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GPR40 protein levels are crucial to the regulation of stimulated hormone secretion in pancreatic islets. Lessons from spontaneous obesity-prone and non-obese type 2 diabetes in rats

Sandra Meidute Abaraviciene[1,2], Sarheed J. Muhammed[1], Stefan Amisten[1], Ingmar Lundquist[1,3] and Albert Salehi[1]

Department of Clinical Science, SUS, Division of Islet Cell Physiology[1], Department of Stem Cell Biology, State Research Institute Centre of Innovative Medicine[2], Vilnius, Lithuania and Department of Experimental Medical Science[3]; University of Lund, Sweden

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Proofs and correspondence:

Dr. Albert Salehi
Department of Clinical Science, SUS
Division of Islet Cell Physiology,
Entrance 72, Building 91, Plan 11,
SE-205 02 Malmö, Sweden
Tel: +46 40 391162
Fax: +46 40 391212
E-mail: S_Albert.Salehi@med.lu.se

Abstract

The role of islet GPR40 protein in the pathogenesis of diabetes is unclear. We explored the influence of GPR40 protein levels on hormone secretion in islets from two rat models of spontaneous type 2 diabetes displaying either hyperlipidaemia or hyperglycaemia.
GPR40 expression was analysed by confocal microscopy, Western blot and qPCR in islets from preobese Zucker (fa/fa) rats, diabetic Goto-Kakizaki (GK) rats, and controls.

Confocal microscopy of control islets showed expression of GPR40 protein in insulin, glucagon and somatostatin cells. GPR40 expression was strongly increased in islets of hyperlipidaemic fa/fa rats and coincided with a concentration-related increase in palmitate-induced release of insulin and glucagon and its inhibition of somatostatin release. Conversely, hyperglycaemic GK islets displayed an extremely faint expression of GPR40 as did high-glucose-cultured control islets. This was reflected in abolished palmitate-induced hormone response in GK islets and high-glucose-cultured control islets. The palmitate antagonist rosiglitazone promoted reappearance of GPR40 in high-glucose-cultured islets and served as partial agonist in glucose-stimulated insulin release.
GPR40 protein is abundantly expressed in pancreatic islets and modulates stimulated hormone secretion. Mild hyperlipidaemia in obesity-prone diabetes creates increased GPR40 expression and increased risk for an exaggerated palmitate-induced insulin response and lipotoxicity, a metabolic situation suitable for GPR40 antagonist treatment. Chronic hyperglycaemia creates abrogated GPR40 expression and downregulated insulin release, a metabolic situation suitable for GPR40 agonist treatment to avoid glucotoxicity. GPR40 protein is interactively modulated by both free fatty acids and glucose and is a promising target for pharmacotherapy in different variants of type 2 diabetes.

1. Introduction

The orphan G protein-coupled receptor 40 (GPR40) has been identified as a receptor for medium to long-chain free fatty acids (FFA). Initial studies showed an abundant expression of GPR40 in the pancreatic β-cells (Briscoe et al., 2003,Itoh et al., 2003,Kotarsky et al., 2003,Salehi et al., 2005). Acute activation of β-cell GPR40 with various long-chain FFA was found to amplify glucose-stimulated insulin release and challenged previous data suggesting that such an effect was mediated by the intracellular metabolism of FFA, generating various lipid-derived signals resulting in augmentation of the release process (Briscoe et al., 2003,Itoh et al., 2003,Latour et al., 2007,Salehi et al., 2005). Previously we (Meidute Abaraviciene et al., 2008) and others (Nolan et al., 2006,Steneberg et al., 2005) showed that long-term exposure of the β-cells to elevated levels of FFA leads to a marked impairment of glucose-stimulated insulin release and that this FFA-induced impairment most conceivably was transduced through GPR40-related events. These results also suggested that β-cell GPR40, during chronic exposure of pancreatic islets to elevated levels of FFA, might play an important role in the pathogenesis of obesity and type 2 diabetes and should be considered a putative target for therapeutic intervention in these worldwide epidemic diseases (Abaraviciene et al., 2008,Meidute Abaraviciene et al., 2008,Morgan et al., 2009,Rayasam et al., 2007,Salehi et al., 2005). In previous experiments we found that the thiazolidinedione derivative rosiglitazone strongly counteracted both short- and long-term β-cell dysfunction induced by palmitate in normal mouse islets at the GPR40 level (Abaraviciene et al., 2008,Meidute Abaraviciene et al., 2008). However, with regard to long-term effects two well recognized studies using genetic ablation of GPR40 in mice have given very controversial results showing on the one hand that such animals were as sensitive as controls to long-term deleterious effects by FFA (Latour et al., 2007) and on the other hand , in contrast, that deletion of GPR40 protected the islets from FFA-induced dysfunction as well as from obesity-induced hyperinsulinaemia (Steneberg et al., 2005). Moreover, most data from several subsequent studies using mice with GPR40 deletion or overexpression suggested that loss of GPR40 did not protect against the deleterious effects of long-term exposure of the islets to elevated FFA or high fat feeding but instead that GPR40 activation could lead to facilitation of glucose-induced insulin secretion (Alquier et al., 2009,Brownlie et al., 2008,Kebede et al., 2009,Nagasumi et al., 2009,Tan et al., 2008). The marked discrepancies between these studies are difficult to explain but different techniques including unwanted side effects of GPR40 deletion and overexpression are likely involved. However, surprisingly and to our knowledge

previous detailed studies did not include basal experiments concerning the relation between GPR40 *protein* expression levels and FFA-induced hormone secretion and did not explore the islets of animal models of spontaneous diabetes. Hence, to avoid possible undesired side effects of genetic manipulation processes the aim of the present investigation was to perform a basic study in a "natural" chronic prediabetic/diabetic state of the relation between palmitate-induced hormone secretion and the expression pattern of GPR40 protein levels in insulin, glucagon and somatostatin cells in spontaneous type 2 prediabetes/diabetes using young (6-8 weeks) hyperlipidaemic, normoglycaemic (ZDF, fa/fa) rats, which show normal blood glucose levels until they start to display rising glucose at ~9-10 weeks of age (Lee et al., 1994), and young hyperglycaemic, normolipidaemic GK rats (Zhou et al., 1995) of the same age and weight. In addition, and for comparison, we explored the role of GPR40 protein levels in relation to palmitate and/or glucose effects on insulin release in islets from normal control rats. It should be recalled that these young fa/fa and GK rats show a normal insulin content and a normal islet size and β-cell number (see e.g. Finegood et al., 2001,Salehi et al., 1999).

2. Materials and methods

2.1. Animals

Male Zucker diabetic fatty fa/fa rats (Charles River Labs Int., Kisslegg, Germany) with normal controls of the same strain and GK rats from the Stockholm colony (Möllegård, Ry, Denmark) with their Wistar controls were used. All animals weighed between 140-175 g and were 6-8 weeks old. There was no difference between the four groups of rats with respect to neither weight nor age. The rats were given a standard pellet diet (B&K, Sollentuna, Sweden) and tap water *ad libitum*. They were housed in metabolic cages with constant temperature (22º C) and 12 h light/dark cycles. The local animal welfare committee, Lund, Sweden, approved the experimental protocols and all procedures using animals.

2.2. Drugs and Chemicals

Collagenase (CLS 4) was from Sigma St. Louis, MO, USA. Fatty acid free bovine serum albumin (BSA) was from Boehringer Mannheim, Germany. The insulin, glucagon and somatostatin radioimmunoassay kits were from Millipore Corporation, MA, USA. Rosiglitazone was from GlaxoSmithKline, UK. All other chemicals were from Merck AG (Darmstadt, Germany) or Sigma (USA).

2.3. Isolation of pancreatic islets

Preparation of rat pancreatic islets was performed by retrograde injection of a collagenase solution *via* the bile-pancreatic duct (Gotoh et al., 1985). Islets were then isolated and handpicked under a stereomicroscope at room temperature. The isolated islets were then subjected to different experimental procedures (see below).

2.4. Detection of mRNA for GPR40 by quantitative PCR

Total RNA from islets was isolated using TRIzol (Invitrogen) according to the manufacturer's instructions with slight modifications *i.e.* after phase separation; the upper aqueous phase was incubated in -20ºC overnight with 10 µg glycogen (Invitrogen) and centrifuged for 15min at 12000 x g, at 4ºC (Amisten, 2012). Each individual RNA pellet was dissolved in RNase-free water and converted into cDNA using TaqMan Reverse transcription (Applied Biosystems, CA, USA) according to the manufacturer's instructions. Expression of rat GPR40 relative GAPDH was determined using the DDCt method (Pfaffl, 2001) using QuantiFast SYBR Green qPCR mastermix and QuantiTect Gpr40 (QT01083719) and Gapdh (QT00199633) primers.

2.5. Confocal microscopy

The islets were fixed with 4% formaldehyde, permeabilized with 5% Triton X-100, and unspecific sites were blocked with 5% Normal Donkey Serum (Jackson Immunoresearch Laboratories Inc, West Grove, PA, USA). Mouse GPR40 was detected with a polyclonal antibody (1:50) in combination with Cy3-conjugated anti-rabbit IgG (1:100) (Jackson Immunoresearch Laboratories Inc. West Grove, PA, USA). The GPR40 antibody was raised in rabbits against the C-terminal peptide: NH2-CVTRTQRGTIQK-COOH (Innovagen, Lund, Sweden). The specificity of this antibody was tested in previous studies and included knock-down experiments in mouse islets (Meidute Abaraviciene et al., 2008,Salehi et al., 2005). For staining of insulin, islets were incubated with a guinea pig-raised anti-insulin antibody (1:1000) (Eurodiagnostica, Malmö, Sweden) followed by an incubation with a Cy5-conjugated anti-guinea pig IgG antibody (Jackson Immunoresearch Laboratories Inc.) (1:150). For the staining of glucagon, islets were incubated with a guinea pig-raised anti-glucagon antibody (1:600) (Eurodiagnostica, Malmö, Sweden) followed by incubation with a Cy5-conjugated anti-guinea pig IgG antibody (1:150) (Jackson Immunoresearch Laboratories Inc.). For the staining of somatostatin, islets were incubated with a mouse-raised anti-somatostatin

antibody (1:500) (Novo-Nordisk, Bagsvaerd, Denmark) followed by incubation with a Cy5-conjugated anti-mouse IgG antibody (1:150) (Jackson Immunoresearch Laboratories Inc). The fluorescence was visualized with a Zeiss LSM510 confocal microscope by sequentially scanning at (excitation/emission) 505-530 nm (Cy3) and 633/>650nm (Cy5). The co-localization of the different hormones and GPR40 was quantified pixel by pixel using Zen 2009 (Carl Zeiss, Oberkochen, Germany) software. All fluorescence intensity measurements were performed with randomly selected islets from 4-7 rats in each group.

2.6. Western blot analysis

Approximately 400 islets were handpicked in Hank's buffer under a stereomicroscope and then suspended in 100 µl of SDS-buffer (Tris-HCl, 50 mmol/l; EDTA 1 mmol/l) containing a mixture of a protease inhibitor cocktail, frozen and sonicated on ice on the day of analysis (Salehi et al., 2001). The protein content of the homogenates was determined according to Bradford (Bradford, 1976). Homogenate samples representing 25 µg of total protein were run on 7.5 % SDS-polyacrylamide gel (Bio-Rad, Hercules, CA, USA). After electrophoresis, proteins were transferred to a nitrocellulose membrane (Bio-Rad). The membrane was blocked in LS-buffer (10mmol/l Tris, pH 7.4, 100 mmol/l NaCl, 0.1% Tween-20) containing 5% non-fat dry milk powder for 40 min at 37ºC. Subsequently the membranes were incubated overnight with a polyclonal anti-GPR40 antibody (1:150) at room temperature. After washing (three times) in LS-buffer the membranes were finally incubated with a horseradish peroxidase-conjugated anti-rabbit antibody (1:500) (StressGen Biotechnologies Corp, Victoria, BC, Canada). Immunoreactivity was detected by an enhanced chemiluminescence reaction (Pierce, Rockford, IL, USA) using AlphaImager imaging system (Alpha Innotech). The results were quantified by densitometric analysis using the Bio-Rad software (Salehi et al., 2001).

2.7. Incubation of pancreatic islets and hormone determination

The freshly isolated islets were preincubated for 30 min at 37°C in Krebs-Ringer bicarbonate buffer, pH 7.4, supplemented with 10 mmol/l HEPES, 0.1 % bovine serum albumin and 1.0 mmol/l glucose. After preincubation the buffer was changed and the islets were incubated for 60 min at 37°C at 5 mmol/l glucose ± different concentrations of palmitate ranging from 1-1000 µmol/l as denoted in the figures. The palmitate/albumin solution was carefully prepared according to a previously described detailed prescription (Meidute Abaraviciene et al., 2008).

Each incubation vial contained 12 islets in 1.0 ml of buffer solution and was gassed with 95% O_2-5% CO_2 to obtain constant pH and oxygenation. In other experiments islets from Wistar rats were cultured for 24 h at 5 or 16.7 mmol/l glucose ± denoted agents. All incubations were performed in an incubation box at 30 cycles/min. Immediately after incubation an aliquot of the medium was removed and frozen for subsequent assay of insulin, glucagon (Millipore, MA, USA) and somatostatin (Euro-Diagnostica, Malmö, Sweden) using commercially available immunoassay kits according to the manufacturer's recommendations.

2.8. Statistics

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

3. Results

3.1. Basal circulating concentrations of glucose, insulin and lipids in fa/fa and GK rats and their controls

The circulating concentrations of glucose, triglycerides, cholesterol, HDL, LDL and insulin in fa/fa and their control rats **(Table 1A)** as well as in GK and their Wistar control rats **(Table 1B)** were first determined. It is shown that our young fa/fa rats were normoglycaemic and tend to have mildly increased levels of triglycerides, cholesterol, HDL and insulin, whereas our young GK rats were hyperglycaemic and normolipidaemic with normal insulin.

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

Figure 1 shows the expression pattern of GPR40 protein in control **(a)** and fa/fa **(b)** islets as determined by immunolabelling and confocal microscopy. In control islets (**Figure 1a**) GPR40 is abundantly expressed in the insulin-producing β-cells (**A-C**) but also in glucagon-producing α-cells (**D-F**) and somatostatin-producing δ-cells (**G-I**). In fa/fa islets (**Figure 1b**) the expression pattern was markedly increased in insulin (**J-L**), glucagon (**M-O**) and somatostatin (**P-R**) cells. Fluorescence intensity measurements comparing GPR40

fluorescence in control *vs* fa/fa islets are depicted in **Figure 1c** and show that all types of the endocrine cells of the fa/fa islets display highly increased fluorescence intensity.

Figure 2 shows the expression pattern of GPR40 mRNA and protein in whole islets as determined with qPCR and Western blot, respectively. Both these parameters were greatly increased in fa/fa islets compared with controls (**Figure 2a and b**). Regarding the Western blots, densitometric quantitative analysis of GPR40 bands from control islet homogenates compared to the bands from fa/fa islets shows an enhanced expression level of GPR40 protein in the fa/fa islets (**Figure 2c**). To correlate these morphological and biochemical observations with FFA-induced hormone secreting capacity of control and fa/fa islets we performed a concentration-response study with palmitate as secretagogue at 5 mmol/l basal glucose. **Figure 3a-c** shows that palmitate-stimulated release of both insulin and glucagon was greatly increased in fa/fa islets compared with the control islets. Moreover, the palmitate-induced inhibition of somatostatin release was more pronounced in fa/fa compared with control islets. These effects could be revealed already at low physiological concentrations of palmitate both in control and fa/fa islets. Hence, the amount of GPR40 protein was closely associated with the effects of palmitate on hormone secretion.

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

Figure 4 illustrates the confocal microscopy picture of the expression pattern of GPR40 protein in Wistar control **(a)** and GK **(b)** islets after immunolabelling. The distribution of GPR40 in insulin-producing β-cells (**A-C**), glucagon-producing α-cells (**D-F**) and somatostatin-producing δ-cells (**G-I**) in the Wistar control islets (**Figure 4a**) was abundant and almost identical to the pattern seen in fa/fa control islets (**Figure 1a**). In GK islets, however, GPR40 expression was almost abolished in insulin (**J-L**), glucagon (**M-O**) and somatostatin (**P-R**) cells (**Figure 4b**). Comparing GPR40 fluorescence intensity in Wistar control *vs* GK islets shows an almost total abolishment of GPR40 protein expression in all endocrine cells (**Figure 4c**). The results of examining the expression of GPR40 mRNA and protein in whole islets are shown in **Figure 5a and b**. Both mRNA and especially protein expression of GPR40 were greatly reduced in GK islets compared with Wistar control islets. Densitometric quantitative analysis of GPR40 bands from Wistar islet homogenates compared to that from GK islets shows a markedly reduced expression level of GPR40 protein in the

GK islets (**Figure 5c**). A concentration-response study with isolated islets showed that the palmitate-induced release of insulin and glucagon seen in Wistar control islets was almost abolished in GK islets and that the basal secretion of somatostatin in GK islets was extremely low and not influenced by palmitate (**Figure 6a-c**). Thus there was a remarkable association between the lack of GPR40 protein in the different endocrine cells and the lack of palmitate effects on hormone secretion suggesting an almost indispensable requirement for GPR40 protein in secretory signalling.

3.4. Expression of GPR40 in Wistar control islets cultured in the absence and presence of different concentrations of glucose or palmitate

To explore the regulation of GPR40 by glucose and FFA also under *in vitro* conditions we cultured Wistar control islets for 24 h in the presence of 5 mmol/l glucose or 16.7 mmol/l glucose and 5 mmol/l glucose + 100 or 1000 μmol/l palmitate. **Figure 7a** shows that the expression of GPR40 protein as illustrated by confocal microscopy in islets cultured at high (16.7 mmol/l) glucose (**D-F**) was almost abolished (**E**) when compared with control islets (**A-C**) cultured at 5 mmol/l glucose (**B**). The fluorescence intensity of GPR40 in islets cultured at 16.7 mmol/l glucose was almost abolished compared with the 5 mmol/l glucose controls (**Figure 7c**). In contrast, culture at 5 mmol/l glucose in the presence of 100 μmol/l palmitate (**Figure 7b, G-I**) increased GPR40 protein expression in the insulin cells (**H**) and the fluorescence intensity was augmented by approximately 50 % (**Figure 7c**). Interestingly, addition of 1000 μmol/l palmitate to 5 mmol/l glucose (**Figure 7b J-L**) did not augment the GPR40 fluorescence intensity compared with the 5 mmol/l glucose controls (**Figure 7c**). Moreover, both mRNA and protein expression (Western blot) of GPR40 was strongly reduced in islets cultured at 16.7 mmol/l glucose (**Figure 8a and b**). Notably, however, in contrast to GPR40 protein expression GPR40 mRNA was significantly and markedly reduced at 5 mmol/l glucose + 1000 μmol/l palmitate compared to 5 mmol/l glucose + 100 μmol/l palmitate, while, similar to the confocal microscopy results (**Figure 7c**), the Western blots showed no quantitative difference. Similar to the fluorescence intensity (**Figure 7c**) densitometric analysis of Western blots for GPR40 protein expression showed a large reduction compared to controls in the presence of high glucose (16.7 mmol/l) and a marked increase for glucose 5 mmol/l + palmitate 100 μmol/l (**Figure 8c**). In contrast to the mRNA expression level which decreased at glucose 5 mmol/l + palmitate 1000 μmol/l concentration

(**Figure 8a**) the protein expression was appreciably the same as for glucose 5 mmol/l + palmitate 100 μmol/l (**Figure 8c**).

3.5. Palmitate-stimulated insulin release in Wistar control islets previously cultured at low or high glucose for 24 h

To explore the functional consequences of the abrogation of GPR40 protein expression in the β-cells of high-glucose-cultured islets we challenged such islets with 250 μmol/l palmitate being an approximately maximal dose according to our concentration-response results in control rats (see **Figure 3a** and **6a**). **Figure 9** shows that palmitate-induced insulin release was almost totally suppressed to basal levels and thus was correlated to the abrogated GPR40 protein expression in the β-cells of high-glucose-cultured islets. In comparison, islets cultured at basal glucose (5 mmol/l) showed a normal insulin response to palmitate (**Figure 9**). Hence, these results are in accordance with our experiments with islets from the *in vivo* hyperglycaemic GK rat (see **Figure 6a**).

3.6. GPR40 protein expression and insulin release at low and high glucose in Wistar control islets following islet culture at high glucose in the absence or presence of palmitate or rosiglitazone

The final series of experiments was then performed in an attempt to elucidate whether the almost total suppression of GPR40 protein expression after culturing the islets at high glucose would influence not only insulin release induced by palmitate but also that induced by glucose. Hence, we cultured the islets for 24 h at high glucose or high glucose + palmitate (1000 μmol/l) or the thiazolidinedione derivative rosiglitazone (ROZ) (1 μmol/l). It should be recalled that we have previously shown that rosiglitazone is a potent palmitate antagonist at the GPR40 level in mouse islets (Abaraviciene et al., 2008,Meidute Abaraviciene et al., 2008). Confocal microscopy and fluorescence intensity measurements showed again that culture of normal Wistar islets in high glucose (16.7 mmol/l) for 24 h induced an almost total abolishment of GPR40 protein expression in the insulin-producing β-cells (**Figure 10 a E**) compared with the control culture in normal glucose (5 mmol/l) (**Figure 10 a B**). Quantitative intensity measurements are shown in **Figure 10 b**. Culture in high glucose + palmitate almost restored GPR40 protein expression in the β-cells (**Figure 10 a H and 10 b**). Finally, culture in high glucose + rosiglitazone (**Figure 10 a K and 10 b**) as well as high glucose + rosiglitazone + palmitate (**Figure 10 a N and 10 b**) restored approximately 50 % of GPR40

protein expression in the β-cells. There were only modest changes in the released amount of insulin secreted during the culture period of 24 h (**Figure 11**). Thus islets cultured with 16.7 mmol/l glucose + rosiglitazone and 16.7 mmol/l glucose + rosiglitazone + palmitate tended to increase insulin secretion while 16.7 mmol/l glucose + palmitate showed a reduction (**Figure 11 a**). A subsequent short-term incubation (60 min) of cultured islets taken from the different groups was then performed at either low (1 mmol/l) or high (16.7 mmol/l) glucose. The results are shown in **Figure 11 b**. As expected there was a basal overproduction of insulin at low glucose (1 mmol/l) following the high-glucose-cultured control group (16.7 mmol/l) (**Figure 11 b A**). Notably, islets cultured together with rosiglitazone (**Figure 11 b B**) or rosiglitazone + palmitate (**Figure 11 b D**) displayed a highly reduced basal insulin release when they subsequently were incubated at 1 mmol/l glucose while the palmitate group (**C**) did not differ from the control group (**A**) in this respect. When the different groups of cultured islets were challenged with high glucose (16.7 mmol/l) the groups cultured with rosiglitazone, palmitate or rosiglitazone + palmitate, which also displayed GPR40 protein expression (**Figure 10 a and b**), tended to secrete more insulin than 16.7 mmol/l glucose cultured controls (**Figure 11 b**). To further evaluate the impact of rosiglitazone on glucose-stimulated insulin release we analysed the stimulation index (ratio of stimulated insulin released at 16.7 mmol/l glucose over basal release at 1 mmol/l glucose). These results are shown in **Figure 11 c**, were calculated from the data displayed in **Figure 11 b** and revealed that rosiglitazone significantly facilitated the glucose-induced insulin response.

4. Discussion

We show here that the FFA receptor GPR40 is located not only to the insulin producing β-cells (Briscoe et al., 2003,Itoh et al., 2003,Kotarsky et al., 2003,Salehi et al., 2005) and glucagon-producing α-cells (Flodgren et al., 2007) but also to the somatostatin producing δ-cells in the pancreatic islets. Moreover, we show for the first time that young mildly hyperlipidaemic, normoglycaemic fa/fa rats display a markedly increased expression of GPR40 protein levels in all three types of their pancreatic endocrine cells as compared with the healthy controls, while the young normolipidaemic, hyperglycaemic GK rats were almost devoid of GPR40 expression in their islets strongly suggesting not only FFA but in particular glucose being of utmost importance for the regulation of GPR40 protein expression and activity. There was no apparent difference in GPR40 expression pattern between the two categories of control rats *i.e.* the fa/fa controls and the GK controls thus excluding any strain

specific factors. Moreover, concentration-response studies with palmitate-stimulated release of insulin and glucagon as well as the suppression of somatostatin secretion showed a striking correlation between the secretory pattern and the GPR40 protein expression pattern in the islets of the two animal models with different *in vivo* metabolic disturbances. We could show, also for the first time, that long-term culture (24 h) of normal rat islets at high glucose abrogated GPR40 protein expression in the β-cells being associated with an almost total suppression of palmitate-stimulated insulin release. Finally, we found that rosiglitazone, which is considered a full antagonist of palmitate-induced insulin secretion in both short-term and long-term aspects (Abaraviciene et al., 2008,Meidute Abaraviciene et al., 2008) could induce GPR40 protein expression and might serve as a partial agonist in glucose-stimulated insulin secretion.

It was shown several years ago (Lee et al., 1994) that the preobese, prediabetic male ZDF (fa/fa) rat displays hyperinsulinaemia, hypertriglyceridaemia and increased circulating levels of FFA (between 1-2 mmol/l) at the age of 6-7 weeks, while their blood glucose level is normal and does not start to rise until at the age of ~9 weeks. It should be recalled that the fa/fa rats lack functional leptin receptors on their β-cells, which, similar to the total lack of leptin as in the obese *ob/ob* mouse, conceivably contributes to elevated insulin levels (Jimenez-Feltstrom et al., 2011). By using fa/fa rats between 6-8 weeks of age we could study GPR40 protein expression and its functional consequences in islet endocrine cells in the hyperlipidaemic, normoglycaemic, prediabetic stage of this animal model under the early development of spontaneous type 2 obese diabetes. Our finding of an abundance of GPR40 protein expression in islet endocrine cells in the young fa/fa rat most likely strongly contributes to the effects of elevated FFA levels to induce hypersecretion of insulin and glucagon. Note that this hypersecretion probably is augmented by an increased FFA-induced inhibition of somatostatin release, a paracrine effect being favourable for a further increment of insulin and glucagon release. Hence GPR40 protein is a most important player in FFA-induced hormone secretion from all three types of these blood glucose regulating endocrine cells. Such a hypothesis was further strengthened by our unexpected finding of an extremely low GPR40 protein expression in insulin, glucagon and somatostatin cells of the hyperglycaemic GK rat. Thus high circulating glucose in these animals was associated with an almost total absence of any effect of palmitate on the secretion of all three hormones. Notably, our young GK rats of the Stockholm colony show a normal content of insulin in their islets (Salehi et al., 1999) and their hyperglycaemia is associated with and most probably the

consequence of a deficient insulin response to glucose (Mosen et al., 2008,Ostenson et al., 1993,Salehi et al., 1999,Salehi et al., 2008). Hence there is an important question whether *in vivo* hyperglycaemia by itself and not only variations in circulating levels of FFA and other lipids is an important regulator of GPR40 protein expression because the plasma lipids (including FFA) in the GK rats are within the same normal range as in their Wistar controls (present data and Briaud et al., 2002,Zhou et al., 1995).

In the present investigation such a hypothesis was indeed further strengthened by our finding that culture of normal Wistar islets for 24 h at high glucose almost abolished the normal pattern of GPR40 protein expression in β-cells seen after culture in a normal physiological glucose concentration (5 mmol/l). Thus, not only long-chain FFA but in particular glucose seems to have a profound regulatory influence on GPR40 protein expression in the β-cells and hence on islet hormone secretion. This is most conceivable since both FFA and glucose are known to influence signalling pathways operating through IP_3-Ca^{2+} and DAG (Abaraviciene et al., 2008,Briaud et al., 2002,Meidute Abaraviciene et al., 2008,Morgan and Dhayal, 2009). Moreover, culture of normal Wistar islets in high glucose almost abrogated a folllowing acute palmitate-induced insulin release thus being in accordance with the great suppression of GPR40 protein expression in their β-cells. Our culture experiments also showed that different concentrations of palmitate can induce unexpected and complex effects on GPR40 mRNA *vs* GPR40 protein expression suggesting that posttranslational regulation and modification at the GPR40 protein level in modulating palmitate-induced hormone secretion is crucial and should be attended to. Interestingly, the very low levels of GPR40 protein in the β-cells of GK rats shown in the present report might explain a previous finding (Briaud et al., 2002) showing that high-fat feeding with ensuing elevation of plasma FFA in such rats resulted in a reduced insulin response to glucose thus suggesting that a FFA-induced facilitation of insulin release was not operating. Moreover, the insulin response to glucose and arginine both *in vitro* and *in vivo* is greatly reduced in young GK rats being a non-obese variant of type 2 diabetes (Mosen et al., 2008,Ostenson et al., 1993,Salehi et al., 1999,Salehi et al., 2008). Although these secretory defects in the β-cells of the GK rat most certainly are of multiple origin including for example excessive islet NO production (Mosen et al., 2008,Salehi et al., 2008) the absence of GPR40 protein could conceivably be contributing since deletion of GPR40 in mice results in a reduced insulin response to both glucose and arginine (Alquier et al., 2009) without affecting intracellular fuel metabolism in the islets. In this context it is highly interesting that

recent studies with isolated human islets (Del Guerra et al., 2010) have shown that the GPR40 gene expression in type 2 diabetes was significantly lower than in control islets as was glucose-induced insulin release.

We have previously found, in normal mouse islets, that palmitate-induced effects on β-cell function were almost fully dependent on GPR40 both in short-term and long-term aspects and that knock-down of GPR40 totally abolished GPR40 staining and palmitate-induced insulin release in parallel (Abaraviciene et al., 2008,Meidute Abaraviciene et al., 2008). We also observed that the long-term effects of palmitate to impair β-cell function via GPR40 include excessive generation of islet NO which is a strong negative modulator of nutrient-stimulated insulin release (Abaraviciene et al., 2008,Meidute Abaraviciene et al., 2008,Mosen et al., 2008,Salehi et al., 2001,Salehi et al., 2008,Shimabukuro et al., 1997). This was associated with a palmitate-induced marked activation of proapoptotic MAPKs *i.e.* SAPK/JNK and p38 as well as also caspase-3 activity (Abaraviciene et al., 2008). Furthermore, we showed that these palmitate-induced deleterious effects on the β-cell could be almost fully counteracted by the antidiabetic drug rosiglitazone, which was found to serve as a GPR40 antagonist against palmitate, and thus that different thiazolidinedione derivatives might be considered of therapeutic and preventive value in the treatment of chronic FFA-induced impairment of β-cell function in hyperlipidaemia-linked type 2 diabetes. This would be in accordance with a previous report (Finegood et al., 2001) showing that treatment of young obese fa/fa rats with rosiglitazone has a preventive action on their β-cells preserving the β-cell mass by maintaining β-cell proliferation and preventing increased β-cell death.

Previous studies have shown highly controversial results concerning whether agonists or antagonists to GPR40 would be of therapeutic value to counteract the gluco- and lipotoxic effects encountered in many patients with type 2 diabetes. Small molecule GPR40 agonists derived from thiazolidinedione compounds were shown to potentiate glucose-stimulated insulin release and reduce blood glucose in normal mice (Tan et al., 2008) while a small-molecule GPR40 antagonist (ANT 203) blocked the acute amplifying effects of palmitate on glucose-stimulated insulin release in mouse islets (Brownlie et al., 2008). These results are in accordance with our data on mouse islets showing that the thiazolidinedione rosiglitazone potentiated acute glucose-stimulated insulin release coinciding with a rise in $[Ca^{2+}]_i$, while the amplifying effect of palmitate was suppressed coinciding with a reduction of $[Ca^{2+}]_i$

(Abaraviciene et al., 2008). Moreover, the long-term iNOS-stimulating detrimental effect of palmitate on glucose-induced insulin release after culture of mouse islets was efficiently counteracted at the GPR40 level in the presence of rosiglitazone (Abaraviciene et al., 2008,Meidute Abaraviciene et al., 2008). However, as mentioned in the Introduction part, an array of different genetic manipulations of β-cell GPR40 (deletion or overexpression) have indeed given contradictory and confusing results as to whether GPR40 agonism or antagonism would be of therapeutic value in type 2 diabetes. Importantly, most of these reports are lacking proof of the extent of GPR40 *protein* expression in the different types of islet endocrine cells, and in addition the functional studies are often restricted to the β-cells. We now show that FFA (palmitate)-induced release of islet hormones is highly dependent on and correlated to the extent of GPR40 protein expression being regulated not only by FFA but in particular to a great extent by glucose. Importantly our present experiments with normal rat islets cultured for 24 h in high glucose show that high glucose almost abolished GPR40 protein expression and consequently also any stimulatory effect on insulin release by a subsequent palmitate challenge in the presence of basal glucose. This finding seems to have its clinical counterpart in the type 2 non-obese diabetic GK rat displaying chronic hyperglycaemia and a remarkable abolishment of almost all palmitate effects on islet hormone release. In contrast, the mildly hyperlipidaemic and normoglycaemic obesity-prone fa/fa rat seems to acquire its strong GPR40 protein expression profile in the islets cells prior to its chronic blood glucose elevation and thus during this early stage develop the more lipid-based type of obese prediabetes being suitable for GPR40 antagonist therapy before any hyperglycaemia ensues.

Such a hypothesis is in line with a recent finding (Tsujihata et al., 2011) showing that the newly developed GPR40 agonist TAK-875 augmented plasma insulin and reduced hyperglycaemia in overt diabetic fa/fa rats underlining our finding that chronic hyperglycaemia reduces GPR40 protein expression and thus requires an agonist to improve the GPR40 expression and insulin secretion. In accordance TAK-875 was also found to facilitate insulin release in isolated human islets at high glucose and there is now preliminary clinical evidence for a glucose-lowering potential of TAK-875 in type 2 diabetes (Tsujihata et al., 2011). These results are in line with our present finding showing that culture at high glucose together with rosiglitazone induced the reappearance of GPR40 protein expression and thus turned this palmitate- and lipotoxicity-antagonist into a partial agonist with regard to glucose-stimulated insulin release. Notably, since rosiglitazone is a highly potent palmitate antagonist it cannot be excluded that our present results showing a marked suppression of

basal insulin release at 1 mmol/l glucose following culture of islets at 16.7 mmol/l glucose is due to an inhibitory effect by this drug on islet-derived FFA mobilized at this low glucose concentration via an autocrine/paracrine mechanism.

Hopefully our initial studies presented here on spontaneous type 2 diabetes in two highly different animal models and the additional finding of a total abolishment of GPR40 protein levels in normal islets cultured at high glucose might pave the way for future more detailed studies concerning the interaction of lipids and glucose on the regulation of GPR40 protein levels and hormone secretion in pancreatic endocrine cells in relation to the pathogenesis of human obesity and different variants of type 2 diabetes.

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Legends to the figures

Figure 1. *Confocal microscopy and fluorescence intensity measurements of freshly isolated islets from control and fa/fa rats.*

The islets from control (**a**) or fa/fa rats (**b**) were double-immunolabelled for insulin (A and J), glucagon (D and M), somatostatin (G and P) and GPR40 protein (B, E, H, K, N and Q). Insulin, glucagon and somatostatin stainings appear as *red* and GPR40 staining appears as *green* fluorescence. Co-localization of insulin/GPR40, glucagon/GPR40 and somatostatin/GPR40 is seen as *orange-yellowish* fluorescence(C, F, I, L, O and R). Bars indicate lengths (20 μm). Intensity measurements (**c**) expressed as percent of controls. (n=15 in each category) *** $p<0.001$.

Figure 2. *Relative GPR40 mRNA quantification of freshly isolated islets from control and fa/fa rats and GPR40 protein expression including densitogram representation.*

Expression of GPR40 mRNA (**a**) in islets isolated from control (white bar) and fa/fa rats (black bar). Data are presented as relative expression of GPR40 to GAPDH for 6 different observations (**a**). A representative example of Western blots of islets taken from control or fa/fa rats and incubated with GPR40 antibody is also shown (**b**). The blots were performed with 25 μg of islet protein on each lane. Arrow indicates the molecular weight of GPR40 (~ 32 kDa). A densitometric quantitative analysis of the GPR40 protein bands are shown in (**c**). (n=6 in each group) ** $p<0.01$.

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

Concentration-dependent effects of palmitate on insulin (**a**), glucagon (**b**) and somatostatin (**c**) secretion measured from freshly isolated islets from control or fa/fa rats incubated for 60 min. The medium glucose concentration was 5 mmol/l. Means ± SEM for 10 incubated batches of islets on each point are shown. * $p<0.05$, ** $p<0.01$, *** $p<0.001$.

Figure 4. *Confocal microscopy and fluorescence intensity measurements of freshly isolated islets from control Wistar and GK rats.*

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

somatostatin/GPR40 are seen as *orange-yellowish* fluorescence (C, F, I, L, O and R). Bars indicate lengths (20 μm). Intensity measurements (**c**) expressed as percent of controls. (n=15 in each category) *** p<0.001.

Figure 5. *Relative GPR40 mRNA quantification of freshly isolated islets from Wistar and GK rats and GPR40 protein expression including densitogram representation.*
Expression of GPR40 mRNA (**a**) in islets isolated from Wistar control (white bar) and GK rats (black bar). Data are presented as relative expression of GPR40 to GAPDH for 6 different observations (**a**). A representative example of Western blots of islets taken from Wistar control or GK rats and incubated with GPR40 antibody is also shown (**b**). The blots were performed with 25 μg of islet protein on each lane. Arrow indicates the molecular weight of GPR40 (~ 32 kDa). A densitometric quantitative analysis of the GPR40 protein bands are shown in (**c**). (n=6 in each group) ** p<0.01.

Figure 6. *Concentration-dependent modulation of hormone release by palmitate from freshly isolated islets from Wistar and GK rats.*
Concentration-dependent effects of palmitate on insulin (**a**), glucagon (**b**) and somatostatin (**c**) secretion measured from freshly isolated islets from Wistar or GK rats incubated for 60 min. The medium glucose concentration was 5 mmol/l. Means ± SEM for 10 incubated batches of islets on each point are shown. ** p<0.01, *** p<0.001.

Figure 7. *Confocal microscopy and fluorescence intensity measurements of isolated islets from Wistar control rats cultured for 24 h at different glucose or palmitate concentrations.*
The islets from Wistar control rats were cultured at (**a**) 5 mmol/l glucose (A-C) or 16.7 mmol/l glucose (D-F) and (**b**) 5 mmol/l glucose + 100 μmol/l palmitate (G-I) or 5 mmol/l glucose + 1000 μmol/l palmitate (J-L). The islets were double-immunolabelled for insulin (A, D, G and J), and GPR40 protein (B, E, H, and K). Insulin, staining appears as *red* and GPR40 staining appears as *green* fluorescence. Co-localization of insulin/GPR40 is seen as *orange-yellowish* fluorescence(C, F, I, and L). Bars indicate lengths (20 μm). Intensity measurements (**c**) expressed as percent of controls (5 mmol/l glucose) (n=10 in each group). *** p<0.001.

Figure 8. *Relative GPR40 mRNA quantification of isolated islets from Wistar controls cultured for 24 h at different glucose or palmitate concentrations and GPR40 protein expression as well as densitometric quantification of the bands.*

Expression of GPR40 mRNA (**a**) in islets isolated from Wistar rats cultured at different glucose or palmitate concentrations. Data are presented as relative expression of GPR40 to GAPDH for 4 different observations (**a**). A representative example of Western blots of GPR40 expression in islets taken from Wistar control rats from the different categories of culture treatments is shown (**b**). The blots were performed with 25 μg of islet protein on each lane. Arrow indicates the molecular weight of GPR40 (~ 32 kDa). A densitometric quantitative analysis of the GPR40 protein bands are shown in (**c**). ** $p<0.01$, *** $p<0.001$.

Figure 9. *The effect of basal or high glucose levels during a 24 h islet culture period on a subsequent palmitate-stimulated insulin release.*

Influence of 24 h culture of Wistar control islets at basal (5 mmol/l) or high (16.7 mmol/l) glucose on a subsequent (60 min) palmitate-stimulated (250 μmol/l) insulin release. Controls at 5 mmol/l glucose are included. Means ± SEM for 12 incubated batches of islets in each group are shown. *** $p<0.001$.

Figure 10. *Confocal microscopy including intensity measurements of isolated islets from Wistar control rats cultured for 24 h at basal glucose, high glucose ± rosiglitazone or high glucose + rosiglitazone ±. palmitate*

(a) The islets from Wistar control rats were cultured at 5 mmol/l glucose (A-C) or 16.7 mmol/l glucose (D-F), 16.7 mmol/l glucose + 1 μmol/l rosiglitazone (G-I), 16.7 mmol/l glucose + 1000 μmol/l palmitate (J-L) or 16.7 mmol/l glucose +1 μmol/l rosiglitazone + 1000 μmol/l palmitate (M-O). The islets were double-immunolabelled for insulin (A, D, G, J and M), and GPR40 protein (B, E, H, K and N). Insulin staining appears as *red* and GPR40 staining appears as *green* fluorescence. Co-localization of insulin/GPR40 is seen as *orange-yellowish* fluorescence(C, F, I, L and O). Bars indicate lengths (20 μm).

(b) Intensity measurements expressed as percent of control (5 mmol/l glucose). (n=5-8 in each group) * $p<0.05$, *** $p<0.001$.

Figure 11. *Insulin release after an islet culture period of 24 h at high glucose or high glucose + palmitate in the absence or presence of rosiglitazone as well as insulin secretion pattern at low (1 mmol/l) or high (16.7 mmol/l) glucose in a subsequent short-time (60 min) incubation*

(a) The effect of high glucose or high glucose + palmitate in the absence or presence of rosiglitazone on insulin release during a 24 h culture period. There were 240 islets in each category.

(b) Insulin release at low (1 mmol/l) or high (16.7 mmol/l) glucose following a short-time (60 min) incubation of the cultured islets from the different experimental groups. There were 8-12 tubes in each group containing 12 islets per tube.

(c) Illustration of the stimulation index (ratio of stimulated insulin release at 16.7 mmol/l glucose over basal release at 1 mmol/l glucose) for isolated islets shown in Figure **11b**. Means ± SEM are shown. * $p<0.05$, *** $p<0.001$

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

	Glucose mmol/l	**Triglycerides mmol/l**	**Cholesterol mmol/l**	**HDL mmol/l**	**LDL mmol/l**	**Insulin pmol/l**
Control	6.1 ± 0.64	0.86 ± 0.14	1.70 ± 0.08	1.22 ± 0.07	0.59 ± 0.14	79.3 ± 8.6
fa/fa	5.7 ± 0.60	1.25 ± 0.11 *	2.10 ± 0.07 *	1.49 ± 0.16 *	0.35 ± 0.13 *	112.1 ± 25.9

Table 1B. Concentrations of serum glucose, triglycerides, cholesterol, HDL, LDL and insulin in Wistar control and GK rats. Means ± SD of 5-7 rats in each group. ***p<0.001 *vs* Wistar control.

	Glucose mmol/l	**Triglycerides mmol/l**	**Cholesterol mmol/l**	**HDL mmol/l**	**LDL mmol/l**	**Insulin pmol/l**
Control	5.7 ± 0.60	0.99 ± 0.10	1.88 ± 0.23	1.15 ± 0.13	0.55 ± 0.13	80.2 ± 11.2
GK	11.3 ± 1.9***	0.88 ± 0.07	1.81 ± 0.13	1.09 ± 0.10	0.53 ± 0.09	91.9 ± 23.0

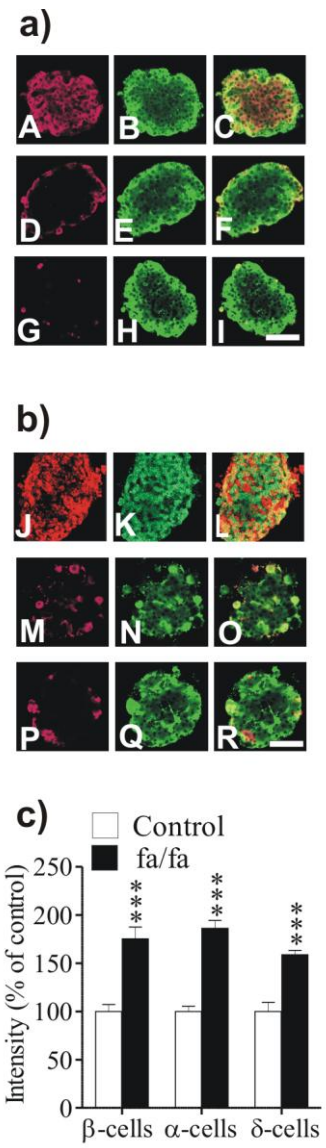
a)
A
B
C
D
E
F
G
H
I
b)
J
K
L
M
N
O
P
Q
R
c)
Control
fa/fa
Intensity (% of control)
250
200
150
100
50
0
***
***
***
β-cells
α-cells
δ-cells

Figure 1

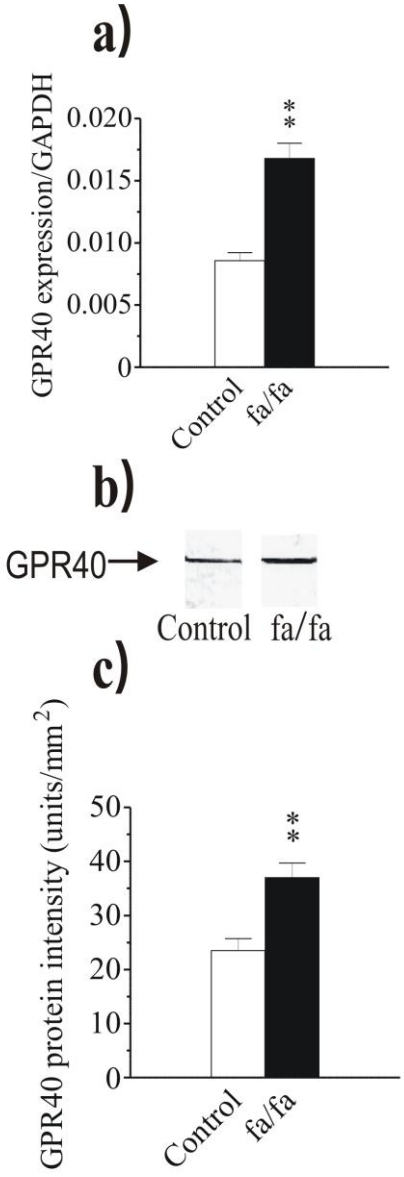
a)
GPR40 expression/GAPDH
0.020
0.015
0.010
0.005
0
**
Control
fa/fa
b)
GPR40
Control
fa/fa
c)
GPR40 protein intensity (units/mm2)
50
40
30
20
10
0
**
Control
fa/fa

Figure 2

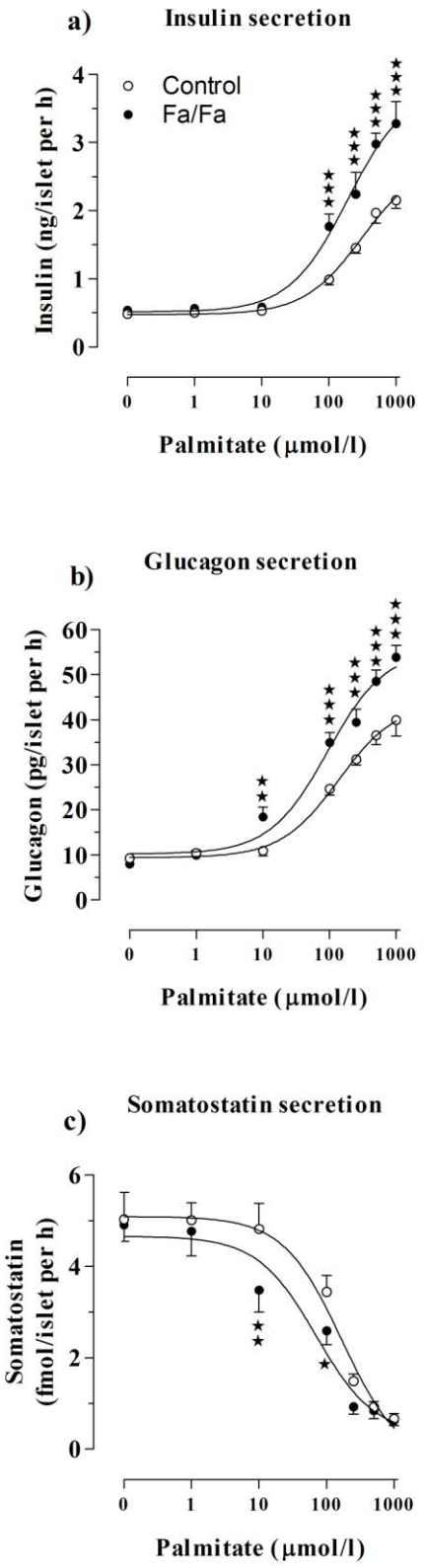
a)
Insulin secretion
Control
Fa/Fa
Insulin (ng/islet per h)
0
1
2
3
4
0
1
10
100
1000
Palmitate (µmol/l)
b)
Glucagon secretion
Glucagon (pg/islet per h)
0
10
20
30
40
50
60
0
1
10
100
1000
Palmitate (µmol/l)
c)
Somatostatin secretion
Somatostatin (fmol/islet per h)
0
2
4
6
0
1
10
100
1000
Palmitate (µmol/l)

Figure 3

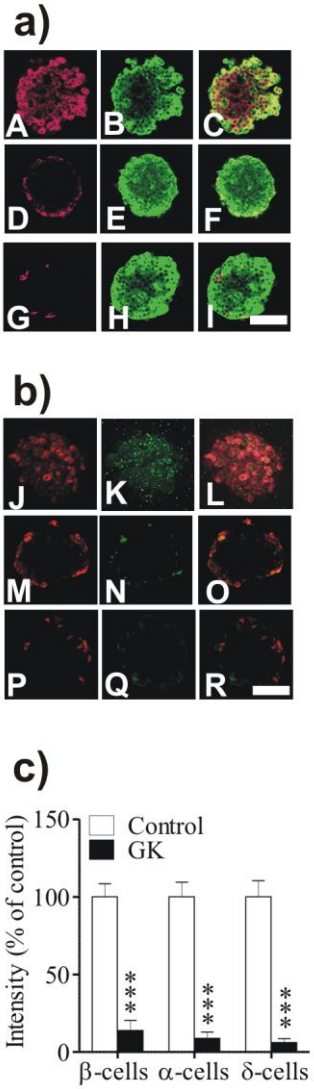
a)
A
B
C
D
E
F
G
H
I
b)
J
K
L
M
N
O
P
Q
R
c)
Control
GK
Intensity (% of control)
150
100
50
0
***
***
***
β-cells
α-cells
δ-cells

Figure 4

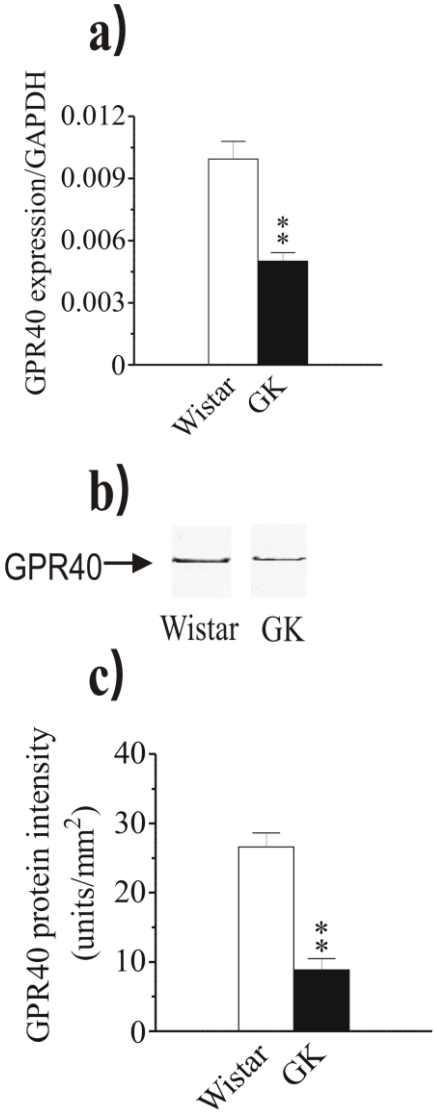
a)
GPR40 expression/GAPDH
0.012
0.009
0.006
0.003
0
**
Wistar
GK
b)
GPR40
Wistar
GK
c)
GPR40 protein intensity
(units/mm2)
40
30
20
10
0
**
Wistar
GK

Figure 5

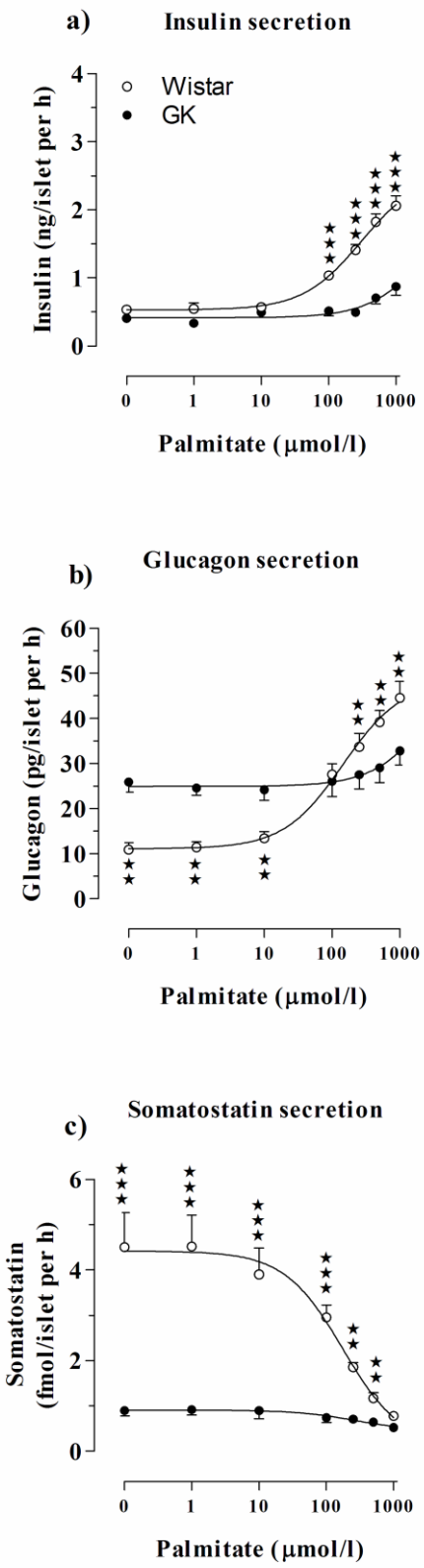
a)
Insulin secretion
Wistar
GK
Insulin (ng/islet per h)
0
1
2
3
4
0
1
10
100
1000
Palmitate (µmol/l)
b)
Glucagon secretion
Glucagon (pg/islet per h)
0
10
20
30
40
50
60
0
1
10
100
1000
Palmitate (µmol/l)
c)
Somatostatin secretion
Somatostatin (fmol/islet per h)
0
2
4
6
0
1
10
100
1000
Palmitate (µmol/l)

Figure 6

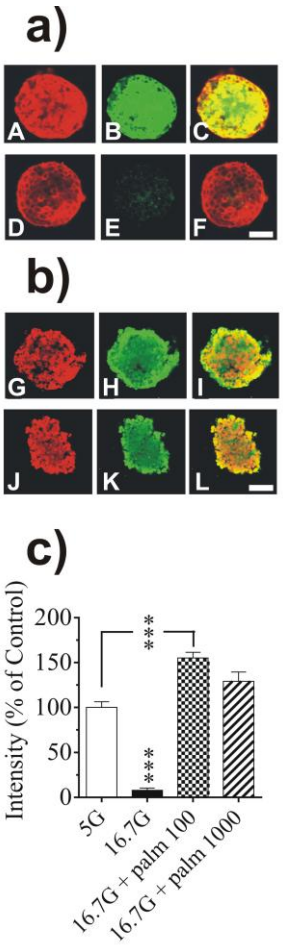
a)
A
B
C
D
E
F
b)
G
H
I
J
K
L
c)
Intensity (% of Control)
200
150
100
50
0
***
***
5G
16.7G
16.7G + palm 100
16.7G + palm 1000

Figure 7

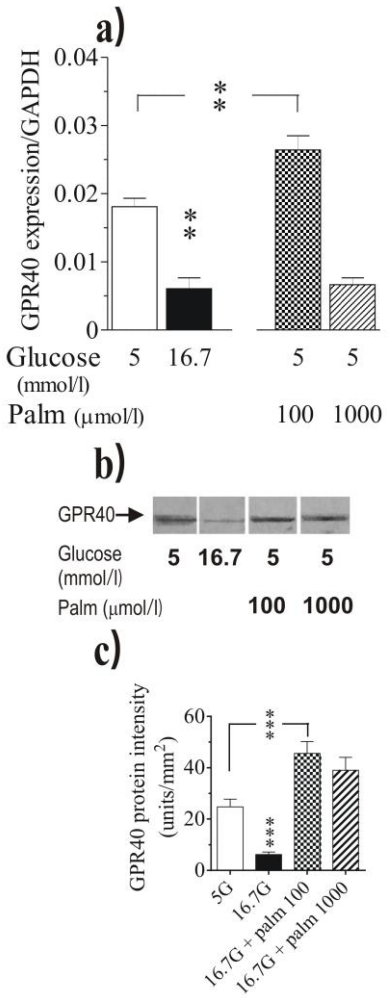
a)
GPR40 expression/GAPDH
0.04
0.03
0.02
0.01
0
**
**
Glucose (mmol/l)
5
16.7
5
5
Palm (μmol/l)
100
1000
b)
GPR40
Glucose (mmol/l)
5
16.7
5
5
Palm (μmol/l)
100
1000
c)
GPR40 protein intensity (units/mm2)
60
40
20
0
***
***
5G
16.7G
16.7G + palm 100
16.7G + palm 1000

Figure 8

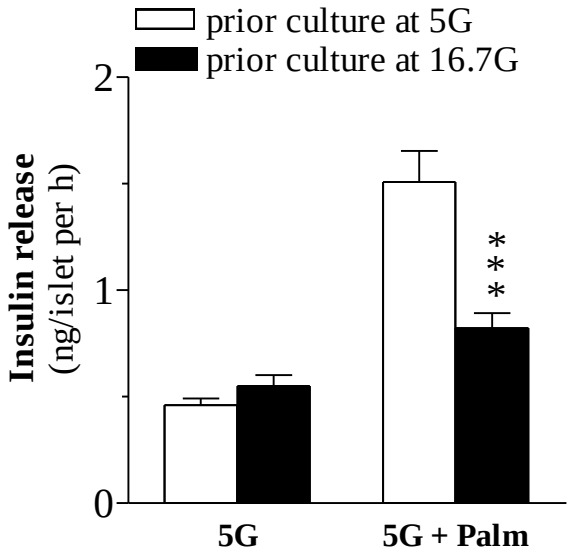
prior culture at 5G
prior culture at 16.7G
Insulin release
(ng/islet per h)
2
1
0
***
5G
5G + Palm

Figure 9

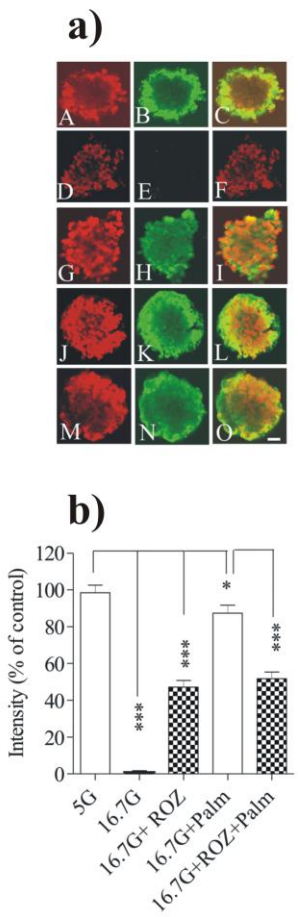
a)
A
B
C
D
E
F
G
H
I
J
K
L
M
N
O
b)
Intensity (% of control)
120
100
80
60
40
20
0
***
***
*
***
5G
16.7G
16.7G+ ROZ
16.7G+Palm
16.7G+ROZ+Palm

Fig10

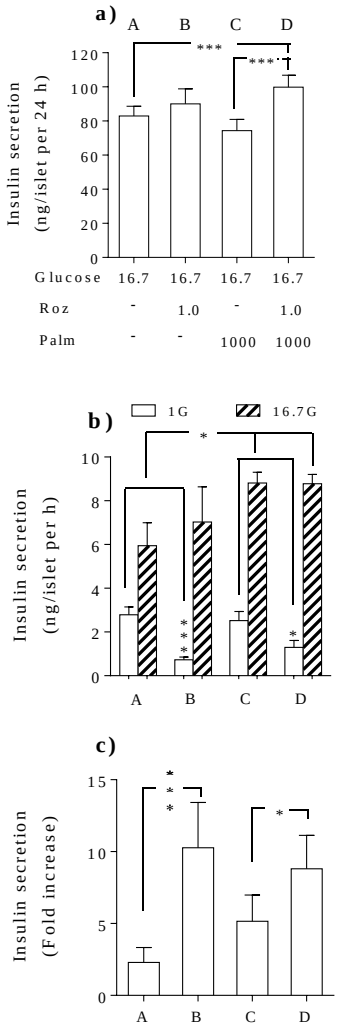
a)
A B C D
***
***
Insulin secretion
(ng/islet per 24 h)
120
100
80
60
40
20
0
Glucose 16.7 16.7 16.7 16.7
Roz - 1.0 - 1.0
Palm - - 1000 1000
b)
1G
16.7G
*
Insulin secretion
(ng/islet per h)
10
8
6
4
2
0
***
*
A B C D
c)
***
*
Insulin secretion
(Fold increase)
15
10
5
0
A B C D

Fig 11

Highlights:

- We studied the GPR40 expression in two different animal models of spontaneous type 2 diabetes.
- Confocal microscopy of pancreatic islets showed expression of GPR40 protein in insulin, glucagon and somatostatin cells.
- GPR40 expression was strongly increased in islets of hyperlipidaemic fa/fa rats which also coincided with increased insulin and glucagon and a reduced somatostatin release.
- Hyperglycaemic GK islets displayed an extremely faint expression of GPR40 which was also reflected in an abolished FFA-induced hormone response in GK islets.
- The thiazolidinedione derivative is a GPR40 antagonist in FFA-induced insulin release but a partial agonist in glucose-stimulated insulin release.

Graphical Abstract (for review)

Islet GPR40 protein expression is regulated by both FFA and glucose

Hyperglycaemia	Mild hyperlipidaemia
↓	↓
Reduced GPR40 expression	Increased GPR40 expression
↓	↓
Reduced FFA- and Glucose-stimulated insulin release	Increased FFA- and Glucose-stimulated insulin release
↓	↓
Risk for GLUCOTOXICITY	Risk for obesity and LIPOTOXICITY

Conflict of Interest

There is no conflict of interest.