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ORIGINAL ARTICLE

The L-α-amino acid receptor GPRC6A is expressed in the islets of Langerhans but is not involved in L-arginine-induced insulin release

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Abstract GPRC6A is a seven-transmembrane receptor activated by a wide range of L-α-amino acids, most potently by L-arginine and other basic amino acids. The receptor is broadly expressed, but its exact physiological role remains to be elucidated. It is well established that L-arginine stimulates insulin secretion; therefore, the receptor has been hypothesized to have a role in regulating glucose metabolism. In this study, we demonstrate that GPRC6A is expressed in islets of Langerhans, but activation of the receptor by L-arginine did not stimulate insulin secretion. We also investigated central metabolic parameters in GPRC6A knockout mice compared with wildtype littermates and found no difference in glucose metabolism or body fat percentage when mice were administered a standard chow diet. In conclusion, our data do not support a role for GPRC6A in L-arginine-induced insulin release and glucose metabolism under normal physiological conditions.

Keywords GPRC6A · L-Arginine · Insulin release

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Introduction

GPRC6A is the newest member of the family C of seven-transmembrane (7TM) receptors, also termed G protein-coupled receptors (GPCRs) (Wellendorph and Bräuner-Osborne 2004). It has been cloned and characterized in human, mouse, and rat, and predicted in several other mammalian species (Wellendorph and Bräuner-Osborne 2004; Kuang et al. 2005; Christiansen et al. 2007; Wellendorph et al. 2007). The deorphanization of GPRC6A revealed that the receptor is activated by L-α-amino acids, most potently by L-arginine and other basic amino acids (Wellendorph et al. 2005). Activation of the receptor by the L-α-amino acids is potentiated by divalent cations such as Ca^{2+} and Mg^{2+} in physiologically relevant concentrations (Kuang et al. 2005; Christiansen et al. 2007; Wellendorph et al. 2007). Recently, GPRC6A has also been suggested to act as receptor for testosterone and osteocalcin (Pi et al. 2010; Pi et al. 2011; Oury et al. 2011).

Several studies have examined the tissue expression in human, mouse, and rat, and a number of tissues evidently express GPRC6A, although with some discrepancies and differences between species (Wellendorph and Bräuner-Osborne 2004; Wellendorph et al. 2007; Kuang et al. 2005; Pi et al. 2005; Regard et al. 2007; Luo et al. 2010; Bystrova et al. 2010; Haid et al. 2011). However, the physiological importance of the receptor is still elusive. GPRC6A is phylogenetically related to the calcium-sensing receptor (CaR) and the widespread expression of GPRC6A is

somewhat similar to that of the CaR (Kuang et al. 2005; Wellendorph et al. 2005; Mitsuma et al. 1999). Thus, given the known physiological importance of the CaR as a metabolic regulator (Tfelt-Hansen and Brown 2005), it is tempting to speculate that GPRC6A could also be implicated in regulation of metabolism. In favour of this hypothesis, mice lacking GPRC6A have been reported to have complex metabolic abnormalities including hyperglycemia, decreased serum insulin levels, glucose intolerance, and insulin resistance (Pi et al. 2008). Moreover, GPRC6A has recently been shown to mediate osteocalcin-induced insulin release in vivo (Pi et al. 2011).

It is well known that amino acids such as L-arginine stimulate insulin release in vitro and in vivo (Palmer and Ensinck 1975; Blachier et al. 1989; Gordin et al. 1977; Floyd et al. 1966). To date, however, the precise molecular mechanisms by which amino acids regulate insulin secretion are still unsolved. In addition, it is well known that protein or amino acid intake stimulates release of glucagon-like peptide 1 (GLP-1) in vivo (Tolhurst et al. 2009; Nilsson et al. 2007), which in turn stimulates the release of insulin.

Overall, this could point to a function of the GPRC6A receptor in metabolic regulation, potentially through mediating L-arginine-induced insulin release.

In the present study, we investigated whether the receptor is expressed in islets of Langerhans and whether it is involved in L-arginine-induced insulin release. In addition, we also performed body composition and metabolic characterization of our global GPRC6A knockout (KO) mice.

Materials and procedures

Animals

For the targeted disruption of GPRC6A, exon 6, containing the entire 7TM domain and C-terminal tail of the gene, was deleted as described previously (Wellendorph et al. 2009). Mice were backcrossed for six generations into the C57BL/6 background, and all studies performed in male mice.

Mice were housed in a climate-controlled animal facility (25 °C, 55–60 % humidity, 12 h light: 12 h darkness cycle) and had free access to regular chow and water. All animal experiments were carried out in accordance with the European Communities Council Resolves of 24 November 1986 (86/609/ECC) and approved by the Danish Animal Experiments Inspectorate (J. No. 2006/561-1232).

RT-PCR

Tissues from 17- to 19-week-old GPRC6A KO and WT littermates were isolated and total RNA was extracted using RNeasy Mini Kit (Qiagen, Sollentuna, Sweden). Total RNA was used for first-strand cDNA synthesis (QuantiTect Reverse Transcription Kit, Qiagen, Sollentuna, Sweden), according to the protocols of the manufacturer. Intron V-spanning primers specific for exon 5 (P4: 5′-gccctggtcaaatgaagaaa-3′) and 6 (P5: 5′-tgatgtagcccag catggta-3′) were used for the RT-PCR using an optimized PCR protocol (94 °C for 5 min predenaturation, then 94 °C for 30 s, annealing at 57 °C for 30 s and extension at 72 °C for 1 min 20 s for 45 cycles). RT-PCR products were separated on 1 % w/v agarose gels and detected with SYBR safe (Invitrogen, Taastrup, Denmark).

Hormone and blood chemistry

Plasma glucose was measured using a portable Glucometer Contour (Bayer, Leverkusen, Germany). Plasma insulin was measured using ultra sensitive mouse insulin ELISA kit (Crystal Chem Inc., Downers Grove, IL, USA).

Islet isolation and culturing

After cervical dislocation of mice, the pancreas was dissected and placed in ice-cold HBSS buffer (Invitrogen, Taastrup, Denmark) supplemented with 10 mM HEPES (pH 7.4). The pancreas was cut into small pieces and digested with 1 mg/ml Collagenase A (Roche, Hvidovre, Denmark) in a shaking water bath at 37 °C for 22 min. Thereafter, washing buffer (HBSS supplemented with 10 mM HEPES (pH 7.4) and 0.5 % NCS) was added and the tissue solution was handshaken vigorously about 20 times. The solution was centrifuged at 1,100 rpm for 1 min and the supernatant removed. The pellet was dissolved in washing buffer by handshaking. Thereafter, the tissue solution was washed twice with washing buffer, allowing the sedimentation for 5 min in between each washing step. The islets of Langerhans were handpicked under a dissecting microscope and incubated in RPMI 1640 cell culture medium supplemented with 10 % newborn calf serum (both from Invitrogen, Taastrup, Denmark) at 37 °C in a humidified atmosphere containing 5 % CO_2.

Insulin secretion in isolated islets

The experiments were carried out in 96-well multiscreen filter plates using five islets per well. The islets were transferred to the plates 2 days after isolation and washed with Krebs–Ringer buffer (129 mM NaCl, 5 mM $NaHCO_3$, 4.8 mM KCl, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4$, 10 mM HEPES, 2.5 mM $CaCl_2$, 0.1 % BSA and 11 mM D-glucose). The islets were pre-incubated with Krebs–Ringer buffer for 1 h at 37 °C followed by incubation with/without 20 mM L-arginine and 11 mM D-glucose in Krebs–Ringer buffer for 60 min at 37 °C. At the end of the incubation

period, supernatants were collected and stored at 80 °C until insulin determination.

In vivo physiological studies

For body composition, mice were analyzed with a quantitative magnetic resonance whole-body composition analyser (EchoMRI, Echo Medical Systems, Houston, TX, USA).

Oral glucose tolerance was assessed at 17–19 weeks of age in 6-h fasted animals by measuring tail blood glucose 0, 15, 30, 60, 90, and 120 min after oral administration of 2 g/kg body weight D-glucose by gavage.

Insulin tolerance was measured at 18–20 weeks of age in 6-h fasted animals by measuring tail blood glucose 0, 15, 30, 60, 90, and 120 min after intraperitoneal injection of 0.3 U/kg body weight insulin (Actrapid, Novo Nordisk, Bagsværd, Denmark).

Insulin secretion in response to intravenous L-arginine (0.05 g/kg body weight) was examined in 16- to 18-week-old overnight fasted animals or non-fasting animals. Mice were anesthetized by intraperitoneal injection with 0.8 ml/100 g body weight Hypnorm-Dormicum (25 % Hypnorm (5 mg/ml) + 25 % Dormicum (5 mg/ml) in water), and L-arginine or saline was injected intravenously in a tail vein. Blood samples were drawn from the retrobulbar intraorbital capillary plexus before (0 min) and 1 min after the injection into EDTA coated blood collection tubes (Franklin Lakes, NJ, USA). Plasma was separated by centrifugation and kept at −80 °C until analysis.

Insulin secretion in response to oral D-glucose (3 g/kg body weight) or L-arginine (1 g/kg body weight) was examined in 16–18 weeks old non-anesthetized animals. Mice were fasted overnight and water was removed 1 h before start of the experiment. D-Glucose, L-arginine or saline was administered by gavage. Blood samples were obtained from the retrobulbar intraorbital capillary plexus before gavage (time 0) and 15 min after gavage into EDTA coated blood collection tubes. Plasma was separated by centrifugation and kept at −80 °C until analysis.

Statistics

All data were analyzed with GraphPad Prism using: two-tailed *t* test or two-way ANOVA followed by Bonferroni post-hoc tests. Data are presented as mean ± SEM. $P < 0.05$ was considered to represent a statistically significant difference.

Results

Expression of GPRC6A in islets of Langerhans

First, we investigated expression of GPRC6A messenger RNA (mRNA) in islets of Langerhans using intron-spanning GPRC6A-specific primers. We observed a cDNA product of the expected size (698 bp) (Fig. 1) in islets of Langerhans in the WT tissue, which was absent in the tissue from GPRC6A KO littermates.

Arginine-induced insulin release

To test if the GPRC6A receptor is implicated in L-arginine-induced insulin secretion, we investigated insulin release after intravenous injection of L-arginine. Insulin secretion in response to L-arginine was not significantly different between KO and WT animals either in fasting mice (Fig. 2a) or in mice fed ad libitum (Fig. 2b). Insulin release was maximal 1 min after the injection of L-arginine, and returned to the basal level after 5 min, both in KO and WT animals (see Supplementary Fig. 1).

To study effects of L-arginine isolated from systemic effects, pancreatic islets were isolated from KO and WT mice and incubated with 20 mM L-arginine in the presence of 11 mM D-glucose. As expected from the in vivo studies, no difference in insulin release between KO and WT was observed (Fig. 3). These data show that GPRC6A is not involved in L-arginine-induced insulin release from the pancreas. However, insulin release can also be stimulated indirectly through incretin hormones, such as GLP-1, released from the gut after meal ingestion (Holst 2004). Therefore, we next investigated insulin release after oral

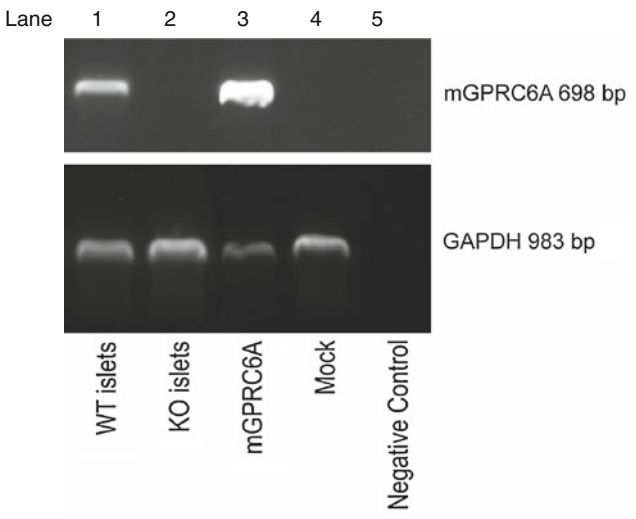

Fig. 1 Evaluation of GPRC6A expression in islet of Langerhans isolated from GPRC6A KO and WT mice. Expression of mGPRC6A and the housekeeping control glycealdehyde-3-phosphate dehydrogenase (GAPDH) was determined by reverse transcriptase PCR (RT-PCR) on mRNA purified from the isolated islets of Langerhans. RT-PCR on tsA201 cells transiently transfected with the mGPRC6A construct (*lane 3*) or empty vector (mock; *lane 4*) were included as controls together with a negative control (PCR amplification without addition of cDNA; *lane 5*)

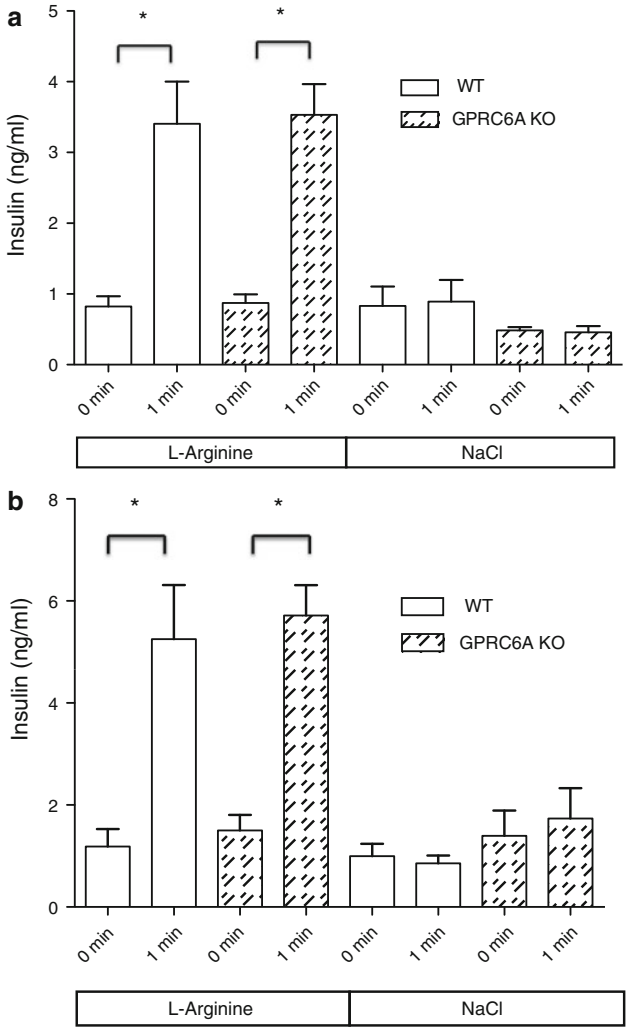

Fig. 2 Insulin secretion after intravenous injection of L-arginine in vivo. L-Arginine (0.05 g/kg) or NaCl (control) was injected intravenously in a tail vein in overnight fasting (**a**) and ad libitum-fed (**b**) male GPRC6A KO and WT mice. Blood samples were drawn from the retrobulbar intraorbital capillary plexus before (0 min) and 1 min after the injection into EDTA coated blood collection tubes, and plasma separated by centrifugation. Data are mean ± SEM, $n = 6$–10 per group; $^*P < 0.05$

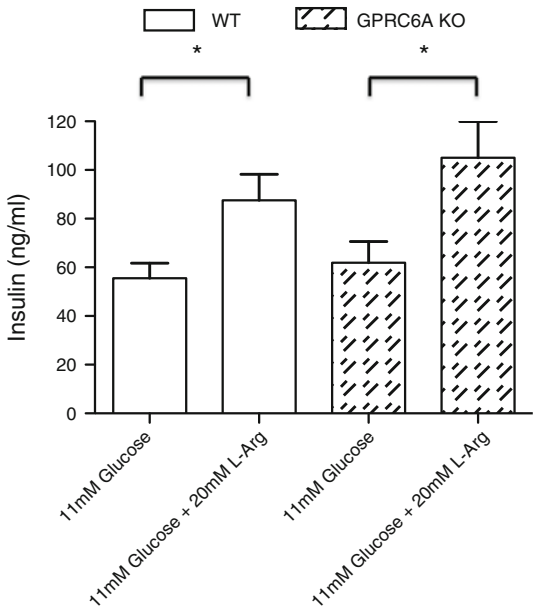

Fig. 3 Arginine-stimulated insulin secretion in the presence of 11 mM glucose in isolated islets of Langerhans from GPRC6A KO and WT male mice. The islets of Langerhans were isolated from five pairs of animals (5 KO, 5 WT) as described in "Materials and procedures". Islets were incubated with 11 mM D-glucose with or without 20 mM L-arginine for 60 min. At the end of the incubation period, supernatants were collected and stored until insulin determination. Data are presented as mean ± SEM in ten incubation wells each with five islets. $^*P < 0.05$

administration of L-arginine or D-glucose. As shown in Fig. 4, insulin concentration increased significantly after stimulation with L-arginine; however, no difference between KO and WT animals was observed.

In conclusion, these data suggest that GPRC6A is not involved in L-arginine-induced insulin release.

Metabolic characteristics of GPRC6A KO mice

Deletion of the gene for GPRC6A had no effect on basal blood glucose and insulin levels in fasting mice or in mice fed ad libitum (data not shown). Glucose clearance curves after oral glucose administration were identical in KO and WT mice (Fig. 5a). Furthermore, no significant difference was observed in insulin sensitivity (Fig. 5b). In conclusion, the absence of GPRC6A does not affect glucose metabolism in mice on normal chow diet.

Body composition of GPRC6A KO mice

Previously, we have shown that GPRC6A KO mice exhibit no significant differences in body weight compared with wildtype (WT) littermates up to an age of 38 weeks when fed on a regular chow diet (Wellendorph et al. 2009). Here, we show that there is no significant difference in body fat percentage between GPRC6A KO mice and their WT littermates (fat percentage at 10 weeks of age: GPRC6A KO 4.4 ± 0.9 %, WT 5.2 ± 0.7 %; fat percentage at 22 weeks of age: GPRC6A KO 7.9 ± 2.9 %, WT 7.0 ± 1.3 %) (Fig. 6).

Discussion

Long-term oral administration of L-arginine has been reported to improve glucose levels, insulin-induced hepatic glucose production, and insulin sensitivity in type 2

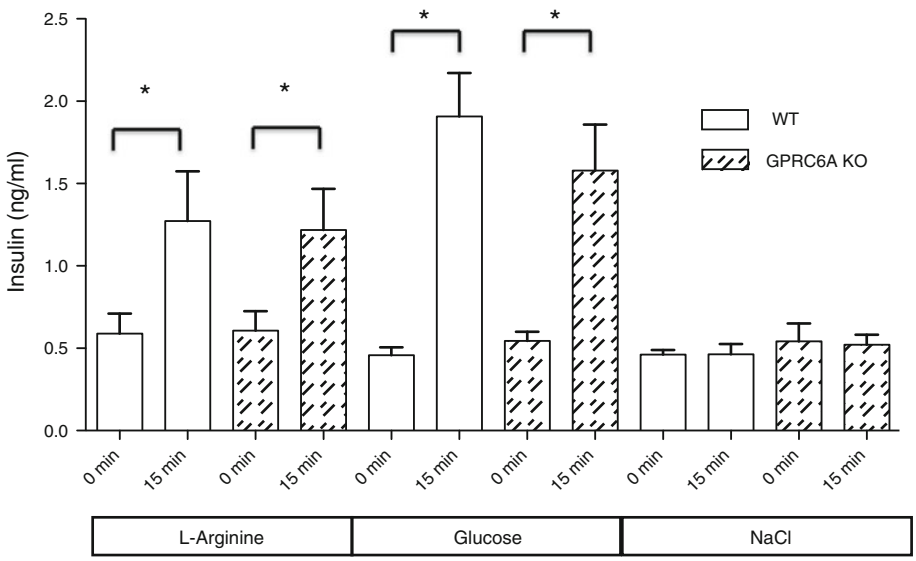

Fig. 4 Insulin secretion after oral administration of L-arginine or D-glucose in vivo. L-arginine (1 g/kg), D-glucose (3 g/kg), or NaCl (control) was administered orally by gavage in overnight fasting male GPRC6A KO and WT mice. Blood samples were obtained from the retrobulbar intraorbital capillary plexus before gavage (time 0) and 15 min after gavage into EDTA coated blood collection tubes, and plasma separated by centrifugation. Data are mean ± SEM, $n = 7$–9 per group; $^*P < 0.05$

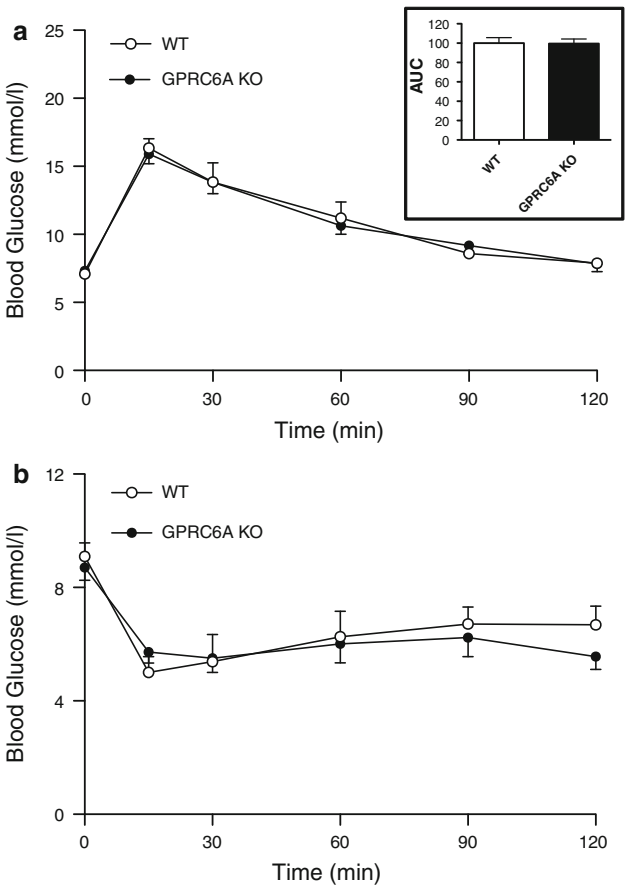

Fig. 5 **a** Oral glucose tolerance test (OGTT) performed with 2 g glucose/kg in 6-h fasted male GPRC6A KO and WT mice. Glucose was measured at the indicated time points in tail blood; Area under the curve (AUC) is shown as an insert. **b** Insulin tolerance test (ITT) performed with 0.5 U insulin/kg administrated i.p. Glucose was measured at the indicated time points in tail blood. Data are mean ± SEM, $n = 6$–10 per group

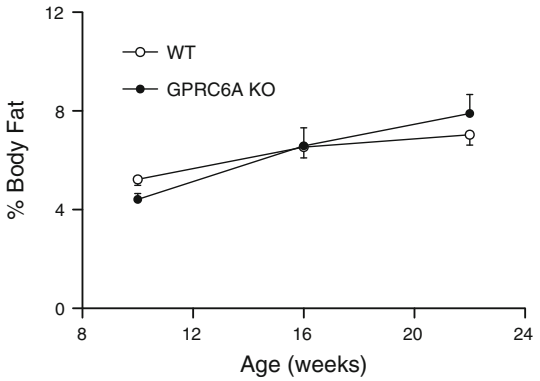

Fig. 6 Percentage body fat in GPRC6A KO and WT mice determined at age 10, 16, and 22 weeks by EchoMRI scans. Data are mean ± SEM, $n = 9$–14 per group

diabetic patients (Lucotti et al. 2006; Piatti et al. 2001), obese rats (Jobgen et al. 2009) and metabolically challenged mice (Clemmensen et al. 2011). It is well known that L-arginine stimulates insulin secretion, but the underlying mechanism remains enigmatic. Several studies have suggested that L-arginine-induced insulin secretion is mediated by β-cell membrane depolarization (Thams and Capito 1999; Henquin and Meissner 1981; Blachier et al. 1989; Hermans et al. 1987; Smith et al. 1997). L-Arginine has been shown to depolarize the plasma membrane in a way that is potentiated by D-glucose (Hermans et al. 1987), and to stimulate Ca^{2+} influx through voltage-sensitive Ca^{2+} channels (Hermans et al. 1987; Smith et al. 1997; Weinhaus et al. 1997). However, L-arginine stimulation of insulin secretion persists in rat islets depolarized by high K^+ (Blachier et al. 1989), suggesting that L-arginine in addition to stimulation of Ca^{2+} influx may stimulate insulin

secretion by other mechanisms. Several GPCRs expressed in islets of Langerhans are known to be involved in regulation of insulin secretion (Ahren 2009) and may thus also mediate the insulin secreting effect of L-arginine.

GPRC6A is a widely expressed GPCR activated by L-amino acids, most potently L-arginine and other basic amino acids (Wellendorph et al. 2005). The main aim of this study was to determine whether GPRC6A is expressed in islets of Langerhans and whether activation of the receptor by L-arginine stimulates insulin secretion.

Discrepant reports about the presence of GPRC6A in islets of Langerhans from mice exist. Regard et al. (2007) reported, using a quantitative real time PCR study, expression of GPRC6A mRNA in mouse and human islets of Langerhans as well as two β-cell lines, and proposed a possible role of the receptor in regulation of insulin secretion. This observation agrees with our previously reported expression of GPRC6A in human pancreas (Wellendorph and Bräuner-Osborne 2004). In addition, a recent study demonstrated GPRC6A mRNA in mouse pancreatic tissue and in a β-cell TC-6 cell line (Pi et al. 2011). However, a study of mouse tissue expression of GPRC6A mRNA investigated by in situ hybridization showed the location of the expression to be mainly in the exocrine tissue of the pancreas and not in the islets of Langerhans (Luo et al. 2010). Therefore, we performed RT-PCR on total RNA from isolated islets of Langerhans. We observed the presence of GPRC6A mRNA in the islets of Langerhans isolated from WT mice, but not from GPRC6A KO mice.

Since we found GPRC6A mRNA to be expressed in the islets of Langerhans and the receptor is known to be activated by L-arginine and other insulin-secreting amino acids, we investigated whether the receptor could have a functional role in regulating the insulin release from the pancreas. The lack of GPRC6A did not have any effect on L-arginine-induced insulin release either in vivo or in isolated islets of Langerhans in our studies. In contrast, a recent study showed that insulin release in response to injection of osteocalcin is markedly attenuated in GPRC6A KO mice compared with WT mice (Pi et al. 2011). These differences might be due to different GPRC6A agonist used and/or different knockout/study models (discussed below).

In addition, we found no differences in basal insulin or glucose levels, glucose tolerance, and insulin sensitivity between GPRC6A KO mice and WT littermates. Interestingly, Pi et al. (2008) found their GPRC6A KO mice to exhibit a mild metabolic syndrome phenotype characterized by hyperglycemia, decreased serum insulin levels, glucose intolerance, and insulin resistance. Albeit their metabolic phenotype was not pronounced, one possible explanation for the observed discrepancies might be the different knockout models used. We created GPRC6A KO mice by deleting exon 6 (containing the entire 7TM domain and C-terminal domain of the receptor), while Pi et al. deleted exon 2 (a small exon that encodes a part of N-terminal tail of the receptor). Different phenotypes between knockout models of the same gene have been reported before (Kaul et al. 2000; Wolfrum et al. 2005; Christoffersen et al. 2008; Persy et al. 2003; Hamamoto et al. 2010). One possible explanation might be that the construction of the knockout gene might affect transcription of neighboring genes. Furthermore, although the knockout models have been made in the same strain (C57BL/6) of mice, the number of backcrossings may vary and there can even be a difference between substrains of the same strain of mice, which may contribute to the discrepancies in phenotypes. A very important difference between the mice from our group and the Quarles group (Pi et al. 2008) is the body composition when fed regular chow diet. They reported their WT mice to have around 20–25 % body fat content, which is high for the C57BL/6 strain of mice on normal chow diet compared with other studies (Guo et al. 2009; Ayala et al. 2008). In contrast, body fat content in our mice was significantly lower (Fig. 1). We speculate whether the observed discrepancies might be because the study by Pi et al. (2008) was conducted in obese mice, representing a pathophysiological condition more than a normal physiological setting, which we have in our study. Furthermore, it is worth noting that the initially reported difference in basal insulin levels between WT and GPRC6A KO mice was highly reduced and almost not present in a recently reported study from the same group (Pi et al. 2011).

In summary, we found the GPRC6A to be expressed in mouse islets of Langerhans. However, although basic amino acids are potent ligands of GPRC6A, the receptor appeared not to be involved in mediating L-arginine-induced insulin secretion, at least not during the conditions used in the present study. This does not rule out that the GPRC6A receptor could have a more subtle role under other conditions. Moreover, no metabolic phenotype was observed in mice lacking the receptor, suggesting that GPRC6A does not possess a vital function in blood glucose homeostasis under normal physiological conditions. However, it should be kept in mind that it is possible that compensatory effects of lifelong global KO of GPRC6A can be effective and mask for a potential role of the receptor in regulation of metabolism. Moreover, the role of GPRC6A was evaluated in a normal physiological setting in this study, and we cannot rule out the appearance of a metabolic phenotype in a pathophysiological condition, such as obesity.

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