured *ex vivo*, at least for a few days. This allows the study and manipulation of the different cell types making up the islet. In **paper II**, FFA-augmented insulin release from islets and the expression of GPR40 in β-cells were assayed after various treatments, while **paper III** illustrates the expression of GPR40 in α-cells of isolated islets and examines its role in glucagon release. Isolated mouse islets were also used in **paper IV** investigating the effect of elevated FFAs on islet cell expression of GPR40 mRNA.

The most complex model system is the *in vivo* setting. **Paper IV** involves the high fat-fed mouse, which is a model of diet-induced insulin resistance and impaired glucose tolerance. Originally introduced by Surwit *et al.* [130,131], this model involves feeding female C57BL/6J mice, a mouse strain genetically pre-disposed for the development of glucose intolerance, a diet with a high fat content. In the high-fat diet (HFD) used, 58% of the calories stem from fat, whereas the corresponding

figure for the control diet is 11%. As expected, the HFD-fed animals have a faster weight-gain and show elevated basal levels of plasma glucose and insulin already after one week on the diet, indicating an established insulin resistance [132]. They also exhibit an impaired GSIS in response to an intra-venous glucose injection. Notably, after 52 weeks on the HFD the mice still display elevated levels of circulating insulin, showing that β-cell death does not seem to be a factor. In fact, in mice fed a diet high in fat, compensatory β-cell hyperplasia was shown [133]. The HFD model is attractive, as it resembles the situation of obesity, insulin resistance, and early diabetes in humans, unlike many other animal models based on the disruption of a specific gene.

Transfections

In order to introduce new genetic material into cells, the cationic lipid tranfection systems, Lipofectamine Plus (**paper I**) and Lipofectamine 2000 (**papers II and III**) from Invitrogen, were used. In brief, the positively charged cationic lipids interact with the negatively charged DNA backbone and then with the negatively charged cell membrane in order to deliver the DNA into the cell. Inside the cell, transcription of the introduced genes takes place in the nucleus.

HFF11 reporter assay

The HeLa HFF11 reporter cells are an assay system based on the activation of transcription factors [134]. Briefly, the cells contain a construct of transcription factor motifs and a promoter driving the transcription of firefly enzyme luciferase. Activation of receptors that can be transfected into the HFF11 cells will lead to expression of luciferase, if the receptor signalling pathway(s) lead to activation of the transcription factors AP-1 and/or NF-κB. This includes the majority of GPCRs, except those coupling to $G\alpha_s$ [135]. To allow transcription of the luciferase gene, receptor stimulation takes place during 6 hours or more. After lysis of the cells, the luciferase substrate, luciferin, is added and the combination of the enzyme and the substrate gives rise to luminescence, which can be detected in a luminometer. This system was used to screen orphan receptors in **paper I**.

Aequorin assay

Originally isolated from luminescent jellyfish, aequorin is photoprotein emitting light in response to increases in $[Ca^{2+}]$. The apoprotein, apoaequorin, can be expressed in cells along with the orphan GPCR in question and the aequorin prosthetic group, coelenterazine, can be added at the time of the assay. If the activated orphan receptor leads to an elevated $[Ca^{2+}]_i$, oxidation of the coelenterazine group will lead to the emission of light, also detectable in a luminometer. Only picking up elevations in $[Ca^{2+}]_i$, the aequorin assay is more specialized than the HFF11 reporter cell assay. Furthermore, as $[Ca^{2+}]_i$ elevation occurs instantly after receptor stimulation, no extended stimulation period is needed. It was used in **paper I** to establish that GPR40 signals, at least in part, via elevations of $[Ca^{2+}]_i$, making $G\alpha_q$ a likely coupling.

Northern blotting

In **paper I**, northern blotting was performed to detect the expression of GPR40 in different tissues. The method is based on the electrophoretic separation of RNA according to size, followed by hybridization to the RNA by a sequence-specific probe after transfer of the separated RNA to a membrane. The probe used in **paper I** was the human *gpr40* open reading frame (ORF), radioactively labelled. In the following papers, gene expression was instead studied using PCR techniques (as described below), but it should be noted that in the northern blotting the probe was able to cross-react with both mouse and rat RNA. For PCR purposes, different sets of species specific primers have to be used, due to the sequence variation of *gpr40* between the species.

PI hydrolysis assay

This is a method to measure PLC-activity. As mentioned, activation of $G\alpha_q$-coupled GPCRs leads to the activation of PLC-β, which in turn hydrolyzes PIP_2 to produce IP_3 and DAG. Loading radioactive $[^3H]$myo-inositol into cells will result in the build-up of radioactive PIP_2 which on PLC-activation will yield radioactive IP_3.

In this assay, cells are loaded with [^{3}H]myo-inositol for 16-20 hours after which any unincorporated radioactivity is washed away. The cells are then subjected to the different agents to be tested for receptor activity for 30 minutes. This time span was chosen to give enough time to generate sufficient levels of IP_3, while limiting the likelihood of inducing major effects on gene regulation (long-chain FFAs are, as mentioned, ligands for the gene regulatory PPARs). After 30 minutes, the cells are lysed and the IP_3 molecules (radioactive as well as non-radioactive) are collected using ion-exchange chromatography. The radioactivity is then counted in a scintillator. To facilitate comparison between different experiments, the results from different ligands are normalized against the basal IP_3 production of untreated control cells and expressed as a multiple thereof. This assay was used in **papers II** and **III** to measure the activation of endogenous or exogenous GPR40 in cells in response to different ligands.

Preparation and incubation of islets

Pancreatic islets were prepared from mice by retrograde infusion of ice-cold collagenase via the bile-pancreatic duct, followed by excision of the pancreas and collagenase digestion at 37°C. The islets were handpicked under a stereo microscope. Islets to be assayed for mRNA expression were immediately frozen in liquid N_2 and then stored at -80°C pending RNA isolation (**paper IV**). For hormone release experiments (**papers II** and **III**), the islets were transferred to a HEPES-buffered Krebs-Ringer bicarbonate buffer with 1 mM glucose and 1% FFA-free bovine serum albumin (BSA) and preincubated for 30 minutes at 37°C. The preincubation medium was then exchanged for the same buffer, but with different concentrations of glucose and with the addition of different test agents or vehicle controls. Hormone release was allowed 60 minutes in a shaking incubator at 37°C, after which aliquots were taken and stored at -20°C. If longer incubations were needed for islet treatments (with antisense morpholinos in **papers II** and **III** or with FFAs in **paper IV**), this was done in RPMI-1640 culture medium in 37°C incubators with the addition of the different agents or controls. Hormone release was then performed as above (**papers II** or **III**) or in a modified buffer

(formulation in **paper IV**) at different glucose concentrations with LA or vehicle for 60 minutes before the drawing and -20°C storage of samples. To generate single β-cells, the islets were shaken at a low [Ca^{2+}] [136], before plating and subjection to the antisense treatment as above (**paper II**).

Hormone concentration measurements

The amounts of secreted insulin or glucagon in the different studies were determined radioimmunochemically using commercially available kits (**papers II** and **IV**: insulin, Linco Research; **paper III**: glucagon, Euro-Diagnostica). In short, in both assays an ^{125}I-labelled hormone (the "tracer") competes with the non-radioactive hormone in the sample or standard for the binding of a hormone specific antibody (Ab). Separation of free and bound radioactivity is accomplished by centrifugation after addition of a secondary Ab. The radioactivity in the resulting pellet is inversely proportional to the amount of hormone in the sample/control and is measured in a γ-counter.

Morpholino antisense in islets

Morpholino antisense oligonucleotides ("morpholinos") were used to achieve a knockdown of GPR40 expression in intact islets. While maintaining Watson-Crick base pairing, a modified backbone makes the morpholinos more resilient to nuclease degradation than DNA- or RNA-based oligos [137]. As such, morpholinos can be used to interfere with the translation or splicing of select mRNA molecules by binding sequence-specifically to complementary stretches of the mRNA, resulting in a lowered expression of the targeted protein. There are no introns in the *gpr40* ORF, so the M40 morpholino used in **papers II** and **III** instead targets a sequence just 5' of the *gpr40* ORF. As opposed to methods that lead to mRNA degradation, *e.g.* RNAi, the effectiveness of the M40 cannot be monitored using RT-PCR, but instead necessitates measuring changes on a protein level.

Confocal microscopy and the GPR40 antibody

Using Abs it is possible to label certain structures on the surface of and inside cells. This was exploited in **papers II** and **III**, in confocal microscopy examinations of GPR40 protein expression. When the specific Ab is coupled to a fluorophore, the target structure can be detected with a high spatial resolution by excitation of the fluorophore. If many specific Abs are used in parallel, co-localization of different structures can be investigated, as was done for GPR40 and insulin in **paper II** and for GPR40 and glucagon in **paper III**.

At the time of **paper II**, there were no Abs to GPR40 commercially available, so a rabbit polyclonal Ab was produced, targeting the C-terminal peptide NH_2-CVTRTQRGTIQK-COOH of the mouse GPR40 protein (Figure 1). Unfortunately, the Ab proved not to work satisfactorily when used for Western blotting (data not shown). Instead, it was used in confocal microscopy applications in **papers II** and **III**. It seems conceivable that the Ab is specific for the three-dimensional epitope(s) produced by the peptide, rather than for the peptide sequence itself, and therefore recognizes only the native receptor and not the polypeptide linearized in a denaturing Western blot SDS-PAGE gel. The specificity of the GPR40 Ab was tested in **paper II** and visualized as the difference in staining between HeLa cells and HeLa cells stably transfected to express mouse GPR40.

RNA preparation and cDNA synthesis

To investigate the levels of GPR40 mRNA expressed under different circumstances, total-RNA was isolated using the QIAgen RNeasy Mini Kit. 1 μg of RNA was used in each reverse transcription (RT) reaction, primed by random hexamers and carried out by either Superscript II or M-MuLV reverse transcriptases. Before the RT, any contaminating DNA was degraded by DNase digestion and control reactions without added reverse trancriptase were always included.

The resulting complementary DNA (cDNA) presents a snapshot of RNA species expressed in a cell or tissue at a given time and cDNA from clonal cells or pancreatic islets was used, albeit for different purposes, in **papers II-IV**.

Real-time PCR

The polymerase chain reaction (PCR) is a sensitive method that can be used to measure the presence of a certain stretch of DNA (*e.g.* a gene) in a sample by the use of sequence-specific primers and the repeated amplification of the sequence in-between. The amplification is logarithmic and, in theory, each cycle in the reaction leads to a doubling of the number of the targeted sequence. The resulting amplification can be visualized in a number of ways. Real-time PCR is a development of the original PCR and allows monitoring of the amplification in real-time as the reaction progresses. The fluorescent dye, SYBR green, increases its fluorescence several hundred-fold upon binding to double-stranded DNA, so the synthesis of more double stranded DNA by the PCR reaction will increase fluorescence proportionally. The C_t-value of a reaction is the cycle number at which the fluorescence (as a function of the number of copies of the DNA) reaches a set threshold value and this is used to compare the amount of starting material in the different samples. To compensate for differences in template concentrations due to sample handling, the C_t-value of the gene of interest is often normalized against the C_t-values of one or more reference genes. The reference genes used in **paper IV** were glyceraldehyd phosphate dehydrogenase (GAPDH), eukaryotic transcription elongation factor 1α (eEF1α) and β-actin.

Glucose and FFA concentrations in plasma

In **paper IV** plasma concentrations of glucose and FFAs were measured using chromogenic assays. The glucose oxidation method [138] oxidizes glucose under the formation of hydrogen peroxide (H_2O_2), which in turn is used to oxidize ABTS in a peroxidase-catalyzed reaction. The resulting green chromogen can be measured photometrically. The FFA assay (the NEFA C kit) from Wako Chemicals is also dependent on the production of H_2O_2, but this is generated in a two-step process via acyl-CoA that is oxidized yielding H_2O_2. Again a peroxidase catalyzes the formation of a (purple) chromogen, allowing photometrical analysis.

SUMMARY

Paper I describes the de-orphanization of the GPCR GPR40. It is promiscuously activated by medium- to long-chain FFAs, including saturated as well as both mono- and polyunsaturated FFAs. EC_{50} values for the different FFAs on the recombinantly expressed receptor are all in the micromolar range. GPR40 is markedly expressed in pancreatic β-cells, as shown in a northern blot that also suggests expression in other major sites for energy metabolism; liver, skeletal and cardiac muscle. Unlike in the case of the β-cellular expression, the relevance of this finding has not been extensively pursued so far. GPR40 couples to the G_q-mediated signalling pathway, and partly to G_i, as indicated by HeLa HFF11 reporter cell activation, partially inhibited by PTX, and elevation of $[Ca^{2+}]_i$ in transfected HeLa and CHO cells as well the insulinoma cell lines, MIN6 and INS-1E, endogenously expressing the receptor. A number of clinically interesting substances were also shown to be GPR40 ligands, including 9-HODE, conjugated linoleic acid, MEDICA16, and antidiabetic drugs of the TZD type. Used clinically in the treatment of diabetes, TZDs have been shown to enhance peripheral insulin sensitivity (among other effects) and to be ligands for nuclear transcription factor PPARγ, which is also a receptor for long-chain FFAs.

Paper II concerns the role of GPR40 in FFA-mediated potentiation of GSIS. Stimulation with LA, shown in **paper I** to activate GPR40, leads to the production of IP_3 in HEK293 cells transfected to express GPR40 as well as in the MIN6c4 mouse insulinoma cell line. IP_3 is a signalling molecule downstream of G_q-coupled GPCR activation. The brominated FFA, 2BrP, has been suggested to influence GSIS via intracellular mechanisms, but is demonstrated to inhibit LA-induced IP_3-production in both cell systems. In isolated mouse islets, an inhibitory effect of 2BrP on the potentiation of GSIS by LA corroborates the evidence of 2BrP interfering with FFA-mediated effects through surface receptor signalling. The augmenting effect of LA on GSIS in isolated islets is dependent on LA concentration and it is also strongly inhibited by the use of antisense morpholino oligonucleotides to specifically lower the surface expression of GPR40.

Paper III presents the expression of GPR40 in α-cells of isolated mouse islets by the use of confocal microscopy. Similar to the previous results from β-cells, stimulation of the In-R1-G9 glucagonoma cell line with LA results in the elevation of intracellular IP_3, the formation of which is increased after transfection-induced overexpression of GPR40 and shown to be dependent on the concentration of LA. A dose-dependent stimulatory effect of LA on glucagon exocytosis is also seen in isolated islets, both at low and elevated glucose concentrations. The use of the same antisense intervention as in **paper II** results in a similar abrogation of the stimulatory effect of LA on hormone release.

Paper IV examines the regulation of GPR40 mRNA by elevation of fat. Islets from high-fat fed mice contain significantly less GPR40 mRNA after five months on the HFD, although no statistically significant change is seen after up to two months. Fasting mice overnight elicits a trend of elevated FFAs, but a dramatic decrease in GPR40 mRNA levels, similar to that seen in the HFD-fed animals. Overnight *in vitro* exposure of mouse islets to elevated concentrations of LA results in a dose-dependent down-regulation of GPR40 mRNA, but the six-carbon caproic acid, which is too short to activate GPR40, is without effect on receptor mRNA levels. With a chain-length of twelve carbon atoms, lauric acid is not a PPARγ ligand but activates GPR40. Its regulatory effect on GPR40 mRNA is comparable to that of LA. Reflecting the relationship of the mRNA levels, LA stimulation of GSIS after overnight incubation with LA is severely blunted, an effect not seen after incubation with caproic acid.

In conclusion, we have shown that GPR40 is a receptor for FFAs, ranging from medium- to long-chain FFAs. Its ligand spectrum also includes other types of substances, including antidiabetic drugs in clinical use. Expressed in both α- and β-cells, it is, at least in part responsible for the reported FFA-induced augmentation of glucagon and insulin. Its expression, at the mRNA level, is negatively regulated by prolonged exposure to agonistic FFAs.

PERSPECTIVES

The discovery and characterization of GPR40 by us [79] and others [81,82] has introduced a new concept to the theories of how FFAs augment GSIS from the β-cell. At the time of the discovery, the present paradigm stated that this happened via intracellular metabolic conversion. On entering the cytoplasm, FFAs are converted to LC-CoA, preventing them from leaving the cell. They are then taken up into the mitochondria via CPT-1 and oxidized to yield energy for the cell. However, after a meal, when plasma glucose levels are high, glucose enters the β-cell and is metabolized via glycolysis and the TCA cycle. Excess TCA cycle intermediates are converted to malonyl-CoA which will inhibit the activity of CPT-1. With CPT-1 inactivated by malonyl-CoA, LC-CoA will build up in the cytoplasm and instead be esterified into complex lipids like TG, PhA, and DAG, which may enhance insulin secretion [139]. The GPCR GPR40 is expressed on the surface of β-cells [81,82,96] and, when stimulated by FFAs, it activates intracellular PLC. This enzyme generates IP_3 and DAG. IP_3 leads to the elevation of $[Ca^{2+}]_i$, a well-known prerequisite for insulin vesicle exocytosis generated via the triggering pathway. There is substantial experimental evidence for both pathways, and the there is an emerging concept that they run in parallel, or maybe even in sequence. Nolan *et al.* even go one step further in their "trident model", also including a third route of influence for FFAs, a TG/FFA cycle [140]. Esterification of LC-CoA to TG and lipolysis of TG to FFAs (which may then be converted LC-CoA) are both stimulated by glucose [141,142] and inhibition of the latter has been shown to inhibit GSIS [143]. Why this happens is not yet settled, but since FFAs can freely cross the plasma membrane, an autocrine effect on GPR40 can not be excluded. Supporting such a mechanism, a recent study reported that GPR40 can in fact be activated by endogenous ligands [95]. Addition of FA-free BSA blocked the "constitutive activity" of GPR40 in a dose-dependent manner. Lipase inhibition in islets lowers insulin secretion[143,144], indicating that FFAs stemming from within the cells expressing the receptor may provide autocrine stimulation.

In contrast to their effects on β-cells, the role of FFAs in acute glucagon release from α-cells has received very little attention. Early studies indicated that FFAs negatively regulate the secretion [111,145], but it can not be excluded that this was due to inhibition of glucagon exocytosis by FFA-mediated insulin augmentation rather than a negative direct impact by the FFAs on the α-cells. However, more recent investigations in isolated islets (with the likely bypassing of the directional blood flow of islet microcirculation) instead indicate that FFAs have a positive influence on glucagon secretion [112-114]. The FFAs displaying glucagonotropic effects in these studies all fall within the chain-length spectrum of established GPR40 agonists and PA-mediated augmentation involved activation of LTCC, similar to what has been shown for GPR40 in β-cells. These observations sparked our study establishing a role for α-cellular expression of GPR40 in the regulation of FFA-mediated glucagon release. Interestingly, a report currently in press [146] confirms that OA in the micromolar range induces $[Ca^{2+}]_i$ elevations in and glucagon secretion from rat α-cells. Ca^{2+} release from the ER is shown to be critical, as is the presence of extra-cellular Ca^{2+}. Interference with K_{ATP}-channels and LTCC activity had only minor effects on $[Ca^{2+}]_i$ and on glucagon release, which is also shown to be strongly dependent on IP_3. The observation that $[Ca^{2+}]_i$ elevation takes twice as long to manifest in α-cells compared to their previous results from β-cells is interpreted by the investigators as suggesting that the effect of OA is not mediated by a cell surface receptor, but the expression of GPR40, and much less the modulation thereof, was never addressed.

Chronic stimulation of GPR40 *in vivo* is not easily studied, as its ligands, FFAs, are involved in a myriad of processes not only concerning glucose homeostasis. However, by overexpressing GPR40 in the β-cells of mice, the situation of chronic stimulation was mimicked [98]. The animals developed glucose intolerance and a severely perturbed insulin secretion with a loss of the first and a marked attenuation of the second phase of insulin secretion. The distorted GSIS was suggested to depend on the combined down-regulation of GLUT2 and up-regulation of CPT-1 and UCP-2 at the mRNA level, possibly lowering glucose uptake and increasing FFA oxidation in the β-cell. A disrupted conversion of proinsulin was also reported.

In a study aimed at examining the effect of long-term elevation of FFAs on GPR40 mRNA levels, we detected a decline in mice kept on a HFD for five months [118]. The same result was seen after fasting mice overnight. In this context, it is noteworthy that Qvigstad *et al.* showed that fasting human subjects for 58 hours resulted in a markedly elevated plasma FFA concentration and a blunted responsiveness to subsequent FFA infusion when GSIS was investigated [147]. Overnight *in vitro* culturing of islets in the presence of elevated FFA concentrations resulted in a dose-dependent down-regulation of GPR40 mRNA. Interestingly, only FFAs unambiguously shown to activate GPR40 caused a decrease in receptor mRNA concentrations. Potentially a protective measure to minimize harmful overstimulation leading to apoptosis of the β-cell [148], a lowered expression of GPR40 mRNA by FFAs does not seem to be enough to prevent the deleterious effects of elevated circulating plasma FFAs in an obese person. A complete β-cell ablation of GPR40, on the other hand, does appear to shield the individual from the harm of a HFD, as evidenced by the aforementioned study [98]. The ongoing attempts to develop small-molecule agonists and antagonists specific for GPR40 [122,123,149] will, if successful when applied *in vivo*, help elucidate the importance of this receptor for glucose homeostasis.

POPULÄRVETENSKAPLIG SAMMANFATTNING

För att kunna fånga upp signaler från omgivningen har en cell speciella mottagarmolekyler på sin yta, så kallade receptorer. I en flercellig organism, som människan, är receptorer en förutsättning för att cellerna ska kunna kommunicera med varandra och samarbeta. Receptorerna kan liknas vid lås på så vis att de bara aktiveras (låses upp) av rätt signal (nyckel). Endast rätt signal aktiverar en viss receptor som då omvandlar signalen till information för cellen. Receptorer kan delas in i olika klasser, till exempel på grundval av hur den omvandlingen sker. I en viss klass av receptorer sker den via speciella så kallade G-proteiner vilka finns inuti cellen. Denna receptorklass kallas därför G-proteinkopplade receptorer, eller GPCR (från engelska). GPCR är inblandade i ett stort antal olika funktioner i kroppen och många av de läkemedel som används idag påverkar GPCR. Många GPCR är dock okända, så till vida att man känner till hur de är uppbyggda, men inte vet hur de fungerar eller vad de har för roll i kroppen. Detta arbete beskriver karaktäriseringen av en sådan tidigare okänd GPCR kallad GPR40.

Ett sätt att dra slutsatser om en receptors funktion är att identifiera vad som aktiverar den (dess nyckel). Vi upptäckte att GPR40 aktiveras av fettsyror som är byggstenar i fettmolekyler. Det talar för att GPR40 är involverad i regleringen av fettomsättningen i kroppen. Detta styrks av att GPR40 finns på, eller ”uttrycks i”, många typer av celler som är inblandade i att upprätthålla energibalansen i kroppen. Bland annat uttrycks GPR40 i de celler i bukspottkörteln som producerar och utsöndrar hormonet insulin. Dessa celler kallas β-celler (beta-celler). Insulins funktion är att stimulera kroppens celler till att ta upp och i vissa fall lagra energi. Exempelvis är insulin av stor betydelse efter födointag, då den energi som finns i födan bland annat skall lagras. Denna process fungerar inte som den ska hos dem som har diabetes och sjukdomen karaktäriseras därför av förhöjda nivåer av glukos (socker) och fett i blodet. Att GPR40 uttrycks i just β-cellerna kan därför innebära att denna receptor är inblandad i kroppens energiomsättning genom insulin och att den kan vara inblandad i uppkomsten av diabetes.

Förekomsten av diabetes typ 2 (även kallad åldersdiabetes) har ökat lavinartat världen över på senare år och spås öka ytterligare i framtiden. Omfattande forskning pågår för att ta reda på mekanismerna bakom sjukdomen, men mycket arbete återstår. En vanlig förklaring till ökningen av typ 2 diabetes är den kraftiga ökning av antalet överviktiga människor som också skett på senare år, eftersom övervikt visat sig vara den största riskfaktorn för att drabbas av typ 2 diabetes. Ökningen av övervikt förklaras i sin tur av att många idag äter mat med ett för högt energiinnehåll i förhållande till den mängd energi de gör av med genom fysik aktivitet. Den överskottsenergi som blir resultatet lagrar kroppen i form av fett. Om kroppen tvingas lagra mer fett än vad som ryms i de avsedda depåerna, fettcellerna, måste fettet istället lagras i andra typer av celler. Detta kan skada dessa celler på olika sätt, till exempel kan deras känslighet för insulin bli avtrubbad. Om det sker måste β-cellerna kompensera genom att utsöndra ytterligare insulin för att kunna lagra all energi och sänka blodsockret. I vissa fall orkar β-cellerna till slut inte kompensera, utan blir utmattade och slutar att fungera. Dessa personer får diabetes och har begränsade möjligheter att hålla blodsockret på en normal nivå, utan att med mediciner stimulera insulinfrisättning eller då det inte räcker, att tillföra kroppen extra insulin utifrån. Högt blodsocker under en längre tid kan leda till stora problem, såsom skador på ögon och njurar samt ökar risken för hjärt-kärlsjukdomar.

Detta arbete, vilket även konfirmerats av andra, visar således att att GPR40 är av stor betydelse för att β-cellerna ska utsöndra insulin när de utsätts för fettsyror, från exempelvis mat. En tänkbar koppling är att ökningen av fettet i kosten (eller den ökade andel fett som cirkulerar i blodet på en överviktig person) ökar aktiveringen av GPR40 på β-cellerna. Det i sin tur leder till ytterligare insulinutsöndring, vilket alltså ursprungligen är bra för att ta hand om blodsockret, men som i förlängningen kan leda till diabetes.

Vad vi också har visat i vårt arbete är att GPR40 även uttrycks i en annan celltyp som kallas α-celler (alfa-celler). Dessa celler uttrycks också i bukspottkörteln och kan i stor utsträckning sägas vara β-cellernas motsats. De utsöndrar inte insulin,

utan istället ett hormon som heter glukagon. Detta frisätts när blodsockret är lågt, till exempel mellan måltider och framför allt vid fasta, och har som huvudsaklig funktion att få levern att utsöndra det socker som levern lagrar, under inflytande av insulin, när blodsockret är högt. Det är mycket viktigt att blodsockret inte sjunker för lågt, för det kan snabbt medföra skador på hjärnan. Våra studier visade att GPR40 är av stor betydelse också för utsöndringen av glukagon. Precis som i fallet med insulin ökar glukagonutsöndringen vid tillsats av de fettsyror som aktiverar GPR40. Det kan tyckas ologiskt att fettsyror skulle öka utsöndringen av både insulin och glukagon som alltså har rakt motsatta effekter. Man måste dock komma ihåg att insulinet bara utsöndras när blodsockret är högt och då hindrar utsöndring av glukagon. När blodsockret istället är lågt utsöndras inget insulin, vilket gör att glukagonutsöndring kan ske. GPR40 kan därför öka utsöndringen av både insulin och glukagon, eftersom detta inte sker samtidigt.

Många personer med diabetes har höga nivåer av glukagon i blodet, trots att ett redan förhöjt blodsocker på grund av sin försämrade insulinutsöndring. En trolig bidragande orsak till denna motsägelsefulla, och kontraproduktiva, glukagonutsöndring skulle kunna vara aktivering av GPR40, eftersom många diabetiker också har förhöjda blodfetter.

Ett sätt för en cell att minska receptoraktiviteten är att reducera antalet receptorer på cellytan. Genom att på olika sätt utsätta celler för förhöjda halter av fettsyror kunde vi i dem se en minskad förekomst av GPR40 mRNA, den ”ritning” cellerna använder för att producera fler kopior av GPR40. Denna effekt är inte så lätt att tolka, men troligen är den ett skydd för cellerna mot de ovan nämnda skadorna från ökade krav på insulinutsöndring.

Resultaten av vårt arbete med att karaktärisera fettsyrereceptorn GPR40 kan leda till en bättre förståelse av de mekanismer som är inblandade i uppkomsten av diabetes. Flera läkemedelsföretag är också intresserade av denna receptor och mot bakgrund av våra resultat verkar det fördelaktigt, ur diabetessynpunkt, att utveckla ett läkemedel som minskar signalerna genom GPR40. Det skulle dels

minska den direkta belastning på β-cellerna, som förhöjda blodfetter utgör, dels begränsa den blodsockerhöjande effekt GPR40-aktiveringen har via stimuleringen av glukagonfrisättning.

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REFERENCES

1. **Pierce KL, Premont RT, Lefkowitz RJ:** Seven-transmembrane receptors. *Nat Rev Mol Cell Biol* 3:639-650, 2002

2. **Lander ES, Linton LM, Birren B, Nusbaum C, Zody MC, Baldwin J, Devon K, Dewar K, Doyle M, FitzHugh W, et al.:** Initial sequencing and analysis of the human genome. *Nature* 409:860-921, 2001

3. **Fredriksson R, Lagerström MC, Lundin LG, Schiöth HB:** The G-protein-coupled receptors in the human genome form five main families. Phylogenetic analysis, paralogon groups, and fingerprints. *Mol Pharmacol* 63:1256-1272, 2003

4. **Schiöth HB, Fredriksson R:** The GRAFS classification system of G-protein coupled receptors in comparative perspective. *Gen Comp Endocrinol* 142:94-101, 2005

5. **Palczewski K, Kumasaka T, Hori T, Behnke CA, Motoshima H, Fox BA, Le Trong I, Teller DC, Okada T, Stenkamp RE, Yamamoto M, Miyano M:** Crystal structure of rhodopsin: A G protein-coupled receptor. *Science* 289:739-745, 2000

6. **Lundström K:** Latest development in drug discovery on G protein-coupled receptors. *Curr Protein Pept Sci* 7:465-470, 2006

7. **Jacoby E, Bouhelal R, Gerspacher M, Seuwen K:** The 7 TM G-protein-coupled receptor target family. *ChemMedChem* 1:761-782, 2006

8. **Offermanns S:** G-proteins as transducers in transmembrane signalling. *Prog Biophys Mol Biol* 83:101-130, 2003

9. **Clapham DE, Neer EJ:** G protein beta gamma subunits. *Annu Rev Pharmacol Toxicol* 37:167-203, 1997

10. **Jordan BA, Devi LA:** G-protein-coupled receptor heterodimerization modulates receptor function. *Nature* 399:697-700, 1999

11. **Kasuga M:** Insulin resistance and pancreatic beta cell failure. *J Clin Invest* 116:1756-1760, 2006

12. **Gerich JE:** Control of glycaemia. *Baillieres Clin Endocrinol Metab* 7:551-586, 1993

13. **Bonner-Weir S:** Anatomy of the Islet of Langerhans. In *The endocrine pancreas* E S, Ed. New York, 1991, p. 15-27

14. **Wierup N, Svensson H, Mulder H, Sundler F:** The ghrelin cell: a novel developmentally regulated islet cell in the human pancreas. *Regul Pept* 107:63-69, 2002

15. **Matschinsky FM, Ellerman JE:** Metabolism of glucose in the islets of Langerhans. *J Biol Chem* 243:2730-2736, 1968

16. **Matschinsky FM, Glaser B, Magnuson MA:** Pancreatic beta-cell glucokinase: closing the gap between theoretical concepts and experimental realities. *Diabetes* 47:307-315, 1998

17. **Henquin JC:** Triggering and amplifying pathways of regulation of insulin secretion by glucose. *Diabetes* 49:1751-1760, 2000

18. **Gembal M, Gilon P, Henquin JC:** Evidence that glucose can control insulin release independently from its action on ATP-sensitive K+ channels in mouse B cells. *J Clin Invest* 89:1288-1295, 1992

19. **Sato Y, Aizawa T, Komatsu M, Okada N, Yamada T:** Dual functional role of membrane depolarization/Ca2+ influx in rat pancreatic B-cell. *Diabetes* 41:438-443, 1992

20. **Crespin SR, Greenough WB, 3rd, Steinberg D:** Stimulation of insulin secretion by long-chain free fatty acids. A direct pancreatic effect. *J Clin Invest* 52:1979-1984, 1973

21. **Gravena C, Mathias PC, Ashcroft SJ:** Acute effects of fatty acids on insulin secretion from rat and human islets of Langerhans. *J Endocrinol* 173:73-80, 2002

22. **Warnotte C, Gilon P, Nenquin M, Henquin JC:** Mechanisms of the stimulation of insulin release by saturated fatty acids. A study of palmitate effects in mouse beta-cells. *Diabetes* 43:703-711, 1994

23. **Sako Y, Grill VE:** A 48-hour lipid infusion in the rat time-dependently inhibits glucose-induced insulin secretion and B cell oxidation through a process likely coupled to fatty acid oxidation. *Endocrinology* 127:1580-1589, 1990

24. **Zhou YP, Grill VE:** Long-term exposure of rat pancreatic islets to fatty acids inhibits glucose-induced insulin secretion and biosynthesis through a glucose fatty acid cycle. *J Clin Invest* 93:870-876, 1994

25. **Straub SG, Sharp GW:** Glucose-stimulated signaling pathways in biphasic insulin secretion. *Diabetes Metab Res Rev* 18:451-463, 2002

26. **Taguchi N, Aizawa T, Sato Y, Ishihara F, Hashizume K:** Mechanism of glucose-induced biphasic insulin release: physiological role of adenosine triphosphate-sensitive K+ channel-independent glucose action. *Endocrinology* 136:3942-3948, 1995

27. **Wood IS, Trayhurn P:** Glucose transporters (GLUT and SGLT): expanded families of sugar transport proteins. *Br J Nutr* 89:3-9, 2003

28. **Nolte W, Hartmann H, Ramadori G:** Glucose metabolism and liver cirrhosis. *Exp Clin Endocrinol Diabetes* 103:63-74, 1995

29. **Zimmermann R, Strauss JG, Haemmerle G, Schoiswohl G, Birner-Gruenberger R, Riederer M, Lass A, Neuberger G, Eisenhaber F, Hermetter A, Zechner R:** Fat mobilization in adipose tissue is promoted by adipose triglyceride lipase. *Science* 306:1383-1386, 2004

30. **Holm C:** Molecular mechanisms regulating hormone-sensitive lipase and lipolysis. *Biochem Soc Trans* 31:1120-1124, 2003

31. **Mojsov S, Kopczynski MG, Habener JF:** Both amidated and nonamidated forms of glucagon-like peptide I are synthesized in the rat intestine and the pancreas. *J Biol Chem* 265:8001-8008, 1990

32. **Svoboda M, Tastenoy M, Vertongen P, Robberecht P:** Relative quantitative analysis of glucagon receptor mRNA in rat tissues. *Mol Cell Endocrinol* 105:131-137, 1994

33. **Göpel SO, Kanno T, Barg S, Weng XG, Gromada J, Rorsman P:** Regulation of glucagon release in mouse -cells by KATP channels and inactivation of TTX-sensitive Na+ channels. *J Physiol* 528:509-520, 2000

34. **Guyton AC, Hall JE:** *Textbook of medical physiology*. Philadelphia, W.B. Saunders, 1996

35. **Ahrén B:** Type 2 diabetes, insulin secretion and beta-cell mass. *Curr Mol Med* 5:275-286, 2005

36. **Kahn BB, Flier JS:** Obesity and insulin resistance. *J Clin Invest* 106:473-481, 2000

37. **Mayor S:** Diabetes affects nearly 6% of the world's adults. *Bmj* 333:1191, 2006

38. **Zimmet PZ:** Diabetes epidemiology as a tool to trigger diabetes research and care. *Diabetologia* 42:499-518, 1999

39. **Bhargava SK, Sachdev HS, Fall CH, Osmond C, Lakshmy R, Barker DJ, Biswas SK, Ramji S, Prabhakaran D, Reddy KS:** Relation of serial changes in childhood body-mass index to impaired glucose tolerance in young adulthood. *N Engl J Med* 350:865-875, 2004

40. **Hales CN, Barker DJ:** Type 2 (non-insulin-dependent) diabetes mellitus: the thrifty phenotype hypothesis. *Diabetologia* 35:595-601, 1992

41. **Dresner A, Laurent D, Marcucci M, Griffin ME, Dufour S, Cline GW, Slezak LA, Andersen DK, Hundal RS, Rothman DL, Petersen KF, Shulman GI:** Effects of free fatty acids on glucose transport and IRS-1-associated phosphatidylinositol 3-kinase activity. *J Clin Invest* 103:253-259, 1999

42. **Haffner SM:** Risk factors for non-insulin-dependent diabetes mellitus. *J Hypertens Suppl* 13:S73-76, 1995

43. **Bollheimer LC, Skelly RH, Chester MW, McGarry JD, Rhodes CJ:** Chronic exposure to free fatty acid reduces pancreatic beta cell insulin content by increasing basal insulin secretion that is not compensated for by a corresponding increase in proinsulin biosynthesis translation. *J Clin Invest* 101:1094-1101, 1998

44. **Lee Y, Hirose H, Ohneda M, Johnson JH, McGarry JD, Unger RH:** Beta-cell lipotoxicity in the pathogenesis of non-insulin-dependent diabetes mellitus of obese rats: impairment in adipocyte-beta-cell relationships. *Proc Natl Acad Sci U S A* 91:10878-10882, 1994

45. **Zhou YP, Grill V:** Long term exposure to fatty acids and ketones inhibits B-cell functions in human pancreatic islets of Langerhans. *J Clin Endocrinol Metab* 80:1584-1590, 1995

46. **Zhou YP, Grill VE:** Palmitate-induced beta-cell insensitivity to glucose is coupled to decreased pyruvate dehydrogenase activity and enhanced kinase activity in rat pancreatic islets. *Diabetes* 44:394-399, 1995

47. **Eitel K, Staiger H, Brendel MD, Brandhorst D, Bretzel RG, Haring HU, Kellerer M:** Different role of saturated and unsaturated fatty acids in beta-cell apoptosis. *Biochem Biophys Res Commun* 299:853-856, 2002

48. **Lupi R, Dotta F, Marselli L, Del Guerra S, Masini M, Santangelo C, Patane G, Boggi U, Piro S, Anello M, Bergamini E, Mosca F, Di Mario U, Del Prato S, Marchetti P:** Prolonged exposure to free fatty acids has cytostatic and pro-apoptotic effects on human pancreatic islets: evidence that beta-cell death is caspase mediated, partially dependent on ceramide pathway, and Bcl-2 regulated. *Diabetes* 51:1437-1442, 2002

49. **Williamson JR, Kreisberg RA, Felts PW:** Mechanism for the stimulation of gluconeogenesis by fatty acids in perfused rat liver. *Proc Natl Acad Sci U S A* 56:247-254, 1966

50. **Boden G, Cheung P, Stein TP, Kresge K, Mozzoli M:** FFA cause hepatic insulin resistance by inhibiting insulin suppression of glycogenolysis. *Am J Physiol Endocrinol Metab* 283:E12-19, 2002

51. **Kovacs P, Stumvoll M:** Fatty acids and insulin resistance in muscle and liver. *Best Pract Res Clin Endocrinol Metab* 19:625-635, 2005

52. **Massillon D, Barzilai N, Hawkins M, Prus-Wertheimer D, Rossetti L:** Induction of hepatic glucose-6-phosphatase gene expression by lipid infusion. *Diabetes* 46:153-157, 1997

53. **Kim JK, Fillmore JJ, Chen Y, Yu C, Moore IK, Pypaert M, Lutz EP, Kako Y, Velez-Carrasco W, Goldberg IJ, Breslow JL, Shulman GI:** Tissue-specific overexpression of lipoprotein lipase causes tissue-specific insulin resistance. *Proc Natl Acad Sci U S A* 98:7522-7527, 2001

54. **Zhou YT, Wang ZW, Higa M, Newgard CB, Unger RH:** Reversing adipocyte differentiation: implications for treatment of obesity. *Proc Natl Acad Sci U S A* 96:2391-2395, 1999

55. **Van Heek M, Compton DS, France CF, Tedesco RP, Fawzi AB, Graziano MP, Sybertz EJ, Strader CD, Davis HR, Jr.:** Diet-induced obese mice develop peripheral, but not central, resistance to leptin. *J Clin Invest* 99:385-390, 1997

56. **Considine RV, Sinha MK, Heiman ML, Kriauciunas A, Stephens TW, Nyce MR, Ohannesian JP, Marco CC, McKee LJ, Bauer TL, et al.:** Serum immunoreactive-leptin concentrations in normal-weight and obese humans. *N Engl J Med* 334:292-295, 1996

57. **Houseknecht KL, Baile CA, Matteri RL, Spurlock ME:** The biology of leptin: a review. *J Anim Sci* 76:1405-1420, 1998

58. **Olson LK, Redmon JB, Towle HC, Robertson RP:** Chronic exposure of HIT cells to high glucose concentrations paradoxically decreases insulin gene transcription and alters binding of insulin gene regulatory protein. *J Clin Invest* 92:514-519, 1993

59. **Poitout V, Hagman D, Stein R, Artner I, Robertson RP, Harmon JS:** Regulation of the insulin gene by glucose and fatty acids. *J Nutr* 136:873-876, 2006

60. **Federici M, Hribal M, Perego L, Ranalli M, Caradonna Z, Perego C, Usellini L, Nano R, Bonini P, Bertuzzi F, Marlier LN, Davalli AM, Carandente O, Pontiroli AE, Melino G, Marchetti P, Lauro R, Sesti G, Folli F:** High glucose causes apoptosis in cultured human pancreatic islets of Langerhans: a potential role for regulation of specific Bcl family genes toward an apoptotic cell death program. *Diabetes* 50:1290-1301, 2001

61. **Efanova IB, Zaitsev SV, Zhivotovsky B, Köhler M, Efendic S, Orrenius S, Berggren PO:** Glucose and tolbutamide induce apoptosis in pancreatic beta-cells. A process dependent on intracellular Ca2+ concentration. *J Biol Chem* 273:33501-33507, 1998

62. **Poitout V, Robertson RP:** Minireview: Secondary beta-cell failure in type 2 diabetes--a convergence of glucotoxicity and lipotoxicity. *Endocrinology* 143:339-342, 2002

63. **Crespin SR, Greenough WB, 3rd, Steinberg D:** Stimulation of insulin secretion by infusion of free fatty acids. *J Clin Invest* 48:1934-1943, 1969

64. **Seyffert WA, Jr., Madison LL:** Physiologic effects of metabolic fuels on carbohydrate metabolism. I. Acute effect of elevation of plasma free fatty acids on hepatic glucose output, peripheral glucose utilization, serum insulin, and plasma glucagon levels. *Diabetes* 16:765-776, 1967

65. **Balasse EO, Ooms HA:** Role of plasma free fatty acids in the control of insulin secretion in man. *Diabetologia* 9:145-151, 1973

66. **Malaisse WJ, Malaisse-Lagae F:** Stimulation of insulin secretion by noncarbohydrate metabolites. *J Lab Clin Med* 72:438-448, 1968

67. **Koyama K, Chen G, Wang MY, Lee Y, Shimabukuro M, Newgard CB, Unger RH:** beta-cell function in normal rats made chronically hyperleptinemic by adenovirus-leptin gene therapy. *Diabetes* 46:1276-1280, 1997

68. **Stein DT, Esser V, Stevenson BE, Lane KE, Whiteside JH, Daniels MB, Chen S, McGarry JD:** Essentiality of circulating fatty acids for glucose-stimulated insulin secretion in the fasted rat. *J Clin Invest* 97:2728-2735, 1996

69. **Dobbins RL, Chester MW, Daniels MB, McGarry JD, Stein DT:** Circulating fatty acids are essential for efficient glucose-stimulated insulin secretion after prolonged fasting in humans. *Diabetes* 47:1613-1618, 1998

70. **Prentki M, Corkey BE:** Are the beta-cell signaling molecules malonyl-CoA and cystolic long-chain acyl-CoA implicated in multiple tissue defects of obesity and NIDDM? *Diabetes* 45:273-283, 1996

71. **Yaney GC, Korchak HM, Corkey BE:** Long-chain acyl CoA regulation of protein kinase C and fatty acid potentiation of glucose-stimulated insulin secretion in clonal beta-cells. *Endocrinology* 141:1989-1998, 2000

72. **Deeney JT, Gromada J, Hoy M, Olsen HL, Rhodes CJ, Prentki M, Berggren PO, Corkey BE:** Acute stimulation with long chain acyl-CoA enhances exocytosis in insulin-secreting cells (HIT T-15 and NMRI beta-cells). *J Biol Chem* 275:9363-9368, 2000

73. **Malaisse WJ, Best L, Kawazu S, Malaisse-Lagae F, Sener A:** The stimulus-secretion coupling of glucose-induced insulin release: fuel metabolism in islets deprived of exogenous nutrient. *Arch Biochem Biophys* 224:102-110, 1983

74. **Chen S, Ogawa A, Ohneda M, Unger RH, Foster DW, McGarry JD:** More direct evidence for a malonyl-CoA-carnitine palmitoyltransferase I interaction as a key event in pancreatic beta-cell signaling. *Diabetes* 43:878-883, 1994

75. **Prentki M, Vischer S, Glennon MC, Regazzi R, Deeney JT, Corkey BE:** Malonyl-CoA and long chain acyl-CoA esters as metabolic coupling factors in nutrient-induced insulin secretion. *J Biol Chem* 267:5802-5810, 1992

76. **Parker SM, Moore PC, Johnson LM, Poitout V:** Palmitate potentiation of glucose-induced insulin release: a study using 2-bromopalmitate. *Metabolism* 52:1367-1371, 2003

77. **Sato Y, Henquin JC:** The K+-ATP channel-independent pathway of regulation of insulin secretion by glucose: in search of the underlying mechanism. *Diabetes* 47:1713-1721, 1998

78. **Olofsson CS, Salehi A, Holm C, Rorsman P:** Palmitate increases L-type Ca2+ currents and the size of the readily releasable granule pool in mouse pancreatic beta-cells. *J Physiol* 557:935-948, 2004

79. **Kotarsky K, Nilsson NE, Flodgren E, Owman C, Olde B:** A human cell surface receptor activated by free fatty acids and thiazolidinedione drugs. *Biochem Biophys Res Commun* 301:406-410, 2003

80. **Richieri GV, Kleinfeld AM:** Unbound free fatty acid levels in human serum. *J Lipid Res* 36:229-240, 1995

81. **Briscoe CP, Tadayyon M, Andrews JL, Benson WG, Chambers JK, Eilert MM, Ellis C, Elshourbagy NA, Goetz AS, Minnick DT, Murdock PR, Sauls HR, Jr., Shabon U, Spinage LD, Strum JC, Szekeres PG, Tan KB, Way JM, Ignar DM, Wilson S, Muir AI:** The orphan G protein-coupled receptor GPR40 is activated by medium and long chain fatty acids. *J Biol Chem* 278:11303-11311, 2003

82. **Itoh Y, Kawamata Y, Harada M, Kobayashi M, Fujii R, Fukusumi S, Ogi K, Hosoya M, Tanaka Y, Uejima H, Tanaka H, Maruyama M, Satoh R, Okubo S, Kizawa H, Komatsu H, Matsumura F, Noguchi Y, Shinohara T, Hinuma S, Fujisawa Y, Fujino M:** Free fatty acids regulate insulin secretion from pancreatic beta cells through GPR40. *Nature* 422:173-176, 2003

83. **Miyazaki J, Araki K, Yamato E, Ikegami H, Asano T, Shibasaki Y, Oka Y, Yamamura K:** Establishment of a pancreatic beta cell line that retains glucose-inducible insulin secretion: special reference to expression of glucose transporter isoforms. *Endocrinology* 127:126-132, 1990

84. **Locht C, Antoine R:** A proposed mechanism of ADP-ribosylation catalyzed by the pertussis toxin S1 subunit. *Biochimie* 77:333-340, 1995

85. **Zhang W, Liu HT:** MAPK signal pathways in the regulation of cell proliferation in mammalian cells. *Cell Res* 12:9-18, 2002

86. **Desvergne B, Wahli W:** Peroxisome proliferator-activated receptors: nuclear control of metabolism. *Endocr Rev* 20:649-688, 1999

87. **Li Y, Lazar MA:** Differential gene regulation by PPARgamma agonist and constitutively active PPARgamma2. *Mol Endocrinol* 16:1040-1048, 2002

88. **Gardner OS, Shiau CW, Chen CS, Graves LM:** Peroxisome proliferator-activated receptor gamma-independent activation of p38 MAPK by thiazolidinediones involves calcium/calmodulin-dependent protein kinase

II and protein kinase R: correlation with endoplasmic reticulum stress. *J Biol Chem* 280:10109-10118, 2005

89. **Brunmair B, Gras F, Neschen S, Roden M, Wagner L, Waldhausl W, Furnsinn C:** Direct thiazolidinedione action on isolated rat skeletal muscle fuel handling is independent of peroxisome proliferator-activated receptor-gamma-mediated changes in gene expression. *Diabetes* 50:2309-2315, 2001

90. **Valledor AF, Ricote M:** Nuclear receptor signaling in macrophages. *Biochem Pharmacol* 67:201-212, 2004

91. **Baek SJ, Wilson LC, Hsi LC, Eling TE:** Troglitazone, a peroxisome proliferator-activated receptor gamma (PPAR gamma) ligand, selectively induces the early growth response-1 gene independently of PPAR gamma. A novel mechanism for its anti-tumorigenic activity. *J Biol Chem* 278:5845-5853, 2003

92. **Yin F, Wakino S, Liu Z, Kim S, Hsueh WA, Collins AR, Van Herle AJ, Law RE:** Troglitazone inhibits growth of MCF-7 breast carcinoma cells by targeting G1 cell cycle regulators. *Biochem Biophys Res Commun* 286:916-922, 2001

93. **Rzonca SO, Suva LJ, Gaddy D, Montague DC, Lecka-Czernik B:** Bone is a target for the antidiabetic compound rosiglitazone. *Endocrinology* 145:401-406, 2004

94. **Milligan G, Stoddart LA, Brown AJ:** G protein-coupled receptors for free fatty acids. *Cell Signal* 18:1360-1365, 2006

95. **Stoddart LA, Brown AJ, Milligan G:** Uncovering the pharmacology of the G protein-coupled receptor GPR40: high apparent constitutive activity in [35S]GTP{gamma}S binding studies reflects binding of an endogenous agonist. *Mol Pharmacol* 2007

96. **Salehi A, Flodgren E, Nilsson NE, Jimenez-Feltstrom J, Miyazaki J, Owman C, Olde B:** Free fatty acid receptor 1 (FFA(1)R/GPR40) and its involvement in fatty-acid-stimulated insulin secretion. *Cell Tissue Res* 322:207-215, 2005

97. **Kenakin T:** Differences between natural and recombinant G protein-coupled receptor systems with varying receptor/G protein stoichiometry. *Trends Pharmacol Sci* 18:456-464, 1997

98. **Steneberg P, Rubins N, Bartoov-Shifman R, Walker MD, Edlund H:** The FFA receptor GPR40 links hyperinsulinemia, hepatic steatosis, and impaired glucose homeostasis in mouse. *Cell Metab* 1:245-258, 2005

99. **Shih DQ, Heimesaat M, Kuwajima S, Stein R, Wright CV, Stoffel M:** Profound defects in pancreatic beta-cell function in mice with combined heterozygous mutations in Pdx-1, Hnf-1alpha, and Hnf-3beta. *Proc Natl Acad Sci U S A* 99:3818-3823, 2002

100. **Assimacopoulos-Jeannet F, Thumelin S, Roche E, Esser V, McGarry JD, Prentki M:** Fatty acids rapidly induce the carnitine palmitoyltransferase I gene in the pancreatic beta-cell line INS-1. *J Biol Chem* 272:1659-1664, 1997

101. **Lameloise N, Muzzin P, Prentki M, Assimacopoulos-Jeannet F:** Uncoupling protein 2: a possible link between fatty acid excess and impaired glucose-induced insulin secretion? *Diabetes* 50:803-809, 2001

102. **Rubí B, Antinozzi PA, Herrero L, Ishihara H, Asins G, Serra D, Wollheim CB, Maechler P, Hegardt FG:** Adenovirus-mediated overexpression of liver carnitine palmitoyltransferase I in INS1E cells: effects on cell metabolism and insulin secretion. *Biochem J* 364:219-226, 2002

103. **Chan CB, De Leo D, Joseph JW, McQuaid TS, Ha XF, Xu F, Tsushima RG, Pennefather PS, Salapatek AM, Wheeler MB:** Increased uncoupling protein-2 levels in beta-cells are associated with impaired glucose-stimulated insulin secretion: mechanism of action. *Diabetes* 50:1302-1310, 2001

104. **Ogawa T, Hirose H, Miyashita K, Saito I, Saruta T:** GPR40 gene Arg211His polymorphism may contribute to the variation of insulin secretory capacity in Japanese men. *Metabolism* 54:296-299, 2005

105. **Hamid YH, Vissing H, Holst B, Urhammer SA, Pyke C, Hansen SK, Glumer C, Borch-Johnsen K, Jorgensen T, Schwartz TW, Pedersen O, Hansen T:** Studies of relationships between variation of the human G protein-coupled receptor 40 Gene and Type 2 diabetes and insulin release. *Diabet Med* 22:74-80, 2005

106. **Park D, Jhon DY, Lee CW, Lee KH, Rhee SG:** Activation of phospholipase C isozymes by G protein beta gamma subunits. *J Biol Chem* 268:4573-4576, 1993

107. **Shapiro H, Shachar S, Sekler I, Hershfinkel M, Walker MD:** Role of GPR40 in fatty acid action on the beta cell line INS-1E. *Biochem Biophys Res Commun* 335:97-104, 2005

108. **Fujiwara K, Maekawa F, Yada T:** Oleic acid interacts with GPR40 to induce Ca2+ signaling in rat islet beta-cells: mediation by PLC and L-type Ca2+ channel and link to insulin release. *Am J Physiol Endocrinol Metab* 289:E670-677, 2005

109. **Rorsman P, Trube G:** Calcium and delayed potassium currents in mouse pancreatic beta-cells under voltage-clamp conditions. *J Physiol* 374:531-550, 1986

110. **Feng DD, Luo Z, Roh SG, Hernandez M, Tawadros N, Keating DJ, Chen C:** Reduction in voltage-gated K+ currents in primary cultured rat pancreatic beta-cells by linoleic acids. *Endocrinology* 147:674-682, 2006

111. **Madison LL, Seyffert WA, Jr., Unger RH, Barker B:** Effect on plasma free fatty acids on plasma glucagon and serum insulin concentrations. *Metabolism* 17:301-304, 1968

112. **Hong J, Abudula R, Chen J, Jeppesen PB, Dyrskog SE, Xiao J, Colombo M, Hermansen K:** The short-term effect of fatty acids on glucagon secretion is influenced by their chain length, spatial configuration, and degree of unsaturation: studies in vitro. *Metabolism* 54:1329-1336, 2005

113. **Olofsson CS, Salehi A, Göpel SO, Holm C, Rorsman P:** Palmitate stimulation of glucagon secretion in mouse pancreatic alpha-cells results from activation of L-type calcium channels and elevation of cytoplasmic calcium. *Diabetes* 53:2836-2843, 2004

114. **Bollheimer LC, Landauer HC, Troll S, Schweimer J, Wrede CE, Scholmerich J, Buettner R:** Stimulatory short-term effects of free fatty acids on glucagon secretion at low to normal glucose concentrations. *Metabolism* 53:1443-1448, 2004

115. **Ginsberg HN:** Insulin resistance and cardiovascular disease. *J Clin Invest* 106:453-458, 2000

116. **Flodgren E, Olde B, Meidute-Abaraviciene S, Winzell MS, Ahrén B, Salehi A:** GPR40 is expressed in glucagon producing cells and affects glucagon secretion. *Biochem Biophys Res Commun* 354:240-245, 2007

117. **Samols E, Stagner JI, Ewart RB, Marks V:** The order of islet microvascular cellular perfusion is B----A----D in the perfused rat pancreas. *J Clin Invest* 82:350-353, 1988

118. **Flodgren E, Winzell MS, Ahrén B:** GPR40 in mouse islets is transcriptionally down-regulated by agonistic free fatty acids. *Manuscript in preparation*

119. **Schnell S, Schaefer M, Schofl C:** Free fatty acids increase cytosolic free calcium and stimulate insulin secretion from beta-cells through activation of GPR40. *Mol Cell Endocrinol* 263:173-180, 2007

120. **Yonezawa T, Katoh K, Obara Y:** Existence of GPR40 functioning in a human breast cancer cell line, MCF-7. *Biochem Biophys Res Commun* 314:805-809, 2004

121. **Hardy S, St-Onge GG, Joly E, Langelier Y, Prentki M:** Oleate promotes the proliferation of breast cancer cells via the G protein-coupled receptor GPR40. *J Biol Chem* 280:13285-13291, 2005

122. **Garrido DM, Corbett DF, Dwornik KA, Goetz AS, Littleton TR, McKeown SC, Mills WY, Smalley TL, Jr., Briscoe CP, Peat AJ:** Synthesis and activity of small molecule GPR40 agonists. *Bioorg Med Chem Lett* 16:1840-1845, 2006

123. **Briscoe CP, Peat AJ, McKeown SC, Corbett DF, Goetz AS, Littleton TR, McCoy DC, Kenakin TP, Andrews JL, Ammala C, Fornwald JA, Ignar DM, Jenkinson S:** Pharmacological regulation of insulin secretion in MIN6 cells through the fatty acid receptor GPR40: identification of agonist and antagonist small molecules. *Br J Pharmacol* 148:619-628, 2006

124. **Hirasawa A, Tsumaya K, Awaji T, Katsuma S, Adachi T, Yamada M, Sugimoto Y, Miyazaki S, Tsujimoto G:** Free fatty acids regulate gut incretin glucagon-like peptide-1 secretion through GPR120. *Nat Med* 11:90-94, 2005

125. **Stewart G, Hira T, Higgins A, Smith CP, McLaughlin JT:** Mouse GPR40 heterologously expressed in Xenopus oocytes is activated by short-, medium-, and long-chain fatty acids. *Am J Physiol Cell Physiol* 290:C785-792, 2006

126. **Poitout V:** The ins and outs of fatty acids on the pancreatic beta cell. *Trends Endocrinol Metab* 14:201-203, 2003

127. **Warnotte C, Nenquin M, Henquin JC:** Unbound rather than total concentration and saturation rather than unsaturation determine the potency of fatty acids on insulin secretion. *Mol Cell Endocrinol* 153:147-153, 1999

128. **Fex M, Lucas S, Winzell MS, Ahrén B, Holm C, Mulder H:** {beta}-Cell Lipases and Insulin Secretion. *Diabetes* 55 Suppl 2:S24-31, 2006

129. **Mulder H, Holst LS, Svensson H, Degerman E, Sundler F, Ahrén B, Rorsman P, Holm C:** Hormone-sensitive lipase, the rate-limiting enzyme in triglyceride hydrolysis, is expressed and active in beta-cells. *Diabetes* 48:228-232, 1999

130. **Surwit RS, Feinglos MN, Rodin J, Sutherland A, Petro AE, Opara EC, Kuhn CM, Rebuffe-Scrive M:** Differential effects of fat and sucrose on the development of obesity and diabetes in C57BL/6J and A/J mice. *Metabolism* 44:645-651, 1995

131. **Surwit RS, Kuhn CM, Cochrane C, McCubbin JA, Feinglos MN:** Diet-induced type II diabetes in C57BL/6J mice. *Diabetes* 37:1163-1167, 1988

132. **Winzell MS, Ahrén B:** The high-fat diet-fed mouse: a model for studying mechanisms and treatment of impaired glucose tolerance and type 2 diabetes. *Diabetes* 53 Suppl 3:S215-219, 2004

133. **Terauchi Y, Takamoto I, Kubota N, Matsui J, Suzuki R, Komeda K, Hara A, Toyoda Y, Miwa I, Aizawa S, Tsutsumi S, Tsubamoto Y, Hashimoto S, Eto K, Nakamura A, Noda M, Tobe K, Aburatani H, Nagai R, Kadowaki T:** Glucokinase and IRS-2 are required for compensatory beta cell hyperplasia in response to high-fat diet-induced insulin resistance. *J Clin Invest* 117:246-257, 2007

134. **Kotarsky K, Antonsson L, Owman C, Olde B:** Optimized reporter gene assays based on a synthetic multifunctional promoter and a secreted luciferase. *Anal Biochem* 316:208-215, 2003

135. **Ye RD:** Regulation of nuclear factor kappaB activation by G-protein-coupled receptors. *J Leukoc Biol* 70:839-848, 2001

136. **Lernmark Å:** The preparation of, and studies on, free cell suspensions from mouse pancreatic islets. *Diabetologia* 10:431-438, 1974

137. **Summerton J, Weller D:** Morpholino antisense oligomers: design, preparation, and properties. *Antisense Nucleic Acid Drug Dev* 7:187-195, 1997

138. **Bruss ML, Black AL:** Enzymatic microdetermination of glycogen. *Anal Biochem* 84:309-312, 1978

139. **Corkey BE, Deeney JT, Yaney GC, Tornheim K, Prentki M:** The role of long-chain fatty acyl-CoA esters in beta-cell signal transduction. *J Nutr* 130:299S-304S, 2000

140. **Nolan CJ, Madiraju MS, Delghingaro-Augusto V, Peyot ML, Prentki M:** Fatty Acid Signaling in the {beta}-Cell and Insulin Secretion. *Diabetes* 55 Suppl 2:S16-23, 2006

141. **Nolan CJ, Leahy JL, Delghingaro-Augusto V, Moibi J, Soni K, Peyot ML, Fortier M, Guay C, Lamontagne J, Barbeau A, Przybytkowski E, Joly E, Masiello P, Wang S, Mitchell GA, Prentki M:** Beta cell compensation for insulin resistance in Zucker fatty rats: increased lipolysis and fatty acid signalling. *Diabetologia* 49:2120-2130, 2006

142. **Winzell MS, Svensson H, Arner P, Ahrén B, Holm C:** The expression of hormone-sensitive lipase in clonal beta-cells and rat islets is induced by long-term exposure to high glucose. *Diabetes* 50:2225-2230, 2001

143. **Mulder H, Yang S, Winzell MS, Holm C, Ahrén B:** Inhibition of lipase activity and lipolysis in rat islets reduces insulin secretion. *Diabetes* 53:122-128, 2004

144. **Masiello P, Novelli M, Bombara M, Fierabracci V, Vittorini S, Prentki M, Bergamini E:** The antilipolytic agent 3,5-dimethylpyrazole inhibits insulin release in response to both nutrient secretagogues and cyclic adenosine monophosphate agonists in isolated rat islets. *Metabolism* 51:110-114, 2002

145. **Campillo JE, Luyckx AS, Lefebvre PJ:** Effect of oleic acid on arginine-induced glucagon secretion by the isolated perfused rat pancreas. *Acta Diabetol Lat* 16:287-293, 1979

146. **Fujiwara K, Maekawa F, Dezaki K, Nakata M, Yashiro T, Yada T:** Oleic acid glucose-independently stimulates glucagon secretion by increasing cytoplasmic Ca2+ via ER Ca2+ release and Ca2+ influx in the rat islet {alpha}-cells. *Endocrinology* [Epub ahead of print], 2007

147. **Qvigstad E, Bjerve KS, Grill V:** Effects of long-term fasting on insulin responses to fatty acids in man. *Scand J Clin Lab Invest* 62:271-277, 2002

148. **Grill V, Björklund A:** Overstimulation and beta-cell function. *Diabetes* 50 Suppl 1:S122-124, 2001

149. **McKeown SC, Corbett DF, Goetz AS, Littleton TR, Bigham E, Briscoe CP, Peat AJ, Watson SP, Hickey DM:** Solid phase synthesis and SAR of small molecule agonists for the GPR40 receptor. *Bioorg Med Chem Lett*, 2007