[Frontiers In Bioscience, Landmark, 22, 1523-1533, June 1, 2017]

Ghrelin doesn't limit insulin release in pregnant rats fed low protein diet

Haijun Gao[1,2], Eric Ho[3], Chandra Yallampalli[1,2]

[1]Department of Obstetrics and Gynecology, Baylor College of Medicine, Houston TX 77030, [2]Texas Children's Hospital, Houston TX 77030, [3]Department of Biochemistry, Rice University, Houston TX 77030

TABLE OF CONTENTS

1. ABSTRACT

To test the hypothesis that insulin secretion in pregnant rats fed LP diet is reduced by ghrelin signaling in pancreatic islets, we investigated plasma insulin levels, expression of ghrelin and its receptor and glucose stimulated insulin secretion (GSIS) of pancreatic islets in response to ghrelin. Plasma insulin levels were lower and ghrelin receptor abundance in islets was unaltered in LP rats. In the presence of both 2.8 and 16.7 mM glucose, GSIS was lower in LP compared to CT rats. In the presence of 2.8 mM glucose, GSIS was unaltered by ghrelin and its antagonist in both CT and LP rats. In the presence of 16.7 mM glucose, GSIS in LP rats was unchanged while in CT rats was reduced by ghrelin and reversed by ghrelin antagonist. These results indicate ghrelin signaling inhibits GSIS of pancreatic islets in pregnant rats fed CT diet, but it is blunted in pregnant rats fed LP diet and thus may not contribute to the reduction of plasma insulin and GSIS of pancreatic islets in late pregnancy.

2. INTRODUCTION

Normal pregnancy, particularly during late stages, requires not only insulin resistance to ensure sufficient glucose for exponential fetal growth and development (1), but also elevated insulin levels to compensate insulin resistance to maintain glucose hemostasis. The elevated insulin levels during pregnancy are achieved by both beta cell expansion and enhanced insulin secretion (2). The proliferation of beta cells peaks in mid-pregnancy, declines thereafter and returns to basal levels before partum (3;4), while plasma insulin levels continue to increase and peak in late pregnancy (5). Thus, insulin secretion from beta cell serves as a major modulator in fine tuning of plasma insulin levels in late pregnancy when insulin resistance is intense (5-7).

Insulin secretion is regulated by multiple factors in addition to glucose. Both *in vivo* and *ex vivo* studies in non-pregnant human or rodents

showed that ghrelin inhibits glucose stimulated insulin secretion (GSIS) (8;9). In rat pancreatic islets, basal insulin release in the presence of low levels of glucose in perfused pancreas and isolated islets is not significantly affected by ghrelin, and in contrast, insulin release in the presence of high levels of glucose is inhibited by ghrelin (9). The currently known ghrelin signaling that is involved in insulin secretion has been reviewed extensively (10). Briefly, ghrelin binds to its receptor GHSR on beta cells and stimulates G protein coupled protein Gαi2, which inhibits PKA and therefore reduces cellular cAMP. The reduction of cAMP activates voltage controlled potassium channel Kv2.1, causing potassium outward flow and blocking cell membrane depolarization. Thus, glucose stimulated cell membrane depolarization via inhibiting ATP sensitive potassium channel is reduced, and as a consequence, glucose stimulated insulin secretion is inhibited by ghrelin.

Pregnant rats fed a low protein diet have been widely used as a model in the study of metabolic programming (11). Protein restriction during gestation not only causes reduced blood flow and insulin secretion in pregnant dams (12-14), but also predisposes obesity and diabetes in offspring (15-17). However, to date, underlying mechanisms of programming have not been understood completely. Recently we found that plasma levels of ghrelin in pregnant rats fed a low protein diet were significantly elevated compared to those in controls in mid-late pregnancy (18), which is coincident with the reduced plasma levels of insulin (19). In this study we hypothesized that insulin secretion in LP rats is reduced by ghrelin signaling in pancreatic islets. In pregnant rats fed a low protein diet, we investigated plasma levels of insulin, expression of ghrelin and ghrelin receptor in pancreatic islets, glucose stimulated insulin secretion in isolated islets in response to ghrelin and its receptor antagonist.

3. MATERIALS AND METHODS

3.1. Diets

The isocaloric low (6% casein)-and normal (20% casein)-protein diets were purchased from Harlan Teklad (Cat. TD.90016 and TD.91352, respectively; Madison, WI, USA). More information about these diets was described in details in our recent publication (18).

3.2. Animals

All procedures were approved by the Animal Care and Use Committee at Baylor College of Medicine and were in accordance with those published by the US National Institutes of Health Guide for the Care and Use of Laboratory Animals (2011). Virgin female Sprague-Dawley rats weighing between 175 and 225 g and male rats weighing between 225 and 249 g were purchased from Harlan Laboratories (Houston, TX, USA). These rats were housed in a room with a controlled light-dark cycle (light phase: 0600 to 2000 and dark phase: 2000 to 0600). Animals were allowed acclimation to our housing conditions for 1 week before breeding. For breeding, 2 virgin female rats were paired with 1 male rat overnight and vaginal smears were checked under microscope next morning. The presence of sperm in vaginal smear indicated positive pregnancy status and this day was designated as Day 1 of pregnancy. Pregnant rats were housed individually, randomly divided into two dietary groups, and received ad libitum either CT or LP diet until they were sacrificed on Day 21 of pregnancy.

3.3. Experimental design

In study one, rats on Day 21 of pregnancy were killed via carbon dioxide inhalation at 8-10 am. Whole blood was collected by left ventricle puncture, injected into BD Vacutainer blood collection tube containing heparin (Cat.367874, BD, Franklin Lakes, NJ). Plasma was prepared and stored at -80ºC for further analysis, following the protocol in our previous publication (18). Pancreatic islets (200-400 per rat) were isolated following collagenase digestion protocol. Isolated islets were either lysed in Trizol reagent for total RNA extraction or snap frozen for total protein extraction (n = 5 rats/diet treatment).

In study two, rats on Day 21 of pregnancy were killed via carbon dioxide inhalation at 8-10 am. Pancreatic islets were isolated and used for glucose stimulated insulin secretion assay *ex vivo* (n = 4-5 rats/diet treatment).

3.4. Pancreatic islet isolation

Pancreatic islets were isolated following collagenase digestion as described by others (20;21). Briefly, 1xHBSS buffer (Cat. 21-023-CV, Corning, Corning, NY 14831) containing 0.1% BSA and collagenase (0.7 mg/ml, Cat. C6885, Sigma-Aldrich, St. Louis, MO USA) was injected through the common bile duct, and then the inflated pancreas was dissected and incubated at 37ºC in the water bath for 27 min. After sedimentation and wash, islets were purified by gradient centrifuge in Histopaque ® -1077 density gradient medium (Cat. 10771, Sigma-Aldrich), followed by recovery in culture medium (Krebs buffer (magnesium sulfate 1.2 mM, potassium phosphate 1.2 mM, potassium chloride 4.7 mM, sodium chloride 119 mM, calcium chloride 2.5 mM, sodium bicarbonate 25 mM, pH7.4.) with 0.25% BSA, 10 mM HEPES and 5.5 mM glucose) at 37ºC for 30 min and manual collection under a microscope. Only healthy islets with a round shape were used for experiments, according to the criteria described by Carter *et al.* (22).

3.5. RNA extraction and RT-PCR

Total RNA was extracted from pancreatic islets by Trizol reagent (Cat. 15596-018; Invitrogen, Carlsbad, CA) combined with Qiagen RNeasy minikit (Cat. 74104; Qiagen Inc., Valencia, CA). The possible genomic DNA in total RNAs was digested with RNase free DNase I (Cat. 79254; Qiagen). In all these procedures manufacturers' instructions were followed. Complementary DNA (cDNA) was synthesized from 0.5. μg of total RNA by reverse transcription in a total volume of 20 μl by using a MyCycler Thermal Cycler (Cat. 170-9703; Bio-Rad Laboratories, Hercules, CA) under the following conditions: One cycle at 28°C for 15 min, 42°C for 50 min, and 95°C for 5 min.

3.6. Quantitative real-time PCR

Real-time PCR detection was performed on a CFX96Real-Time PCR Detection System (Cat. 184-5096; Bio-Rad). iTaq™ Universal Probes Supermix (Cat. 1725135; Bio-Rad) was used for amplification of *Ghrl, Ghsr, Mboat4, Bche* and *Actb*. Sequences of these primers were described previously (23;24). The reaction mixture was incubated at 95°C for 10 min and cycled according to the following parameters: 95°C for 30 seconds and 60°C for 1 min for a total of 40 cycles. Negative control without cDNA was performed to test primer specificity. The relative gene expression was calculated by use of the threshold cycle (C_T) *Actb* /C_T target gene.

3.7. Protein extraction and Western blotting

Protein was extracted from frozen pancreatic islets (n = 5 rats/diet group) using RIPA buffer (Cat. 9806, Cell Signaling, Danvers, MA) with protease inhibitor cocktails (Cat. 11697498001; Roche, Indianapolis, IN), phosphatase inhibitor cocktails 2 and 3 (Cat. P5726 and P0044; Sigma, St. Louis, MO), according to the manufacturers recommendations. Aliquots of 10 μg proteins were added with 4X Sample Buffer (Cat. NP0007, Invitrogen), followed by incubation at 70ºC for 10 minutes. The separated proteins in NuPAGE 4-12% Bis-Tris Gel (Cat. NP0321BOX; Invitrogen) were transferred onto a PVDF membrane at 4ºC overnight. After blocking in 5% BSA in TBST buffer, a rabbit anti- GHSR polyclonal IgG (Cat.AB15159; EMD Millipore, Billerica, MA) at 1:160 dilutions was added to nitrocellulose membrane and incubated at 4°C overnight. The blot were washed and incubated with HRP-conjugated goat anti-rabbit IgG (Cat. 4030-05; Southern Biotech, Birmingham, AL) at 1:2000 dilutions at room temperature for 1 h. ACTB (β-actin) was used as an internal control. Primary antibody, mouse monoclonal antibody for ACTB (Cat. 3700; Cell Signaling), and secondary antibody, HRP-conjugated goat antimouse IgG (Cat. 1030-05; Southern Biotech) were used at 1:10000 dilutions. Proteins in the blot were visualized with ODYSSEY FC Imaging System (LI-COR Biotechnology) according to the manufacturer's recommendations. The relative amount of target protein was expressed as a ratio to ACTB analyzed by western blotting.

3.8. *Ex vivo* Glucose stimulated insulin secretion

Ten islets of medium size were seeded to each well of 12-well cell culture plate and incubated in 500 μl culture medium (Krebs buffer with 0.25% BSA, 10 mM HEPES and 2.8 mM glucose) only (NONE), or supplemented with ghrelin (G, 10nM; Cat.1465, Tocris Bioscience, UK), ghrelin antagonist (A, 1μM; GHRP-6 (D-Lys3)); Cat. 031-22, Phoenix Pharmaceuticals, Inc.) and their combination (G+A), respectively for 30 min. These doses were determined based upon previous reports (21;25). Fifty microliter of culture medium was removed and stored for future measurement of insulin, then 50 μl of culture medium with concentrated glucose was added to increase working glucose concentration up to 16.7 mM. After incubation for another 30 min, culture medium was collected and stored at -80ºC for insulin determination. Pancreatic islets were placed in 50 μl water and stored at –80ºC for DNA measurement.

3.9. Insulin ELISA and DNA measurement

Insulin concentration in plasma and culture medium collected in GSIS assay was measured by ELISA (Cat. 10-1250-01, Mercodia, Uppsala, Sweden). All procedures were conducted according to the instructions in the assay kit. Frozen islets were sonicated and the concentration of DNA was measured by NanoDrop 1000 (Thermo Scientific, Wilmington, DE).

3.10. Statistical analysis

All quantitative data were subjected to least-squares analysis of variance (ANOVA) using the general linear models procedures of the Statistical Analysis System (Version 9.4., SAS Institute, Cary, NC). Data on gene expression and abundance of proteins were analyzed for the effect of diet treatment. Data on *ex vivo* insulin secretion were analyzed for the effects of ghrelin, its antagonist and their combination within and between the two diet groups. Log transformation of variables was performed when the variance of data were not homogenous among treatment groups, as assessed by the Levene's test. A P-value ≤ 0.05 was considered significant; a P-value > 0.05 and ≤ 0.10 was considered a trend toward significance. Data were presented as least-squares means (LSMs) with overall standard errors (SE).

4. Results

4.1. Plasma levels of insulin

To confirm the reduction of plasma insulin levels in response to a low protein diet, we first measured plasma levels of insulin in both CT and LP

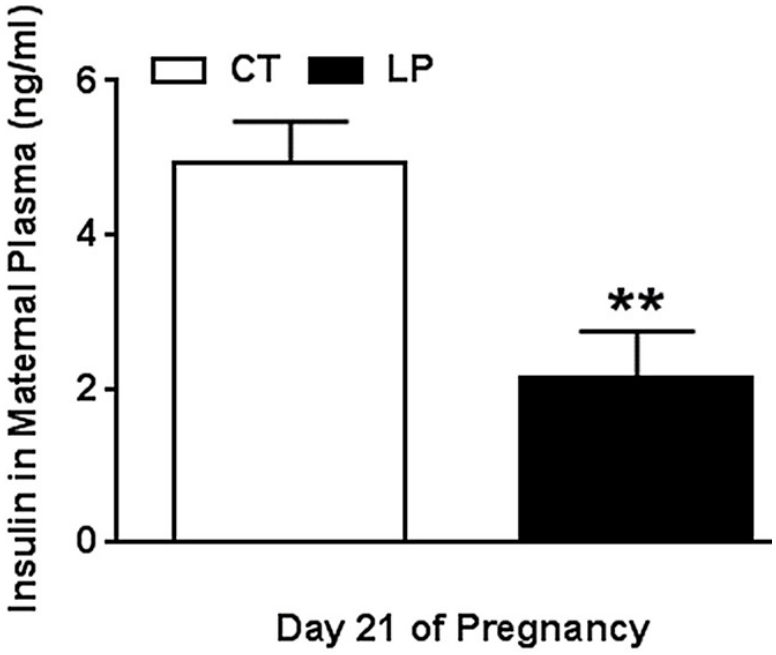

Figure 1. Plasma levels of insulin in pregnant rats fed a low protein diet. CT: control; LP, low protein. The error bar represents the mean ± SEM (n = 8-10 rats/diet group). **, $P < 0.01$.

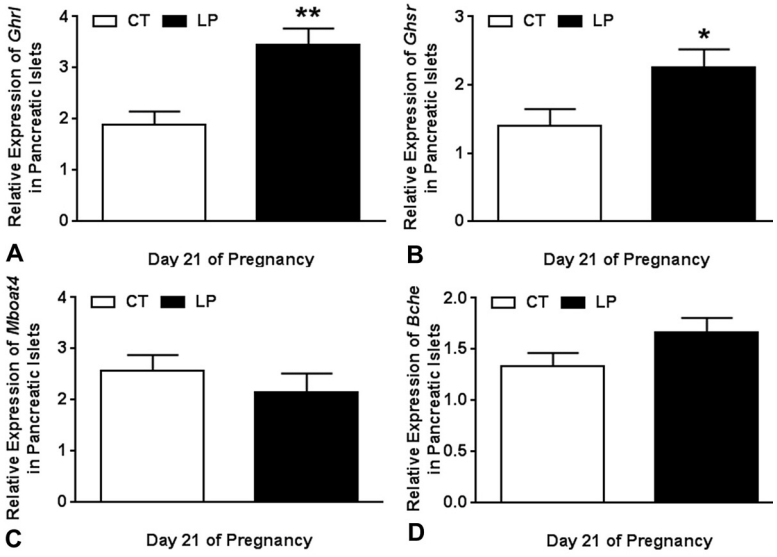

Figure 2. Quantitative real-time PCR analysis of expression of *Ghrl* (A), *Ghsr* (B), *Mboat4* (C) and *Bche* (D) in pancreatic islets of pregnant rats fed a low protein diet. CT: control; LP, low protein. The error bar represents the mean ± SEM expressed as relative units of mRNA standardized against *Actb* (n = 5 rats/diet group).*, $P < 0.05$;**, $P < 0.01$.

rats. Plasma insulin levels were reduced ($P < 0.05$) by 2.3 -fold in LP compared to CT rats when measured on Day 21 of pregnancy (Figure 1).

4.2. Ghrelin and its related genes in pancreatic islets

Besides primary production in the stomach, ghrelin is also produced locally in the delta cells of pancreatic islets and exerts its inhibitory effect on GSIS via ghrelin receptors on β-cells (10;26). We measured gene expression of *Ghrl*, *Ghsr*, *Mboat4* (the enzyme catalyzing octanoylation of ghrelin and making active ghrelin which can bind to ghrelin receptor) and *Bche* (the enzyme inactivating active ghrelin by deacetylation) in pancreatic islets. mRNA levels of *Ghrl* and *Ghsr* were higher by 1.8 -($P < 0.01$) and 1.6 ($P < 0.05$)-fold in LP rats compared to those in CT rats,

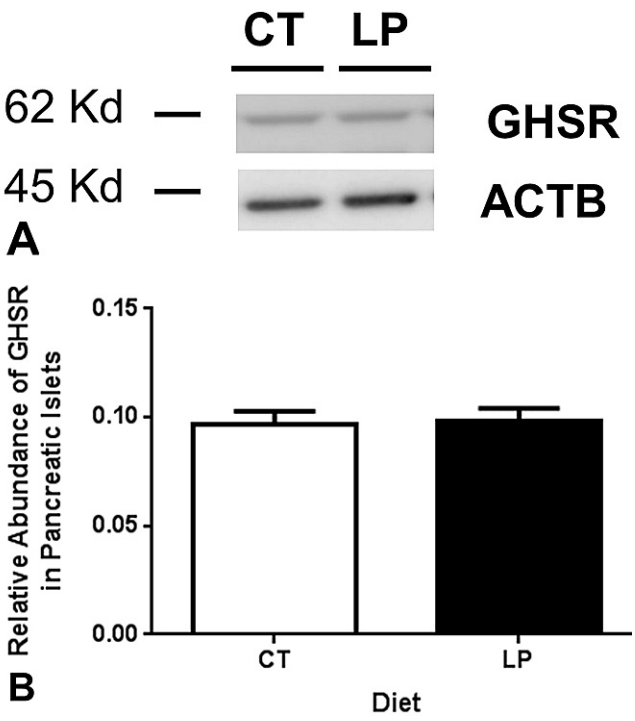

Figure 3. Western blotting analysis of ghrelin receptor proteins in pancreatic islets from pregnant rats fed a low protein diet. A) GHSR proteins shown as 62-kDa bands. B) Relative abundance of ACE protein normalized to ACTB. GHSR: ghrelin receptor; ACTB: beta-actin; CT: control; LP, low protein. The error bar represents the mean ± SEM expressed as the ratio of density of the GHSR band to that of ACTB (n = 5 rats/diet group).

respectively, while mRNA levels of *Mboat4* and *Bche* were unchanged in LP rats (Figure 2).

4.3. Abundance of ghrelin receptor protein was unchanged in pancreatic islets

As *Ghsr* mRNA levels were increased in pancreatic islets in LP rat (Figure 2), we investigated whether the abundance of ghrelin receptor protein in islets is also increased in response to LP diet. However, the abundance of ghrelin receptor proteins in islets was unchanged in LP rats compared to CT rats (Figure 3).

4.4. Glucose stimulated insulin secretion in pregnant rats fed low protein diet

To investigate the sensitivity of pancreatic islets to the changes in glucose levels, we measured insulin secretion from pancreatic islets in presence of low and high glucose concentrations. In the presence of 2.8 and 16.7 mM glucose, insulin secretion from isolated pancreatic islets was 3.8- ($P < 0.001$) and 2.3- ($P < 0.05$) fold higher in CT rats compared to LP rats, respectively. In CT rats, insulin secretion was 6.0.-fold higher in presence of 16.7 mM compared to 2.8 mM glucose. Similarly, in LP rats, insulin secretion was 9.8-fold higher in presence of 16.7 mM compared to 2.8 mM glucose (Figure 4).

4.5. Glucose stimulated insulin secretion in response to ghrelin

In presence of 2.8 mM glucose, insulin secretion was not changed by ghrelin (G) and its antagonist (A) in both CT and LP rats, although insulin secretion was higher in CT rats for each treatment except combination of ghrelin and its antagonist (G+A) (Figure 5A). In presence of 16.7 mM glucose, in LP rats insulin secretion was unchanged by ghrelin and its antagonist; in CT rats insulin secretion with the treatment of ghrelin was reduced to 69.8% of untreated group, but insulin secretion with the treatment of A and G+A was increased ($P < 0.001$) by 2.8- and 2.6 -fold, respectively (Figure 5B). With both NONE and G treatments, fold-changes of insulin secretion with 2.8 and 16.7 mM glucose were similar between CT and LP rats, while with treatments of A and G+A, these values were 2.61- and 2.65-fold higher ($P < 0.001$) in CT compared to LP rats, respectively. In CT rats, fold-changes of insulin secretion from 2.8 and 16.7 mM glucose were higher ($P < 0.05$) with the treatment of A (Groups A and G+A), compared to those without A (Groups NONE and G) (Figure 5C), while in LP rats, these values were similar among all treatment groups.

5. DISCUSSION

Insulin resistance is required during pregnancy to ensure adequate glucose supply to

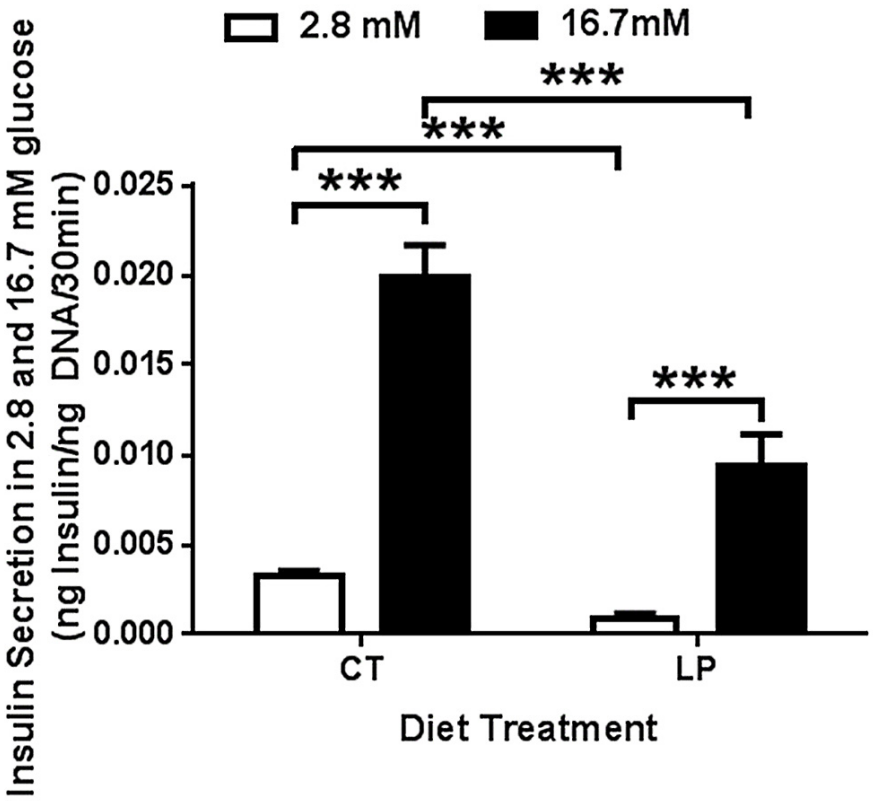

Figure 4. Glucose stimulated insulin secretion in pancreatic islets from pregnant rats fed a low protein diet in presence of 2.8 and 16.7 mM glucose. CT: control; LP, low protein. The error bar represents the mean ± SEM expressed as the ratio of amount of insulin secreted in medium during 30 minutes' incubation to total amount of DNA in pancreatic islets (n = 4-5 rats/diet group). ***, $P < 0.001$.

support fetal growth, but it has to be compensated by enhanced insulin secretion to maintain glucose homeostasis. This study and others (19) found plasma insulin levels were significantly lower in pregnant rats fed LP diet. This decrease in insulin levels not only reflects the reduction of insulin release from pancreatic beta cells (Figure 4), but also represents the dysregulation between insulin and blood glucose. Despite increases in plasma levels of ghrelin in LP rats during late phase of pregnancy (18) and local expression of *Ghrl* gene in pancreatic islets (Fig.2), ghrelin signaling fails to inhibit insulin secretion in pancreatic islets in LP rats. In contrast, ghrelin exerts its inhibition on GSIS in pregnant rats fed normal protein diet in obvious dose-dependent manner, thus we propose that ghrelin resistance in pancreatic islets may occur in LP rats during late phase of pregnancy similar to that occurs in the hypothalamus (24).

Low protein di*et al*ters glucose metabolism and insulin secretion during gestation. This study confirms that plasma insulin levels were reduced in LP rats compared to CT rats during late gestation (19). This reduction in insulin levels is supported by reduced GSIS of pancreatic islets from LP rats in both low and high concentrations of glucose (Figure 4). However, what causes the reduced GSIS in response to the LP diet during gestation remains unclear. In non-pregnant rats low protein diet reduces GSIS by hypotrophy of β-cells (27), decreased coupling among β-cells (28), inappropriate glucose metabolism (29), and disrupted signaling pathways (30-32). Unlike non-pregnant rats, beta cell proliferation peaks at mid-pregnancy and returns to basal levels at term (3;4), thus insulin secretion from islets may be a major regulator in insulin homeostasis during late pregnancy. This is supported by the reported higher pancreatic insulin content in pregnant rats fed a LP diet (33). Considering that ghrelin inhibits insulin secretion in both *in vivo* and *ex vivo* studies (8;9), and plasma levels of ghrelin are significantly increased in pregnant rats fed the LP diet (18), we originally expected that the elevated levels of ghrelin contributes to the reduction of plasma insulin levels in LP rats.

Our study is the first to show ghrelin signaling reducing the GSIS in isolated islets of pregnant rats, as most of previous studies, if not all, investigated the effects of ghrelin on GSIS in males. In presence of high glucose, ghrelin inhibits GSIS of islets from pregnant rats fed the CT diet, while both ghrelin

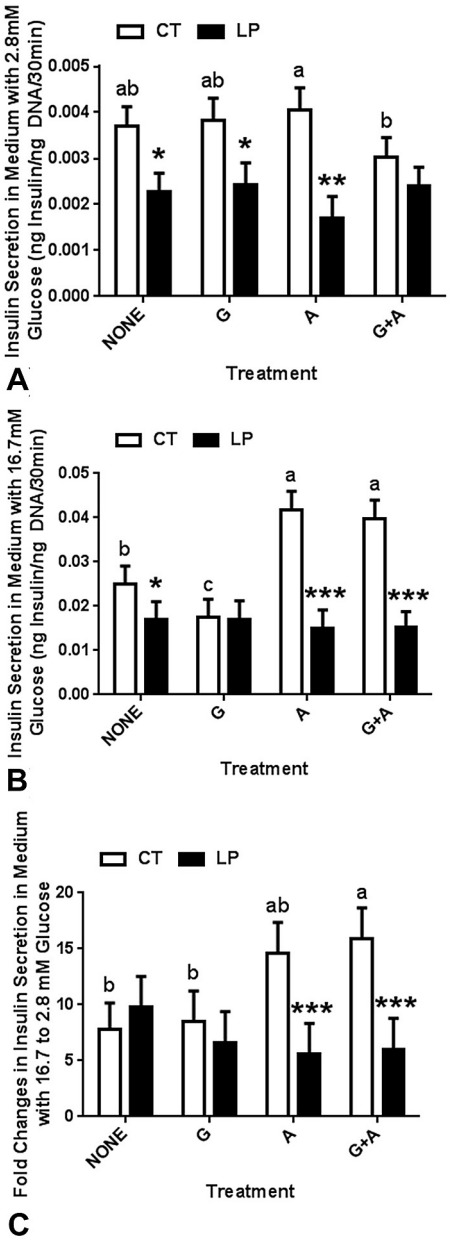

Figure 5. Effect of ghrelin and ghrelin antagonist on glucose stimulated insulin secretion in pancreatic islets of pregnant rats fed a low protein diet. (A) Insulin secretion in media in presence of 2.8 mM glucose; (B) Insulin secretion in media in presence of 16.7 mM glucose; The error bar represents the mean ± SEM expressed as the ratio of amount of insulin secreted in medium during 30 minutes' incubation to total amount of DNA in pancreatic islets (n = 4-5 rats/diet group). (C) Fold changes in insulin secretion in media with 16.7 to 2.8 mM glucose. CT: control; LP, low protein. NONE: culture medium without supplementation of ghrelin and its antagonist; G, ghrelin; A: ghrelin antagonist; G+A: ghrelin and its antagonist. Asterisks denote the statistical difference in each treatment between CT and LP rats. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$. Different letters denote statistical differences among all treatments within CT or LP group.

antagonist alone and the combination of ghrelin and its antagonist reverses the reduction in GSIS by ghrelin (Figure 5B). Therefore the inhibitory effect of ghrelin on GSIS is mediated by GHSR, the only known ghrelin receptor, which is abundantly expressed in beta cells of pancreatic islets (26;34). Our findings are consistent with previous studies in male rodents and also *Ghrl* or *Ghsr* ablated mice (35;36). Thus, the inhibitory effect of ghrelin on GSIS is maintained in pregnant dams. In contrast, ghrelin and its antagonist did not affect GSIS in pregnant rats fed the LP diet (Figs. 5A, 5B) regardless of glucose concentrations, although elevated mRNA levels of *Ghrl* and *Ghsr* may represent a positive adaptation (Figure 2). These indicate functional ghrelin receptor may not be present in beta cells of LP rats. Ghrelin receptor is constitutively

activated in cell membrane and accounts for 50% of its full activity when it is not bound by ligands (37). Its full activation by ghrelin requires the recruitment of Gαq protein to form an active receptor:Gαq complex (38). Moreover, the function of this complex in pancreatic islets is modified by somatostatin (SST) (39), cortistatin (40) and the energy balance (39). For instance, in low energy balance, GHSR/SST5 heterodimer is preferably bound to Gαi/o and inhibits insulin secretion; however, in high energy balance, the dissociation of SST5 from GHSR facilitates the formation of GHSR:Gαq complex (39). Pregnant rats fed the LP diet consumed significantly less diet with very little body weight gain in late gestation (18), and consequently were in obvious low energy balance. Therefore we could expect that the coupling between ghrelin receptor to Gαq proteins have been interrupted. In addition, ghrelin signaling pathway following ghrelin receptor activation in beta cells includes reduced cAMP production and activation of voltage-dependent Kv channels (Kv2.1), which causes the outward flow of potassium, attenuating membrane excitability and consequently suppressing calcium influx and insulin release. Whether these events are interrupted in beta cells of LP rats are unclear, but mRNA expression of Kv2.1 was increased in islets of LP rats (data not shown). The enhanced function of Kv2.1 could partly, if not entirely explain the reduced insulin secretion in islets of LP rats, as the activity of Kv. 2.1. could contribute to a background current which negatively affect the action potential of plasma membrane of beta cells in response to high extracellular glucose (41). In addition, long-term exposure to excess endogenous ghrelin in LP rats during mid- and late pregnancy (18) may lead to the blunted ghrelin signaling *in vivo*, and therefore pancreatic islets were less sensitive to ghrelin treatment *ex vivo*. All these proposed mechanisms responsible for blunted ghrelin signaling warrant future studies.

Insulin plays a critical role in suppressing blood glucose to avoid glucotoxicity in both pregnant and non-pregnant individuals and elevated insulin levels are required for compensating insulin resistance especially during late pregnancy (5-7). The reduction of plasma insulin levels in LP rats during late pregnancy may cause elevated blood glucose, as liver gluconeogenesis is enhanced during normal pregnancy, thus LP diet may cause gestational diabetes during late pregnancy. In addition, reduced insulin levels may also contribute to the impaired GSIS of pancreatic islets in LP rats and thus, further reduce GSIS by a positive feedback, as knockout of insulin receptor causes reduced insulin response to glucose and amino acids (42). In addition, trophoblast cells are highly sensitive to insulin (43), as insulin stimulates placental growth (44), while hyperglycemia slows down trophoblast growth (45). Therefore, lower insulin levels may negatively affect placental growth as well as function and thus, playing an important role in placental programming in response to low protein diet during gestation.

In summary, in this study we report that ghrelin signaling is involved in regulating beta cell functions, and it inhibits GSIS of pancreatic islets in pregnant rats fed CT diet. Ghrelin signaling is blunted in pregnant rats fed LP diet and thus may not contribute to the reduction of plasma insulin and GSIS of pancreatic islets in late pregnancy of rats fed LP diet.

6. ACKNOWLEDGEMENTS

The authors have nothing to declare. This research was supported by National Institutes of Health grants R01HL102866 and R01HL58144. The authors thank Dr. Vijay Yechoor, Dr. Daniel Simoes and Mrs. Jong Kyung Lee in Division of Diabetes, Endocrinology and Metabolism, Department of Medicine, Baylor College of Medicine for their technical supports and also Mrs. Sandra Dale for editorial work on this manuscript and administrative support.

7. REFERENCES

1. KY Lain, PM Catalano: Metabolic changes in pregnancy. *Clin Obstet Gynecol* 50, 938-948 (2007)
DOI: 10.1097/GRF.0b013e31815a5494

2. NF Butte: Carbohydrate and lipid metabolism in pregnancy: normal compared with gestational diabetes mellitus. *Am J Clin Nutr* 71, 1256S-1261S (2000)

3. JA Parsons, TC Brelje, RL Sorenson: Adaptation of islets of Langerhans to pregnancy: increased islet cell proliferation and insulin secretion correlates with the onset of placental lactogen secretion. *Endocrinology* 130, 1459-1466 (1992)

4. RL Sorenson, TC Brelje: Adaptation of islets of Langerhans to pregnancy: beta-cell growth, enhanced insulin secretion and the role of lactogenic hormones. *Horm Metab* Res 29, 301-307 (1997)
DOI: 10.1055/s-2007-979040

5. C Munoz, P Lopez-Luna, E Herrera: Glucose and insulin tolerance tests in the rat on different days of gestation. *Biol Neonate* 68, 282-291 (1995)
DOI: 10.1159/000244248

6. CG Di, R Miccoli, L Volpe, C Lencioni, PS Del: Intermediate metabolism in normal pregnancy and in gestational diabetes.

Diabetes Metab Res Rev 19, 259-270 (2003)
DOI: 10.1002/dmrr.390

7. RH Knopp, HJ Ruder, E Herrera, N Freinkel: Carbohydrate metabolism in pregnancy. VII. Insulin tolerance during late pregnancy in the fed and fasted rat. *Acta Endocrinol (Copenh)* 65, 352-360 (1970)

8. F Broglio, E Arvat, A Benso, C Gottero, G Muccioli, M Papotti, AJ van der Lely, R Deghenghi, E Ghigo: Ghrelin, a natural GH secretagogue produced by the stomach, induces hyperglycemia and reduces insulin secretion in humans. *J Clin Endocrinol Metab* 86, 5083-5086 (2001)
DOI: 10.1210/jcem.86.10.8098

9. K Dezaki, H Hosoda, M Kakei, S Hashiguchi, M Watanabe, K Kangawa, T Yada: Endogenous ghrelin in pancreatic islets restricts insulin release by attenuating Ca2+ signaling in beta-cells: implication in the glycemic control in rodents. *Diabetes* 53, 3142-3151 (2004)
DOI: 10.2337/diabetes.53.12.3142

10. T Yada, B Damdindorj, RS Rita, T Kurashina, A Ando, M Taguchi, M Koizumi, H Sone, M Nakata, M Kakei, K Dezaki: Ghrelin signalling in beta-cells regulates insulin secretion and blood glucose. *Diabetes Obes Metab* 16 Suppl 1, 111-117 (2014)
DOI: 10.1111/dom.12344

11. A Kautzky-Willer, A Handisurya: Metabolic diseases and associated complications: sex and gender matter! *Eur J Clin Invest* 39, 631-648 (2009)
DOI: 10.1111/j.1365-2362.2009.02161.x

12. V Iglesias-Barreira, MT Ahn, B Reusens, S Dahri, JJ Hoet, C Remacle: Pre- and postnatal low protein diet affect pancreatic islet blood flow and insulin release in adult rats. *Endocrinology* 137, 3797-3801 (1996)

13. MA de-Mello, L Cury: Effects of protein-calorie malnutrition on endocrine pancreatic function in young pregnant rats. *Braz J Med Biol Res* 22, 791-794 (1989)

14. MA de Mello, E Luciano, EM Carneiro, MQ Latorraca, CA Machado de Oliveira, AC Boschero: Glucose homeostasis in pregnant rats submitted to dietary protein restriction. *Res Commun Mol Pathol Pharmacol* 113-114, 229-246 (2003)

15. AR Pinheiro, ID Salvucci, MB Aguila, CA Mandarim-de-Lacerda: Protein restriction during gestation and/or lactation causes adverse transgenerational effects on biometry and glucose metabolism in F1 and F2 progenies of rats. *Clin Sci (Lond*) 114, 381-392 (2008)
DOI: 10.1042/CS20070302

16. E Zambrano, PM Martinez-Samayoa, CJ Bautista, M Deas, L Guillen, GL Rodriguez-Gonzalez, C Guzman, F Larrea, PW Nathanielsz: Sex differences in transgenerational alterations of growth and metabolism in progeny (F2) of female offspring (F1) of rats fed a low protein diet during pregnancy and lactation. *J Physiol* 566, 225-236 (2005)
DOI: 10.1113/jphysiol.2005.086462

17. H Guan, E Arany, JP van Beek, A Chamson-Reig, S Thyssen, DJ Hill, K Yang: Adipose tissue gene expression profiling reveals distinct molecular pathways that define visceral adiposity in offspring of maternal protein-restricted rats. *Am J Physiol Endocrinol Metab* 288, E663-E673 (2005)
DOI: 10.1152/ajpendo.00461.2004

18. H Gao, DT Tanchico, U Yallampalli, MP Balakrishnan, C Yallampalli: Appetite regulation is independent of the changes in ghrelin levels in pregnant rats fed low-protein diet. *Physiol Rep* 3, 4 (2015)
DOI: 10.14814/phy2.12368

19. N Jansson, J Pettersson, A Haafiz, A Ericsson, I Palmberg, M Tranberg, V Ganapathy, TL Powell, T Jansson: Down-regulation of placental transport of amino acids precedes the development of intrauterine growth restriction in rats fed a low protein diet. *J Physiol* 576, 935-946 (2006)

20. PE Lacy, M Kostianovsky: Method for the isolation of intact islets of Langerhans from the rat pancreas. *Diabetes* 16, 35-39 (1967)
DOI: 10.2337/diab.16.1.35

21. K Dezaki, H Hosoda, M Kakei, S Hashiguchi, M Watanabe, K Kangawa, T Yada: Endogenous ghrelin in pancreatic islets restricts insulin release by attenuating Ca2+ signaling in beta-cells: implication in the glycemic control in rodents. *Diabetes* 53, 3142-3151 (2004)
DOI: 10.2337/diabetes.53.12.3142

22. JD Carter, SB Dula, KL Corbin, R Wu, CS Nunemaker: A practical guide to rodent islet isolation and assessment. *Biol Proced Online* 11, 3-31 (2009)
DOI: 10.1007/s12575-009-9021-0

23. H Gao, DT Tanchico, U Yallampalli, MP Balakrishnan, C Yallampalli: Appetite regulation is independent of the changes in ghrelin levels in pregnant rats fed low-protein diet. *Physiol Rep* 3, 12 (2015)
DOI: 10.14814/phy2.12368

24. H Gao, S Sisley, C Yallampalli: Blunted hypothalamic ghrelin signaling reduces diet intake in rats fed a low-protein diet in late pregnancy. *Physiol Rep* 3, 12 (2015)
DOI: 10.14814/phy2.12629

25. H Ni, WK De, P Walia, JP Chanoine: *In vitro* and *in vivo* effect of acylated and unacylated ghrelin on neonatal glucose homeostasis. *Pediatr Res* 67, 609-613 (2010)
DOI: 10.1203/PDR.0b013e3181da463a

26. H Kageyama, H Funahashi, M Hirayama, F Takenoya, T Kita, S Kato, J Sakurai, EY Lee, S Inoue, Y Date, M Nakazato, K Kangawa, S Shioda: Morphological analysis of ghrelin and its receptor distribution in the rat pancreas. *Regul Pept* 126, 67-71 (2005)
DOI: 10.1016/j.regpep.2004.08.031

27. RH Rao: Chronic undernutrition may accentuate the beta cell dysfunction of type 2 diabetes. *Diabetes Res Clin Pract* 8, 125-130 (1990)
DOI: 10.1016/0168-8227(90)90022-L

28. A Marcal-Pessoa, C Bassi-Branco, CD Salvatierra, L Stoppiglia, L Ignacio-Souza, RS de Lima, R Veloso, RM de Barros, E Carneiro, A Boschero, V Arantes, M Latorraca: A low-protein diet during pregnancy prevents modifications in inter-cellular communication proteins in rat islets. *Biol Res* 48, 3 (2015)
DOI: 10.1186/0717-6287-48-3

29. J Rasschaert, B Reusens, S Dahri, A Sener, C Remacle, JJ Hoet, WJ Malaisse: Impaired activity of rat pancreatic islet mitochondrial glycerophosphate dehydrogenase in protein malnutrition. *Endocrinology* 136, 2631-2634 (1995)

30. MQ Latorraca, EM Carneiro, MA Mello, AC Boschero: Reduced insulin secretion in response to nutrients in islets from malnourished young rats is associated with a diminished calcium uptake. *J Nutr Biochem* 10, 37-43 (1999)
DOI: 10.1016/S0955-2863(98)00080-1

31. Ferreira F, Barbosa HC, Stoppiglia LF, Delghingaro-Augusto V, Pereira EA, Boschero AC, Carneiro EM 2004 Decreased insulin secretion in islets from rats fed a low protein diet is associated with a reduced PKAalpha expression. *J Nutr* 134, 63-67

32. F Ferreira, E Filiputti, VC Arantes, LF Stoppiglia, EP Araujo, V Delghingaro-Augusto, MQ Latorraca, MH Toyama, AC Boschero, EM Carneiro: Decreased cholinergic stimulation of insulin secretion by islets from rats fed a low protein diet is associated with reduced protein kinase calpha expression. *J Nutr* 133, 695-699 (2003)

33. MA de Mello, E Luciano, EM Carneiro, MQ Latorraca, CA Machado de Oliveira, AC Boschero: Glucose homeostasis in pregnant rats submitted to dietary protein restriction. *Res Commun Mol Pathol Pharmacol* 113-114, 229-246 (2003)

34. S Gnanapavan, B Kola, SA Bustin, DG Morris, P McGee, P Fairclough, S Bhattacharya, R Carpenter, AB Grossman, M Korbonits: The tissue distribution of the mRNA of ghrelin and subtypes of its receptor, GHS-R, in humans. *J Clin Endocrinol Metab* 87, 2988 (2002)
DOI: 10.1210/jcem.87.6.8739

35. T Kurashina, K Dezaki, M Yoshida, RR Sukma, K Ito, M Taguchi, R Miura, M Tominaga, S Ishibashi, M Kakei, T Yada: The beta-cell GHSR and downstream cAMP/TRPM2 signaling account for insulinostatic and glycemic effects of ghrelin. *Sci Rep* 5, 14041 (2015)
DOI: 10.1038/srep14041

36. Y Sun, M Asnicar, PK Saha, L Chan, RG Smith: Ablation of ghrelin improves the diabetic but not obese phenotype of ob/ob mice. *Cell Metab* 3, 379-386 (2006)
DOI: 10.1016/j.cmet.2006.04.004

37. M Damian, J Marie, JP Leyris, JA Fehrentz, P Verdie, J Martinez, JL Baneres, S Mary: High constitutive activity is an intrinsic feature of ghrelin receptor protein: a study with a functional monomeric GHS-R1a receptor reconstituted in lipid discs. *J Biol Chem* 287, 3630-3641 (2012)
DOI: 10.1074/jbc.M111.288324

38. M Damian, S Mary, M Maingot, C M'Kadmi, D Gagne, JP Leyris, S Denoyelle, G Gaibelet, L Gavara, CM Garcia de Souza, D Perahia, E Trinquet, B Mouillac, S Galandrin, C Gales, JA Fehrentz, N Floquet, J Martinez, J Marie, JL Baneres: Ghrelin receptor conformational dynamics regulate the transition from a preassembled to an active receptor:Gq complex. *Proc Natl Acad Sci U S A* 112, 1601-1606 (2015)
DOI: 10.1073/pnas.1414618112

39. S Park, H Jiang, H Zhang, RG Smith: Modification of ghrelin receptor signaling by somatostatin receptor-5 regulates insulin release. *Proc Natl Acad Sci U S A* 109, 19003-19008 (2012)
DOI: 10.1073/pnas.1209590109

40. B Chanclon, RM Luque, J Cordoba-Chacon, MD Gahete, AI Pozo-Salas, JP Castano, F Gracia-Navarro, AJ Martinez-Fuentes: Role of endogenous cortistatin in the regulation of ghrelin system expression at pancreatic level under normal and obese conditions. *PLoS One* 8, e57834 (2013)
DOI: 10.1371/journal.pone.0057834

41. JC Henquin, M Nenquin, MA Ravier, A Szollosi: Shortcomings of current models of glucose-induced insulin secretion. *Diabetes Obes Metab* 11 Suppl 4, 168-179 (2009)
DOI: 10.1111/j.1463-1326.2009.01109.x

42. RN Kulkarni, JN Winnay, M Daniels, JC Bruning, SN Flier, D Hanahan, CR Kahn: Altered function of insulin receptor substrate-1-deficient mouse islets and cultured beta-cell lines. *J Clin Invest* 104, R69-R75 (1999)
DOI: 10.1172/JCI8339

43. L Lassance, M Haghiac, P Leahy, S Basu, J Minium, J Zhou, M Reider, PM Catalano, MS Hauguel-de: Identification of early transcriptome signatures in placenta exposed to insulin and obesity. *Am J Obstet Gynecol* 212, 647-11 (2015)
DOI: 10.1016/j.ajog.2015.02.026

44. U Hiden, E Glitzner, M Ivanisevic, J Djelmis, C Wadsack, U Lang, G Desoye: MT1-MMP expression in first-trimester placental tissue is upregulated in type 1 diabetes as a result of elevated insulin and tumor necrosis factor-alpha levels. *Diabetes* 57, 150-157 (2008)
DOI: 10.2337/db07-0903

45. JD Frohlich, B Huppertz, PM Abuja, J Konig, G Desoye: Oxygen modulates the response of first-trimester trophoblasts to hyperglycemia. *Am J Pathol* 180, 153-164 (2012)
DOI: 10.1016/j.ajpath.2011.09.012

Key Words: Ghrelin, Insulin, Pancreatic Islet, Pregnancy, Low Protein Diet

Send correspondence to: Chandra Yallampalli, Baylor College of Medicine, Department of Obstetrics and Gynecology, 1102 Bates Street, Suite # 450, Houston, Texas 77030, Tel: 832-824 4188, Fax: 832-825 7946, E-mail: cyallamp@bcm.edu