GPRC6A Mediates the Effects of L-Arginine on Insulin Secretion in Mouse Pancreatic Islets

Min Pi, Yunpeng Wu, Nataliya I Lenchik, Ivan Gerling, and L. Darryl Quarles

Department of Medicine, University of Tennessee Health Science Center, Memphis, Tennessee 38163

L-Arginine (L-Arg) is an insulin secretagogue, but the molecular mechanism whereby it stimulates insulin secretion from β-cells is not known. The possibility that L-Arg regulates insulin secretion through a G protein-coupled receptor (GPCR)-mediated mechanism is suggested by the high expression of the nutrient receptor GPCR family C group 6 member A (GPRC6A) in the pancreas and TC-6 β-cells and the finding that *Gprc6a*$^{-/]minus]}$ mice have abnormalities in glucose homeostasis. To test the direct role of GPRC6A in regulating insulin secretion, we evaluated the response of pancreatic islets derived from *Gprc6a*$^{-/]minus]}$ mice to L-Arg. We found that the islet size and insulin content were decreased in pancreatic islets from *Gprac6a*$^{-/]minus]}$ mice. These alterations were selective for β-cells, because there were no abnormalities in serum glucagon levels or glucagon content of islets derived from *Gprac6a*$^{-/]minus]}$ mice. Significant reduction was observed in both the pancreatic ERK response to L-Arg administration to *Gprc6a*$^{-/]minus]}$ mice *in vivo* and L-Arg-induced insulin secretion and production *ex vivo* in islets isolated from *Gprc6a*$^{-/]minus]}$ mice. L-Arg stimulation of cAMP accumulation in isolated islets isolated from *Gprc6a*$^{-/]minus]}$ mice was also diminished. These findings suggest that L-Arg stimulation of insulin secretion in β-cells is mediated, at least in part, through GPRC6A activation of cAMP pathways. **(*Endocrinology* 153: 4608–4615, 2012)**

G-protein-coupled receptors (GPCR) are involved in normal pancreatic islet physiology and may be important in the pathophysiology and treatment of diabetes (1). Mouse pancreatic islets express high levels of at least 28 different GPCR, of which several have been shown to regulate insulin secretion in response to a variety of ligands, including adrenergic factors, melatonin, serotonin, acetylcholine, somatostatin, glucagon and glucagon-like peptide, and free fatty acids (2). In general, GPCR that signal through the $G\alpha_q$ and $G\alpha_s$ pathways increase insulin secretion, whereas GPCR that signal through the $G\alpha_i$ pathway inhibit insulin secretion (2).

GPCR family C group 6 member A (GPRC6A) was identified among the 28 receptors expressed in pancreatic islets (3–5). GPRC6A belongs to the C family of GPCR, which are structurally characterized by a very large primitive Venus fly trap extracellular domain that permits these receptors to sense diverse ligands (6–11). Although closely related to the calcium-sensing receptor, GPRC6A has different amino acid and cation specificities (6–13). Ligands for GPRC6A include basic amino acids, such as L-arginine (L-Arg), L-lysine, L-ornithine, and extracellular cations, such as calcium, magnesium, and strontium, as well as calcimimetics, osteocalcin, and testosterone (5, 14–17). There is evidence that GPRC6A is coupled to $G\alpha_i$, $G\alpha_q$, and $G\alpha_s$ pathways in various cell culture models (5, 18).

In addition to expression in pancreatic islets, GPRC6A is present in multiple tissues. *Gprc6a*$^{-/-}$ mice have complex metabolic abnormalities, including obesity, glucose intolerance, hepatic steatosis, insulin resistance, hyperphosphatemia, impaired mineralization, and defects in osteoblast function as well as several hormonal abnormalities, including decreased testosterone, IGF-I, and insulin and increased estradiol, LH, GH, and leptin (4, 19). These complex abnormalities in of *Gprc6a*$^{-/-}$ mice and the wide tissue distribution of GPRC6A suggest that the GPCR

ISSN Print 0013-7227 ISSN Online 1945-7170
Printed in U.S.A.

doi: 10.1210/en.2012-1301 Received March 16, 2012. Accepted July 12, 2012.
First Published Online August 7, 2012

Abbreviations: GPCR, G-Protein-coupled receptor; GPRC6A, GPCR family C group 6 member A, MetS, metabolic syndrome.

plays multiple biological functions, including regulation of energy metabolism and sex hormone production (4, 18).

There is emerging evidence for a direct role for GPRC6A in regulating β-cell functions. In this regard, *Gprc6a* is highly expressed in β-cells and pancreas (4, 15). In addition, as noted above, ablation of *Gprc6a* results in elevated serum glucose concentrations and decreased serum insulin levels in $Gprc6a^{-/-}$ mice (4). GPRC6A also mediates the effects of osteocalcin, a bone-derived insulin-regulating hormone (20). These findings suggest that activation of GPRC6A directly stimulates insulin secretion.

L-Arg was discovered to be a potent insulin secretagogue over 45 yr ago (21) and has been used to evaluate β-cell dysfunction in type 2 diabetes (22). The mechanism whereby L-Arg regulates insulin secretion remains unknown. L-Arg has multiple metabolic effects (23, 24), and the prevailing notion is that amino acids, like glucose, regulate insulin secretion in response to their catabolism as metabolic fuels (25); however, L-Arg regulation of insulin secretion does not appear to involve nitric oxide or metabolic products (26, 27). In addition, L-Arg and glucose regulation of insulin secretion appear to be through distinct pathways, because β-cell-specific ablation of the insulin receptor attenuated glucose-mediated insulin secretion but had no effect on L-Arg stimulation of insulin secretion (28). Because L-Arg is a ligand for GPRC6A, it is possible that the effect of this amino acid on insulin secretion is mediated by GPRC6A expressed in β-cells (5, 14, 15). In the current study, we examined the role of GPRC6A in regulating insulin secretion and mediating the response to L-Arg.

Materials and Methods

Animals

Mice were maintained and used in accordance with recommendations as described in the Guide for the Care and Use of Laboratory Animals (Institute on Laboratory Animal Resources, Rockville, MD) and following guidelines established by the University of Tennessee Health Science Center Institutional Animal Care and Use Committee. The animal study protocol used in these studies was approved by the University of Tennessee Health Science Center's Institutional Animal Care and Use Committee. The $Gprc6a^{-/-}$ mouse model was created and genotyped as previously described (4). We used $Gprc6a^{flox/flox}$ mice that we generated and Ins2-Cre to selectively delete exons 2 and 3 of *Gprc6a* in β-cells (18, 29).

Cell culture, reagents, and antibodies

A human embryonic kidney (HEK-293) cell line was obtained from the American Type Culture Collection (Manassas, VA). The cells were maintained in DMEM supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin (Invitrogen, Carlsbad, CA) in humidified 5% CO_2 at 37 C. L-Arg and forskolin were purchased from Sigma Chemical Co. (St. Louis, MO). Human full-length recombinant osteocalcin protein was purchased from Novus Biologicals (Littleton, CO). BSA (fraction V) was obtained from Roche Applied Science (Indianapolis, IN). The anti-phospho-ERK1/2 MAPK antibody and anti-ERK1/2 MAPK antibody were purchased from Cell Signaling Technology (Danvers, MA). Mouse Ultrasensitive Insulin ELISA kit was obtained from ALPCO Immunoassays (Salem, NH). The cAMP enzyme immunoassay kit was obtained for Cayman Chemical (Ann Arbor, MI).

Immunofluorescence staining

The paraffin sections were deparaffinized and blocked in 5% normal goat serum for 1 h at room temperature. For double-fluorescence immunohistochemical localization of glucagon and insulin, the rabbit antiglucagon and guinea pig antiinsulin antibodies (1:100; Invitrogen) were applied after blocking and revealed using Alexa Fluor 488 goat antirabbit IgG and Alexa Fluor 594 goat anti-guinea pig antiinsulin antibodies (1:400; Invitrogen). Sections were placed in *SlowFade* Gold antifade reagent (Molecular Probes, Eugene, OR) with a cover glass and were examined under an Olympus BX51 microscope (Olympus Optical, Tokyo, Japan) at a magnification of ×200.

Mouse islets isolation and ligand stimulation

Primary islets were isolated using a modified method as described (30, 31). Briefly, after dissection and mincing, pancreata were digested with 3 ml/pancreas of a collagenase P (1 mg/ml; Roche) solution in complete Hanks' balanced salt solution [1× supplemented with 20 mM HEPES (pH 7.4) and 2 mM $CaCl_2$] for 15 min in a 37 C shaking water bath. Islets were subsequently purified through a Histopaque 1083 density centrifugation (Sigma). After centrifugation, the islet layer was transferred into petri dishes with wash buffer (Hanks' balanced salt solution with 10 mM HEPES and 1% fetal bovine serum; Invitrogen) then handpicked and cultured in low-glucose medium (RPMI 1640 with 5.6 mM glucose; Invitrogen) for 1 h before being treated for 1 h with high glucose (16.7 mM) or L-Arg (10 mM) as indicated. The insulin stimulation index was calculated as the ratio of media insulin concentrations in high glucose divided by the insulin concentration in low-glucose conditions.

Real-time RT-PCR

For quantitative real-time RT-PCR assessment of insulin and glucagon gene expression, we isolated and reverse transcribed 2.0 μg total RNA from the islets of $Gprc6a^{+/+}$ and $Gprc6a^{-/-}$ mice after 1 h stimulated with low glucose (5.6 mM), high glucose (16.7 mM), and 10 mM arginine. PCR contained 100 ng template (cDNA or RNA), 300 nM each of forward and reverse primer, and 1× iQ SYBR Green Supermix (Bio-Rad Laboratories, Hercules, CA) in 50 μl. Samples were amplified for 40 cycles in an iCycler iQ real-time PCR detection system (Bio-Rad) with an initial melt at 95 C for 10 min, followed by 40 cycles of 95 C for 15 sec and 60 C for 1 min. PCR product accumulation was monitored at multiple points during each cycle by measuring the increase in fluorescence caused by the binding of SybrGreen I to double-stranded DNA. The threshold cycle of tested-gene product from the indicated genotype was normalized to the threshold

cycle for cyclophilin A. The primers for mouse insulin consisted of mIns1.For313: ggggagcgtggcttcttcta and mIns1.Rev452: acaatgccacgcttctgcc; for mouse glucagon consisted of mGCG.For145: gaagacaaacgccactcaca and mGCG.Rev472: tggtgctcatctcgtcagag; and for the cyclophilin A consisted of CycA.For: ctgcactgccaagactgaat and CycA.Rev: ccacaatgttcatgccttct. Dissociation analysis was used to confirm the presence of a single transcript and lack of primer-dimer amplification in all PCR.

Measurement of total and phospho-ERK by Western blot

To prepare total protein from mouse pancreas, L-Arg (at concentration from 0.4, 2, and 4 g/kg) or PBS vehicle was injected into the ip cavity of wild-type (*Gprc6a*$^{+/+}$) and *Gprc6a*$^{-/-}$ mice, and the pancreas was harvested after 20 min for assessment of ERK phosphorylation (32, 33). ERK activation will be assessed by immunoblotting using anti-phospho-ERK1/2 MAPK antibody (Cell Signaling Technology) corrected for the total amount of ERK using an anti-ERK1/2 MAPK antibody (Cell Signaling Technology).

Measurement of cAMP accumulation

Untransfected HEK-293 (controls) and HEK-293 transfected with mouse *Gprc6a* cDNA (17) were cultured (10^5 cells per well) in triplicate in DMEM supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin. Cells were cultured for 48 h followed by overnight incubation in DMEM/F12 containing 0.1% BSA to achieve quiescence. Quiescent cells were treated with 20 mM L-Arg and 100 nM forskolin for 30 min at 37 C. Then the reaction was stopped, and cells were lysed by replacing 0.5 ml 0.1 N HCl. cAMP levels were measured by using a cAMP enzyme immunoassay kit (Cayman Chemical) under the manufacturer's protocol.

Statistics

We evaluated differences between groups by one-way ANOVA, followed by a *post hoc* Tukey's test. Significance was set at $P < 0.05$. All values are expressed as means ± SEM. All computations were performed using the Statgraphic statistical graphics system (STSC Inc., Rockville, MD).

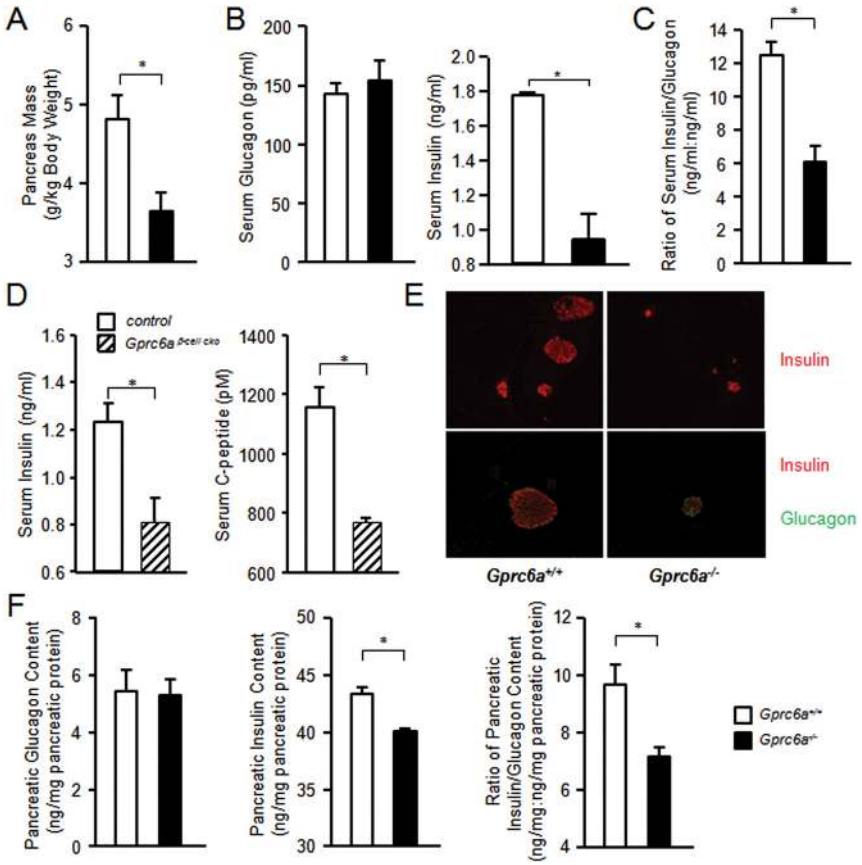

FIG. 1. Serum levels and pancreatic content of glucagon and insulin from wild-type and *Gprc6a*$^{-/-}$ mice. A, Comparison of pancreas mass in 16-wk-old male *Gprc6a*$^{+/+}$ and *Gprc6a*$^{-/-}$ mice. B and C, Comparison of serum glucagon and insulin levels (B) and ratio of insulin to glucagon (C) from wild-type and *Gprc6a*$^{-/-}$ mice at age 16 week. D, Comparison of serum insulin and C-peptide levels in mice of selectively deleted *Gprc6a* in β-cell (*Gprc6a*$^{\beta\text{-cell-cko}}$). E, Fluorescence image of islets from wild-type and *Gprc6a*$^{-/-}$ mice. Sections of pancreas were deparaffinized and stained with antibodies for insulin (*red*) and glucagon (*green*). F, Comparison of pancreatic content of glucagon, insulin, and ratio of insulin to glucagon from wild-type and *Gprc6a*$^{-/-}$ mice at the age of 16 wk. Values represent single determinations and the mean ± SEM. *, Significantly different between wild-type and *Gprc6a*$^{-/-}$ mice ($P < 0.05$; n ≥ 4).

Results

Deletion of *Gprc6a* attenuated pancreas mass, islet size, serum levels, and pancreatic contents of insulin

We found that pancreas mass was significantly reduced in 16-wk-old *Gprc6a*$^{-/-}$ mice compared with wild-type littermates (Fig. 1A). We found no difference in serum glucagon concentrations between *Gprc6a*$^{-/-}$ mice compared with wild-type mice, but we observed a 44% reduction in the serum insulin level (Fig 1B), similar to previous reports (4), and a 2-fold-reduction in the ratio of serum insulin to glucagon in *Gprc6a*$^{-/-}$ mice compared with wild-type mice (Fig. 1C). We also used *Ins2-Cre* and *Gprc6a*$^{\text{floxed/floxed}}$ mice to selectively delete *Gprc6a* in β-cells *in vivo*. We found that both insulin and C-peptide levels were significantly reduced in *Gprc6a*$^{\beta\text{-cell-cko}}$ mice (Fig 1D), which is similar to reductions in insulin levels we found in global *Gprc6a*$^{-/-}$ mice (4). In addition to reduction of pancreatic mass (Fig 1A), immunohistological analyses showed an apparent decrease in number of islets, islet size, and intensity of insulin immunoreactivity (Fig. 1E, *upper panel*), whereas α-cells, identified by glucagon expression, were reduced to a lesser degree

(Fig. 1E, *bottom panel*). Quantitative data will be needed to assess the contribution of reduced β-cell mass to the reductions in serum insulin levels; however, the pancreatic content of insulin in *Gprc6a*$^{-/-}$ mice was significantly lower, whereas the glucagon content was not changed in *Gprc6a*$^{-/-}$ mice compared with wild-type littermates (Fig. 1F). The ratio of pancreatic content of insulin to glucagon was also significantly lower in *Gprc6a*$^{-/-}$ mice compared with wild-type littermates (Fig. 1F). Combined, these results indicated that deletion of *Gprc6a* leads to abnormalities only in β-cells without significant effects on α-cells.

Expression of *Gprc6a* in pancreas

We quantified *Gprc6a* expression in the pancreas with other tissues by quantitative real-time PCR. *Gprc6a* was most highly expressed in salivary glands, pancreas, lungs, and mammary tissues (Fig. 2A). We found that *Gprc6a* was more highly expressed in pancreas compared with other tissues involved in regulating energy metabolism. In this regard, expression of GPRC6A message in the pancreas (0.0011 ± 0.00008) was 8.8-, 9.8-, and 27.8-fold greater than liver (0.00013 ± 0.000013), muscle (0.00012 ± 0.000008), and fat (0.00004 ± 0.000006), respectively (Fig. 2A).

Pancreatic ERK response to L-Arg administration *in vivo* in wild-type and *Gprc6a*$^{-/-}$ mice

To establish a linkage between GPRC6A receptor activation and pancreatic response *in vivo*, we examined the impact of loss of *Gprc6a* on the capacity of L-Arg to stimulate phospho-ERK activity in pancreas isolated from wild-type and *Gprc6a*$^{-/-}$ mice after the ip administration of L-Arg. We found that L-Arg treatment at the dose of 0.4, 2, and 4 g/kg stimulated ERK activity in pancreas of wild-type mice, but this response was markedly attenuated in *Gprc6a*$^{-/-}$ mice (Fig. 2B).

L-Arg regulates insulin secretion through GPRC6A in isolated mouse islets

We assessed *Gprc6a* message expression by quantitative RT-PCR in islets derived from wild-type and *Gprc6a*$^{-/-}$ mice. We found that independent of the culture conditions [*i.e.* both in the presence of high glucose (16.7 mM) or 10 mM L-Arg and 5.6 mM glucose], insulin message expression was significantly lower in islets derived from *Gprc6a*$^{-/-}$ mice compared with wild-type mice (Fig. 3A, *left panel*). This response was selective for insulin, because the glucagon expression was not attenuated in *Gprc6a*$^{-/-}$ mice by these treatments (Fig. 3A, *right panel*).

Next, we examined the response of pancreatic islets isolated from wild-type and *Gprc6a*$^{-/-}$ mice to changes in media glucose or L-Arg concentrations. In low-glucose media (5.6 mM), insulin concentrations were 27.6 ± 4.1 and 60.7 ± 8.32 ng/ml, respectively in islets derived from wild-type and *Gprc6a*$^{-/-}$ mice (Fig. 3B, *left panel*). Incubation of islets with high glucose resulted in media insulin concentrations of 93.5 ± 28.2 and 85.8 ± 9.51 ng/ml, re-

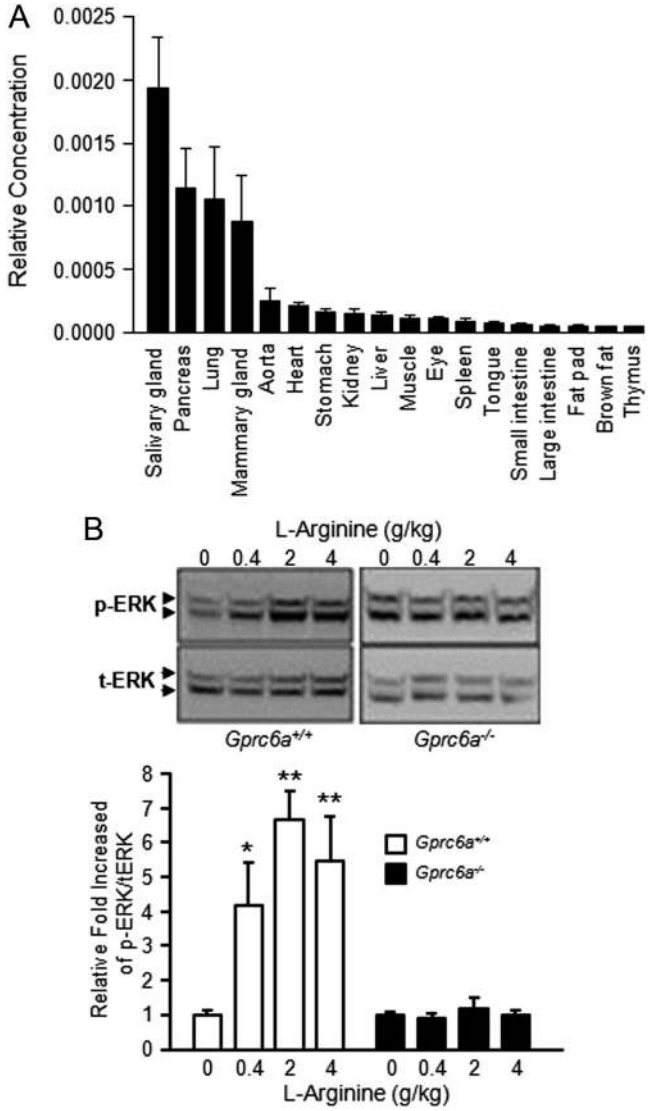

FIG. 2. *Gprc6a* message levels in normal male mouse tissues. A, *Gprc6a* was predominantly expressed in salivary gland, pancreas, lung, and mammary gland in male mice. Expression was assessed by real-time PCR using total RNA derived from wild-type male mouse tissues as indicated. Values represent single determinations and the mean ± SE (n ≥ 3). *Gprc6a* expression is relative to the level of the cyclophilin control gene. B, *Gprc6a* deficiency attenuated the pancreatic response to systemic L-Arg administration by ERK phosphorylation. Western blot analysis of ERK phosphorylation in pancreatic tissue assessed 20 min after injection of L-Arg at concentrations as indicated shows loss of arginine-mediated ERK activation in the pancreas obtained from *Gprc6a*$^{-/-}$ mice but markedly stimulated ERK activity in the pancreas of wild-type mice. Representative blots are shown. The results were verified in three or more independent experiments. Values represent the mean ± SE. *, $P < 0.05$; **, $P < 0.001$ significantly difference between control and L-Arg administration mice.

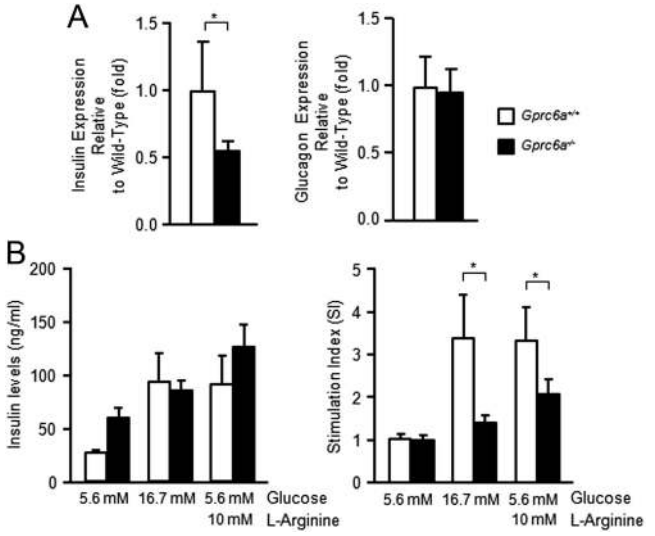

FIG. 3. β-Cell dysfunction in *Gprc6a*$^{-/-}$ mice. A, Expression of insulin (*left*) and glucagon (*right*) message levels in isolated islets from wild-type and *Gprc6a*$^{-/-}$ mice by quantitative real-time PCR. B, Islets from *Gprc6a*$^{-/-}$ mice showed impaired glucose and L-Arg stimulation index. Stimulation index was attenuated in response to high glucose and L-Arg in isolated islets from *Gprc6a*$^{-/-}$ mice. Expression of insulin but not glucagon was attenuated in islets from *Gprc6a*$^{-/-}$ mice compared with the islets from wild-type mice. Values represent single determinations and the mean ± SE. *, Significantly difference between wild-type and *Gprc6a*$^{-/-}$ mice ($P < 0.05$; n ≥ 4).

spectively, in wild-type and *Gprc6a*$^{-/-}$ mice. This resulted in a stimulation index that was significantly lower in *Gprc6a*$^{-/-}$ mice compared with wild-type controls (1.4 *vs.* 3.4, respectively) (Fig. 3B, *right panel*). We have previously shown 10 mM L-Arg activates GPRC6A in cell culture (15). Treatment of isolated islets from wild-type mice with L-Arg at concentrations of 10 mM in the presence of low glucose resulted in a stimulation index of 3.3, but islets isolated from *Gprc6a*$^{-/-}$ mice displayed a stimulation index of only 2.2 in response to L-Arg, which was significantly less than wild-type controls (Fig. 3B).

L-Arg stimulates accumulation of cAMP through GPRC6A

cAMP is involved in the regulation of insulin secretion in β-cells (34). To elucidate whether stimulation of GPRC6A mediates cAMP accumulation, we investigated cAMP second messenger signaling in HEK-293 cells stably transfected with GPRC6A, a model in which L-Arg, osteocalcin, and Zn^{2+}, the ligands of GPRC6A, have been previously shown to activate ERK (5, 14). We found that 20 mM L-Arg, 60 ng/ml human recombinant osteocalcin, and 100 mM Zn^{2+} resulted in significant increments in cAMP accumulation in HEK-293 cells expressing *Gprc6a* but not in untransfected control HEK-293 cells that do not express *Gprc6a* (Fig. 4A). We also assessed L-Arg-stimulated cAMP accumulation in isolated islets from wild-type

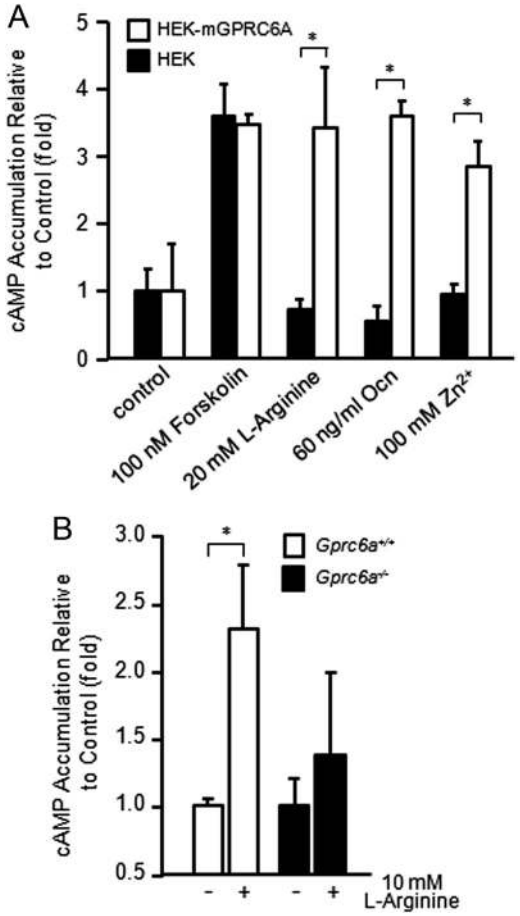

FIG. 4. L-Arg stimulated GPRC6A-mediated intracellular cAMP accumulation. A, The ligands of GPRC6A simulated cAMP accumulation in HEK cells transfected with *Gprc6a*. The HEK cells transfected with *Gprc6a* or without the plasmid cDNA of *Gprc6a* were incubated in DMEM containing 0.1% BSA quiescence media and exposed to the extracellular L-Arg, osteocalcin (Ocn), and Zn^{2+} at indicated concentrations for 20 min, and cAMP levels were determined. Nontreated cells were used as control. All results were verified in at least three independent experiments. *, Significantly difference between control HEK cells and HEK cells transfected with Gprc6a ($P < 0.05$; n = 6). B, cAMP accumulation was altered in isolated islets from *Gprc6a*$^{-/-}$ mice. Isolated islets were incubated in low-glucose media for 1 h, and then the islets were exposed to 10 mM L-Arg. After 20 min, the islets were lysed, and cAMP levels were measured as described in *Materials and Methods*. *, Significantly difference between no treated and treated 10 mM L-Arg islets from wild type mice ($P < 0.05$; n ≥ 4).

and *Gprc6a*$^{-/-}$ mice. We found that the level of cAMP was significantly increased in wild-type islets stimulated with 10 mM L-Arg, but this response was attenuated in the islets isolated from *Gprc6a*$^{-/-}$ mice (Fig. 4B).

Discussion

There is growing evidence supporting a role of the nutrient receptor GPRC6A in regulating pancreatic β-cell function (35). In the current study, we show that *Gprc6a* transcripts

are expressed at higher levels in pancreas relative to other tissues. In addition, we found that L-Arg administration, a known ligand for GPRC6A, stimulated ERK activation in the pancreas *in vivo* and that L-Arg added to cells expressing GPRC6A *in vitro* and to pancreatic islets *ex vivo* stimulated cAMP accumulation through GPRC6A-dependent mechanisms. L-Arg in the presence of low-glucose media also stimulated insulin secretion in islets from wild-type mice similar to that of high glucose; however, the L-Arg-stimulated insulin secretion index was significantly attenuated in islets isolated from *Gprc6a*$^{-/-}$ mice. The low insulin and C-peptide levels in *Gprc6a*$^{\beta\text{-cell-cko}}$ mice also are consistent with a direct role of GPRC6A in β-cells to regulate serum insulin. The observation that L-Arg regulates insulin secretion through GPRC6A is similar to osteocalcin, another known GPRC6A ligand, regulation of insulin secretion through GPRC6A-dependent mechanisms (15).

L-Arg activation of GPRC6A only partially accounted for L-Arg effects on insulin secretion. Because glucose and other amino acids stimulate insulin secretion through non-receptor mechanisms, L-Arg may also regulate insulin secretion through these metabolic pathways (23, 24, 36, 37). The facts that L-Arg and osteocalcin activate GPRC6A in pancreas and TC-6 mouse β-cells and regulate insulin secretion *in vitro* and *in vivo* (15), however, indicate that GPRC6A is also important in mediating L-Arg effects on β-cell function. Taken together, these data provide the compelling evidence that GPRC6A is a biologically relevant GPCR in β-cells that mediates the effects of L-Arg and other GPRC6A ligands on insulin secretion.

In vivo studies in *Gprc6a*$^{-/-}$ mouse models also found reduced size of pancreatic islets and reduced insulin content in the whole pancreas and reduced insulin message expression in isolated islets, along with reduced circulating insulin levels, but no alterations in serum glucagon levels or glucagon content of pancreas or glucagon message expression in isolated islets. This suggests for the first time that GPRC6A may also regulate β-cell mass in addition to insulin secretion. We also found that the stimulatory effect of L-Arg on insulin secretion and its attenuation in islets from *Gprc6a*$^{-/-}$ mice was correlated with commensurate increases and attenuated cAMP response in islets isolated from wild-type and *Gprc6a*$^{-/-}$, respectively. We also showed that GPRC6A is coupled to generation of cAMP in HEK-293 cells overexpressing GPRC6A. Thus, GPRC6A is coupled to signaling pathways known to regulate insulin production and secretion and survival of pancreatic β-cells (17, 18, 38).

Despite the compelling evidence of impaired insulin secretion and low β-cell mass in *Gprc6a*$^{-/-}$ mice, basal insulin secretion was unexpectedly increased *ex vivo* in islets from *Gprc6a*-deficient mice. The fact that *Gprc6a*$^{-/-}$ mice have higher basal insulin levels per islet *ex vivo*, but low serum insulin levels *in vivo*, suggests a complex relationship between GPRC6A regulation of insulin secretion and the capacity of the islets to produce insulin, which is determined by insulin content and number. Alternatively, differences between β-cell function *in vivo* and *ex vivo* may reflect differences in GPRC6A coupling to $G\alpha_s$, $G\alpha_i$, and $G\alpha_q$ pathways, which have different effects on insulin secretion and, depending on which pathway predominates in the *in vivo* and *ex vivo* setting, could either stimulate or inhibit insulin secretion. The loss of GPRC6A in fat, muscle, liver, or other tissues leading to alterations in insulin resistance could also lead to compensatory changes in insulin secretion by β-cells *in vivo* that are not reflected in islets *ex vivo*. Additional studies on insulin secretion in β-cell-specific knockouts of *Gprc6a* and assessment of GPRC6A signaling pathways will be necessary to assess potential different roles of GPRC6A in regulating basal and ligand-stimulated insulin secretion.

The clinical significance of L-Arg activation of GPRC6A is not yet clear. *Gprc6a* is expressed in multiple tissues in addition to pancreatic β-cells, and L-Arg has multiple metabolic effects other than regulation of insulin secretion. Indeed, treatment of mice with L-Arg, but not L-alanine, prevents the effects of high-fat diet or mutant leptin receptors to induce metabolic syndrome (MetS) (39, 40). L-Arg supplementation also attenuates the severity of MetS in humans (41, 42), whereas low arginine concentrations increase the risk factor for MetS (43). In addition, arginine, ornithine, and lysine (the three principal amino acid ligands for GPRC6A) (14), but not other amino acids, stimulate GH release (44, 45), suggesting GPRC6A, which is also expressed in the hypothalamus and pituitary gland, may regulate the secretion of additional hormones (45).

In conclusion, the islets from *Gprc6a*$^{-/-}$ mice exhibit impaired glucose- and L-Arg-mediated insulin secretion *ex vivo*. GPRC6A, which is activated by osteocalcin, basic amino acids, calcium, and testosterone (5, 15, 17), may be a common receptor mediating the responses of β-cells to multiple insulin secretagogues.

Acknowledgments

Address all correspondence and requests for reprints to: Min Pi, Ph.D., or or L. Darryl Quarles, M.D., Department of Medicine, University of Tennessee Health Science Center, 19 South Manassas Street, Memphis, Tennessee 38163. E-mail: mpi@uthsc.edu or dquarles@uthsc.edu.

This work was supported by National Institutes of Health Grant R01-AR037308.

M.P., I.G., and L.D.Q. contributed to the research design; M.P., Y.W., and N.L. performed research; and M.P. and L.D.Q contributed to the writing of the manuscript.

Disclosure Summary: The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

References

1. **Winzell MS, Ahrén B** 2007 G-protein-coupled receptors and islet function: implications for treatment of type 2 diabetes. Pharmacol Ther 116:437–448
2. **Regard JB, Kataoka H, Cano DA, Camerer E, Yin L, Zheng YW, Scanlan TS, Hebrok M, Coughlin SR** 2007 Probing cell type-specific functions of Gi in vivo identifies GPCR regulators of insulin secretion. J Clin Invest 117:4034–4043
3. **Pi M, Quarles LD** 2005 Osteoblast calcium-sensing receptor has characteristics of ANF/7TM receptors. J Cell Biochem 95:1081–1092
4. **Pi M, Chen L, Huang MZ, Zhu W, Ringhofer B, Luo J, Christenson L, Li B, Zhang J, Jackson PD, Faber P, Brunden KR, Harrington JJ, Quarles LD** 2008 GPRC6A null mice exhibit osteopenia, feminization and metabolic syndrome. PLoS ONE 3:e3858
5. **Pi M, Faber P, Ekema G, Jackson PD, Ting A, Wang N, Fontilla-Poole M, Mays RW, Brunden KR, Harrington JJ, Quarles LD** 2005 Identification of a novel extracellular cation-sensing G-protein-coupled receptor. J Biol Chem 280:40201–40209
6. **Brown EM, Gamba G, Riccardi D, Lombardi M, Butters R, Kifor O, Sun A, Hediger MA, Lytton J, Hebert SC** 1993 Cloning and characterization of an extracellular Ca^{2+}-sensing receptor from bovine parathyroid. Nature 366:575–580
7. **Möhler H, Fritschy JM** 1999 $GABA_B$ receptors make it to the top: as dimers. Trends Pharmacol Sci 20:87–89
8. **Pin JP, Duvoisin R** 1995 The metabotropic glutamate receptors: structure and functions. Neuropharmacology 34:1–26
9. **Nelson G, Chandrashekar J, Hoon MA, Feng L, Zhao G, Ryba NJ, Zuker CS** 2002 An amino-acid taste receptor. Nature 416:199–202
10. **Robbins MJ, Michalovich D, Hill J, Calver AR, Medhurst AD, Gloger I, Sims M, Middlemiss DN, Pangalos MN** 2000 Molecular cloning and characterization of two novel retinoic acid-inducible orphan G-protein-coupled receptors (GPRC5B and GPRC5C). Genomics 67:8–18
11. **Bräuner-Osborne H, Krogsgaard-Larsen P** 2000 Sequence and expression pattern of a novel human orphan G-protein-coupled receptor, GPRC5B, a family C receptor with a short amino-terminal domain. Genomics 65:121–128
12. **Nelson G, Hoon MA, Chandrashekar J, Zhang Y, Ryba NJ, Zuker CS** 2001 Mammalian sweet taste receptors. Cell 106:381–390
13. **Calver AR, Michalovich D, Testa TT, Robbins MJ, Jaillard C, Hill J, Szekeres PG, Charles KJ, Jourdain S, Holbrook JD, Boyfield I, Patel N, Medhurst AD, Pangalos MN** 2003 Molecular cloning and characterisation of a novel $GABA_B$-related G-protein coupled receptor. Brain Res Mol Brain Res 110:305–317
14. **Wellendorph P, Hansen KB, Balsgaard A, Greenwood JR, Egebjerg J, Bräuner-Osborne H** 2005 Deorphanization of GPRC6A: a promiscuous L-α-amino acid receptor with preference for basic amino acids. Mol Pharmacol 67:589–597
15. **Pi M, Wu Y, Quarles LD** 2011 GPRC6A mediates responses to osteocalcin in β-cells in vitro and pancreas in vivo. J Bone Miner Res 26:1680–1683
16. **Christiansen B, Hansen KB, Wellendorph P, Bräuner-Osborne H** 2007 Pharmacological characterization of mouse GPRC6A, an L-alpha-amino-acid receptor modulated by divalent cations. Br J Pharmacol 150:798–807
17. **Pi M, Parrill AL, Quarles LD** 2010 GPRC6A mediates the non-genomic effects of steroids. J Biol Chem 285:39953–39964
18. **Oury F, Sumara G, Sumara O, Ferron M, Chang H, Smith CE, Hermo L, Suarez S, Roth BL, Ducy P, Karsenty G** 2011 Endocrine regulation of male fertility by the skeleton. Cell 144:796–809
19. **Pi M, Zhang L, Lei SF, Huang MZ, Zhu W, Zhang J, Shen H, Deng HW, Quarles LD** 2010 Impaired osteoblast function in GPRC6A null mice. J Bone Miner Res 25:1092–1102
20. **Pi M, Chen J, Zhu W, Quarles L** 2010 Dominant negative effect of the extracellular domain of CASR. J Receptor, Ligand Channel Res 3:15–23
21. **Floyd Jr JC, Fajans SS, Conn JW, Knopf RF, Rull J** 1966 Stimulation of insulin secretion by amino acids. J Clin Invest 45:1487–1502
22. **Ozbek M, Erdogan M, Karadeniz M, Cetinkalp S, Ozgen AG, Saygili F, Yilmaz C, Tuzun M** 2009 Evaluation of beta cell dysfunction by mixed meal tolerance test and oral L-arginine in patients with newly diagnosed type 2 diabetes mellitus. Exp Clin Endocrinol Diabetes 117:573–576
23. **Wu G, Bazer FW, Davis TA, Kim SW, Li P, Marc Rhoads J, Carey Satterfield M, Smith SB, Spencer TE, Yin Y** 2009 Arginine metabolism and nutrition in growth, health and disease. Amino Acids 37:153–168
24. **Fisker S, Nielsen S, Ebdrup L, Bech JN, Christiansen JS, Pedersen EB, Jørgensen JO** 1999 L-Arginine-induced growth hormone secretion is not influenced by co-infusion of the nitric oxide synthase inhibitor *N*-monomethyl-L-arginine in healthy men. Growth Horm IGF Res 9:69–73
25. **Odegaard ML, Joseph JW, Jensen MV, Lu D, Ilkayeva O, Ronnebaum SM, Becker TC, Newgard CB** 2010 The mitochondrial 2-oxoglutarate carrier is part of a metabolic pathway that mediates glucose- and glutamine-stimulated insulin secretion. J Biol Chem 285:16530–16537
26. **Sener A, Blachier F, Rasschaert J, Mourtada A, Malaisse-Lagae F, Malaisse WJ** 1989 Stimulus-secretion coupling of arginine-induced insulin release: comparison with lysine-induced insulin secretion. Endocrinology 124:2558–2567
27. **Thams P, Capito K** 1999 L-arginine stimulation of glucose-induced insulin secretion through membrane depolarization and independent of nitric oxide. Eur J Endocrinol 140:87–93
28. **Kulkarni RN, Brüning JC, Winnay JN, Postic C, Magnuson MA, Kahn CR** 1999 Tissue-specific knockout of the insulin receptor in pancreatic β-cells creates an insulin secretory defect similar to that in type 2 diabetes. Cell 96:329–339
29. **Gannon M, Shiota C, Postic C, Wright CV, Magnuson M** 2000 Analysis of the Cre-mediated recombination driven by rat insulin promoter in embryonic and adult mouse pancreas. Genesis 26:139–142
30. **Liu M, Shapiro ME** 1995 A new method for isolation of murine islets with markedly improved yields. Transplant Proc 27:3208–3210
31. **Gerling IC, Serreze DV, Christianson SW, Leiter EH** 1992 Intrathymic islet cell transplantation reduces β-cell autoimmunity and prevents diabetes in NOD/Lt mice. Diabetes 41:1672–1676
32. **Urakawa I, Yamazaki Y, Shimada T, Iijima K, Hasegawa H, Okawa K, Fujita T, Fukumoto S, Yamashita T** 2006 Klotho converts canonical FGF receptor into a specific receptor for FGF23. Nature 444:770–774
33. **Ferron M, Hinoi E, Karsenty G, Ducy P** 2008 Osteocalcin differentially regulates beta cell and adipocyte gene expression and affects the development of metabolic diseases in wild-type mice. Proc Natl Acad Sci USA 105:5266–5270
34. **Grill V, Cerasi E** 1973 Activation by glucose of adenyl cyclase in pancreatic islets of the rat. FEBS Lett 33:311–314
35. **Pi M, Quarles LD** 2012 Multiligand specificity and wide tissue expression of GPRC6A reveals new endocrine networks. Endocrinology 153:2062–2069
36. **Schmidt HH, Warner TD, Ishii K, Sheng H, Murad F** 1992 Insulin

secretion from pancreatic B cells caused by L-arginine-derived nitrogen oxides. Science 255:721–723
37. **Blachier F, Mourtada A, Sener A, Malaisse WJ** 1989 Stimulus-secretion coupling of arginine-induced insulin release. Uptake of metabolized and nonmetabolized cationic amino acids by pancreatic islets. Endocrinology 124:134–141
38. **Seino S** 2012 Cell signalling in insulin secretion: the molecular targets of ATP, cAMP and sulfonylurea. Diabetologia 55:2096–2108
39. **Jobgen W, Meininger CJ, Jobgen SC, Li P, Lee MJ, Smith SB, Spencer TE, Fried SK, Wu G** 2009 Dietary L-arginine supplementation reduces white fat gain and enhances skeletal muscle and brown fat masses in diet-induced obese rats. J Nutr 139:230–237
40. **Fu WJ, Haynes TE, Kohli R, Hu J, Shi W, Spencer TE, Carroll RJ, Meininger CJ, Wu G** 2005 Dietary L-arginine supplementation reduces fat mass in Zucker diabetic fatty rats. J Nutr 135:714–721
41. **McKnight JR, Satterfield MC, Jobgen WS, Smith SB, Spencer TE, Meininger CJ, McNeal CJ, Wu G** 2010 Beneficial effects of L-arginine on reducing obesity: potential mechanisms and important implications for human health. Amino Acids 39:349–357
42. **Lucotti P, Setola E, Monti LD, Galluccio E, Costa S, Sandoli EP, Fermo I, Rabaiotti G, Gatti R, Piatti P** 2006 Beneficial effects of a long-term oral L-arginine treatment added to a hypocaloric diet and exercise training program in obese, insulin-resistant type 2 diabetic patients. Am J Physiol Endocrinol Metab 291:E906–E912
43. **Jo I, Han C, Ahn Jo S, Seo JA, Park MH, Kim NH** 2010 Low levels of plasma agmatine in the metabolic syndrome. Metab Syndr Relat Disord 8:21–24
44. **Biller BM, Samuels MH, Zagar A, Cook DM, Arafah BM, Bonert V, Stavrou S, Kleinberg DL, Chipman JJ, Hartman ML** 2002 Sensitivity and specificity of six tests for the diagnosis of adult GH deficiency. J Clin Endocrinol Metab 87:2067–2079
45. **van Vught AJ, Nieuwenhuizen AG, Veldhorst MA, Brummer RJ, Westerterp-Plantenga MS** 2010 The effects of dietary protein on the somatotropic axis: a comparison of soy, gelatin, α-lactalbumin and milk. Eur J Clin Nutr 64:441–446