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ORIGINAL ARTICLE

Differential expression of gonadotropin-releasing hormone (GnRH) in pancreas during rat pregnancy

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Abstract Many studies have shown that there is a relationship between gonadotropin-releasing hormone (GnRH) and glucose metabolism, but little is known about the effects of GnRH on the pancreas. Our experiment investigated the effect of GnRH on pancreatic islet cell in Sprague–Dawley (SD) rats fed with high-cholesterol diet before and during pregnancy. We found that although high-cholesterol diet led to no significant difference of GnRH mRNA levels in pancreas in nonpregnant rats, it led to a marked increase of those in pregnant rats. Furthermore, in rats fed with standard laboratory chow, no significant differences were apparent in GnRH mRNA levels before and during gestation; however, when fed with high-cholesterol diet, the GnRH mRNA levels increased significantly in pregnant rats. As results indicated both diets could lead to increase of PG mRNA in pancreas of pregnant rats. It is also demonstrated that the GnRH mRNA levels are positively associated with PG mRNA levels. Moreover, our data showed a significant increase in fasting insulin level in the Gestation group compared with Control. Such changes were contrary to the changes of GnRH level in the pancreas. This may imply that GnRH influences hormones secretion in the pancreas by autocrine and paracrine effects on islet cells.

Keywords FIN · GnRH · High-cholesterol diet · Pregnancy

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Introduction

Several studies have shown that gonadotropin-releasing hormone (GnRH) and its receptor (GnRHR) existed and played a biological effect in several tissues other than hypothalamus-pituitary-gonad (HPG) Axis tissues. Khodr et al. [1] first confirmed the presence of GnRH in placenta. After that, other groups reported that GnRH as well as gene expression and synthesis have been shown in many reproductive organs such as ovary, mammary glands, and endometrium [2–5]. GnRH synthesized by gonads could regulate the steroid hormone synthesis and metabolism in mammals' reproductive system as an autocrine factor [6]. Moreover, GnRH was found to widely exist in various tumors [7–9] and immune system [10, 11]. It was also noticed that GnRH analogs could inhibit the proliferation of liver cancer and other tumor cells, which suggested that GnRH may be involved in the regulation of tumor process [12–14]. GnRH agonist administration leads to increased mRNA level of GnRHR in the thymus, spleen, peripheral lymphocyte, and other immune cells and increased r chain mRNA expression of the IL-2 receptor in peripheral blood mononuclear cell [15, 16].

We previously demonstrated that GnRH and its receptor are synthesized in rat gastrointestinal system, pancreas

and submaxillary glands, and modulate the digestive tract function as a kind of gastrointestinal hormone [17–19]. The results of immunohistochemistry, in situ hybridization, and gene sequencing confirmed that GnRH, which has the same mRNA sequence with that of hypothalamus, widely existed in the exocrine pancreas [20]. However, GnRH receptors (GnRHR) exist in both exocrine and endocrine pancreas [21–23].

Smith et al. [24, 25] found that GnRH agonist administration led to insulin resistance syndrome and decreased the insulin sensitivity in patients with prostate cancer, which suggested that GnRH agonists were positively associated with diabetes mellitus and cardiovascular disease risk. GnRH agonists also increased insulin resistance in premenopausal women with symptomatic uterine leiomyomas [26]. Clinical study showed the increased prevalence of gestational diabetes mellitus (GDM) in in vitro fertilization pregnancies inadvertently conceived during treatment with long-acting GnRH analogs [27]. Insulin resistance (IR) is increased in middle and late pregnancy stage; however, only about 5% of pregnant women develop gestational diabetes mellitus, which suggests that GDM is linked to pancreatic islet B cell function and that postreceptor defects in insulin signaling may contribute to the pathogenesis of GDM [28, 29]. Although many studies have shown that there was a relationship between GnRH and glucose metabolism, little is known about the effects of GnRH and its receptor on pancreas. We noticed that GnRH existed in the exocrine pancreas and its receptor existed in islet cell. We presumed that GnRH may play a role in pancreas tissues through paracrine or autocrine effects.

Since serum GnRH levels changed significantly during pregnancy, we wondered whether the GnRH levels of pancreas changed also and whether the GnRH levels affected the pancreatic islet cell function during pregnancy. In this report, we describe the changes of insulin secretion, GnRH and its receptor mRNA levels and proglucagon (PG) mRNA levels in pancreas tissues of rats feeding high-cholesterol diet during pregnancy. In addition, in order to investigate the effect of GnRH on pancreatic islet cell, the correlation between insulin secretion and the levels of pancreatic GnRH and its receptor was assessed.

Results

Body weight and blood biochemical parameters

The body weight and blood biochemical parameters of rats are summarized in Table 1 and Fig. 1. At 14 days (starting the experimental diets for 18 weeks) after gestation, the body weight, CH and TG were significantly higher in HCh group than those in Control. The same significant differences were found between HCh + Gestation group and other groups. The body weight in the Gestation group was significantly higher than that of Control group; however, there were no differences in CH and TG between the groups. The serum LH concentration significantly increased in the Gestation group compared to the Control group. Similarly, the serum LH concentration was higher in HCh + Gestation than that of HCh group. The rats in HCh group had significantly greater GnRH concentration than control rats. The serum GnRH concentration was higher in HCh + Gestation than that of Gestation group (Table 1).

Figure 1 illustrates the changes in the blood glucose levels and insulin levels during oral glucose tolerance test (OGTT) in rats. The results showed that the glucose concentration of rats in each group reached peak values at 30 min after glucose administration. However, the insulin secretion reached the peak at 15 min in Control, and at 30 min in HCh, Gestation and HCh + Gestation groups, which noted the peak of insulin secretion could be delayed by both high-cholesterol diet and pregnancy status. The fasting concentration of insulin was significantly higher in the Gestation group than that of Control, while no significant differences were apparent in the insulin area under the curve (AUC) between the two groups. This noted insulin resistance in pregnancy is mainly on the increase of basal insulin. The insulin AUC was significantly increased in the HCh group compared with Control. However, insulin sensitivity index (ISI) value in HCh group was significantly lower than in Control (Table 1). This showed insulin resistance existed in hyperlipemia.

The fasting concentration of insulin in HCh + Gestation group was significantly higher than that of Control, while there was no significant difference compared with that of

Table 1 Body weight and blood biochemical parameters of the rats fed experimental diets

Groups	Control	HCh	Gestation	HCh + Gestation
Diet	Standard chow	High cholesterol	Standard chow	High cholesterol
Gestation	−	−	+	+
Weight (g)	298.13 ± 10.26	332.5 ± 12.03[a]	337.5 ± 6.12[a]	403.13 ± 7.07[a,b,c]
CH (mmol/l)	2.25 ± 0.22	3.43 ± 0.27[a]	2.41 ± 0.20	4.10 ± 0.20[a,b,c]
TG (mmol/l)	1.28 ± 0.17	2.15 ± 0.23[a]	1.60 ± 0.19	3.00 ± 0.13[a,b,c]
GnRH (μg/l)	10.59 ± 0.51	12.09 ± 0.73[a]	10.17 ± 0.23	12.33 ± 0.72[a,c]
LH (μg/l)	5.17 ± 0.19	5.55 ± 0.33	6.12 ± 0.27[a]	6.95 ± 0.30[a,b,c]
ISI	4.95 ± 0.56	2.84 ± 0.52[a]	4.64 ± 0.90	2.21 ± 0.32[c]

Values are means ± SE, $n = 8$

[a] Versus Control

[b] Versus HCh

[c] Versus Gestation ($P < 0.05$)

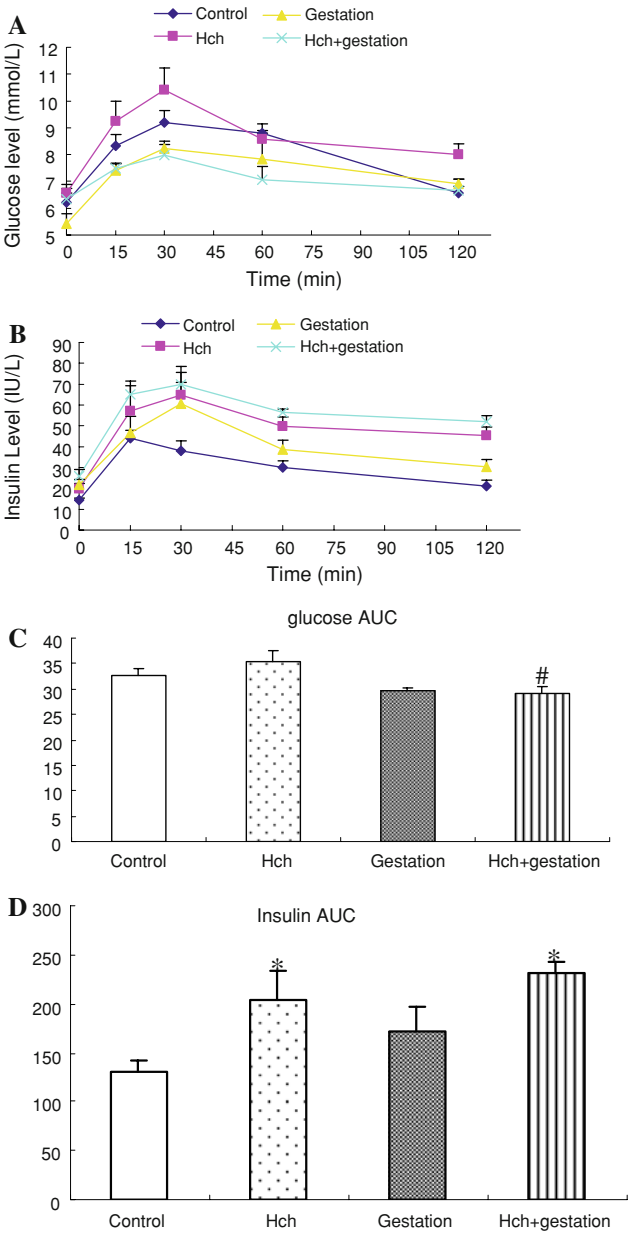

Fig. 1 Variation in the serum glucose and insulin concentration and their AUC after feeding experimental diets in rats. **a** Changes of blood glucose levels at 0, 15, 30, 60, and 120 min after glucose administration. **b** Changes of blood insulin levels at 0, 15, 30, 60, and 120 min after glucose administration. **c** Glucose AUC. **d** Insulin AUC. Values are means ± SE, $n = 8$. * Versus Control, # versus HCh. Differences were considered significant for $P < 0.05$

the HCh group. The glucose AUC in the HCh + Gestation group was significantly lower than that of the HCh group. Moreover, the insulin AUC showed significant increase in the HCh + Gestation group than Control, and the ISI value in HCh + Gestation group was significantly lower than that in Control and Gestation groups. These results noted that hyperinsulinemia and insulin resistance could already appear in middle pregnancy with high-cholesterol diet.

Expression of pancreatic tissue genes

The results from the RT–PCR analysis of the total RNA prepared from the pancreatic tissue of the rats are shown in Fig 2. In Fig 2a, there were 28S, 18S and 5S distinct straps in 3% agarose gel electrophoresis (AGE) of total RNA, and there was insignificant DNA contamination and degradation.

The GnRH mRNA levels did not differ significantly between the Control and HCh group or Gestation group. The GnRH mRNA level tended to be higher in the HCh + Gestation group than that of the Gestation or HCh groups (Fig. 2b). In this study, no difference in GnRHR mRNA expression was found between all groups (Fig. 2c).

The level of PG mRNA was found to be significantly higher in the Gestation group than that of Control. The PG mRNA level did not differ significantly between the Control and HCh groups. We also could not find the difference in the expression of PG mRNA between the HCh + Gestation group and Gestation group. The expression of PG mRNA significantly increased in the HCh + Gestation group than that of the HCh group (Fig. 2d).

No difference in glucagon-like peptide-1 (GLP-1) mRNA expression was found between all groups (Fig. 2e).

The correlation between the GnRH mRNA and proglucagon mRNA in rat pancreas

The association between the GnRH mRNA levels and proglucagon mRNA levels in pancreas is shown in Fig. 3. A positive association was observed between the GnRH mRNA levels and proglucagon mRNA levels. The correlation coefficient (r) was 0.521 ($P = 0.01$).

Expression of GnRH protein in rat pancreas

As shown in Fig. 4 we could not find a difference in the expression of GnRH protein of rat pancreas between the Control and HCh group or Gestation group, whereas the GnRH protein level of rat pancreas was up-regulated in the HCh + Gestation group compared to the HCh or Gestation groups.

Discussion

Our results showed that insulin secretion increased and reached maximum at 15 min in control rats after glucose administration; however, the peak was delayed to 30 min in rats in the other three groups. This prompted the peak of insulin secretion could be delayed by both the disorder of lipid metabolism and pregnancy status. At the same time, insulin sensitivity decreased in normal pregnancy, and

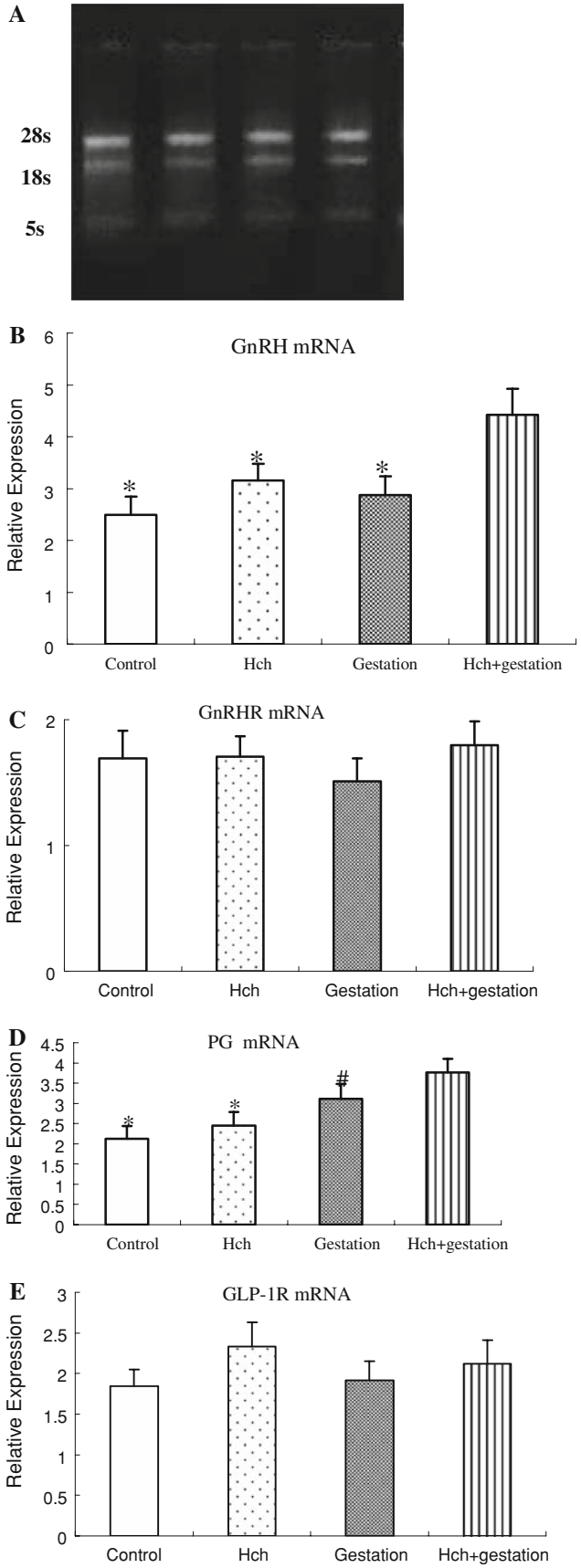

Fig. 2 Expression of GnRH, GnRHR, PG and GLP-1 mRNA. The mRNA levels were analyzed by RT-PCR.**a** Electrophoresis of the total mRNA in pancreatic tissue. **b** Levels of GnRH mRNA. **c** Levels of GnRHR mRNA. **d** Levels of PG. **e** Levels of GLP-1 mRNA. * Versus HCh + Gestation, # versus Control. Differences were considered significant for $P < 0.05$

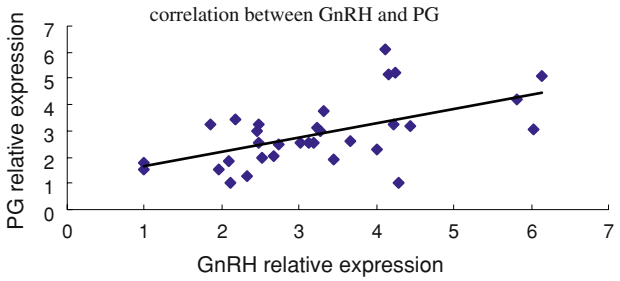

Fig. 3 The association between the GnRH mRNA levels and proglucagon mRNA levels in pancreas. $r = 0.5219$, $P = 0.01$

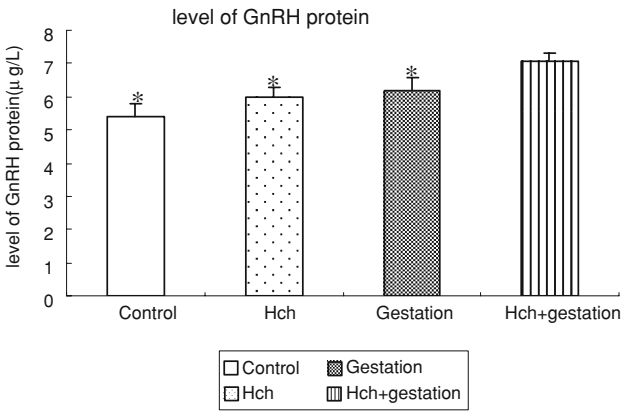

Fig. 4 The expression of GnRH protein in rat pancreas in the four groups. * Versus HCh + Gestation. Differences were considered significant for $P < 0.05$

especially in abnormal pregnancy with metabolic disorder [30, 31]. The fasting insulin level (FIN) in normal pregnancy rats was higher than that of non-pregnant rats. However, after feeding with high-cholesterol diet, there were no significant differences in the fasting insulin level between pregnancy rats and non-pregnant rats. Moreover, the insulin AUC in pregnancy rats with high-cholesterol diet was significantly higher than that of control rats, while no significant differences were apparent compared with that of the non-pregnant rats with high-cholesterol diet. The glucose AUC in pregnancy rats with high-cholesterol diet was significantly lower than that of non-pregnant rats with high-cholesterol diet, while no significant differences were apparent compared with that of control rats. In addition, high-cholesterol diet led to reduction of the ISI value in both pregnant and non-pregnant rats. This

suggested insulin resistance would be increased in pregnancy with high-fat diet, and its glucose metabolism has its own particularity.

In the present study, we carried out semi-quantitative RT–PCR to observe the mRNA expression of GnRH, GnRHR, proglucagon and GLP-1 in pancreas of rats, using β-actin gene as the endogenous reference gene. In addition, GnRH protein level was detected by ELISA. We found that although high-cholesterol diet led to no significant difference of GnRH mRNA levels in pancreas in non-pregnant rats (HCh group compared with Control), it could lead to markedly increase of GnRH mRNA levels in pancreas in pregnant rats (HCh + Gestation group compared with Gestation group). Furthermore, in rats fed with standard laboratory chow, no significant differences were apparent in the GnRH mRNA levels before and during gestation; however, when fed with high-cholesterol diet, the GnRH mRNA levels increased significantly in pregnant rats compared with non-pregnant rats. The changes in the mRNA levels were consistent with those of the protein levels. We found it very interesting that a high-cholesterol diet led to different GnRH expression changes in rats before and during pregnancy and the pregnant rats had different GnRH expression changes after being fed with standard laboratory chow and high-cholesterol diet, respectively. The changes of GnRH levels in pancreas have no relevance to fasting serum levels of GnRH. As our results indicated that there were no obvious changes in GLP-1 receptor mRNA levels among the groups; however, both standard laboratory chow and high-cholesterol diet could lead to a significant increase of PG mRNA in pancreas of pregnant rats compared with non-pregnant rats. This suggested that glucagon level in pregnancy was higher than non-pregnant.

It has also been demonstrated that the GnRH mRNA levels are positively associated with the PG mRNA levels. A recent study had revealed that glucagon was regulated mainly through endocrine and paracrine effects, and neural regulation is of secondary significance [32]. Many factors had participated in regulation of glucagon, including insulin, zinc, GABA, glutamate, etc. [33]. Our previous studies have clearly demonstrated that GnRH existed in exocrine cell of rat pancreas, and the GnRHR distribution is the same as that of islet cell [20–23]. We presume that activated GnRH in exocrine cells of pancreas may act on islet cell through paracrine effects and influence its hormones secretion such as glucagon secretion.

It was well demonstrated that there was a relationship between GnRH and glucose metabolism. Several investigators have reported GnRH agonists could increase the risk of diabetes mellitus and cardiovascular disease [24, 25]. In premenopausal women with symptomatic uterine leiomyomas, GnRH agonists also increased insulin resistance [26]. Ansari et al. [34] found castration of the male non-obese mouse model of autoimmune diabetes (NOD mouse) led to an increased incidence of diabetes, and GnRH administration exerted reciprocal effects, leading to earlier timing of onset of diabetes. In addition, GnRH receptor antagonist prevented the increased incidence of diabetes in the castrated male mice. GnRH modulates the expression of diabetes in the NOD mouse independently of gonadal steroids. These studies focus on the effect of GnRH analogues on the blood glucose and insulin. Although data about the effect of endogenous GnRH on glucose metabolism are scarce, in the PCOS patients study, it showed that 40% of the patients existed impaired glucose tolerance, 10% with diabetes [35]. Many studies reported that IR led to the disorder of glucose metabolism in PCOS patients, but the causal relationship between IR and disorder of glucose metabolism in patients is not clear [36]. At the same time, these patients showed a higher rhythmic release of GnRH, which leads to increased levels of GnRH. It is unclear whether abnormal secretion of GnRH leads to glucose metabolism disorders in POCS women. But the data of the relationship between exogenous GnRH and glucose metabolism suggested that endogenous GnRH played an important role in glucose metabolism.

Endogenous GnRH, a sex hormone, has a physiological effect at all stages of human life, especially in women pregnancy. Many experiments showed the relationship between changes of sex hormones and changes of glucose metabolism [37–39]. Diamond et al. [40, 41] concluded that glucose metabolism was impaired in the luteal phase of the menstrual cycle and the mechanism of this defect was unclear. Others have found that during menstruation, diabetic women experienced worse control of their blood sugar and an increased incidence of ketoacidosis. Physiological IR appeared in pregnancy [42]. Although the levels of insulin hormone antagonists such as placenta lactogen, prolactin, and progesterone and glucocorticoid hormones increase progressively during pregnancy, relatively few pregnant women developed IR-related diseases such as gestational diabetes mellitus [43]. These results suggested that there are impaired islet cell functions in those patients. However, it is not clear how islet function changed during pregnancy. Clinical trials have confirmed that the prevalence of the metabolic syndrome in women with previous gestational diabetes mellitus (GDM) is three-fold higher than in general population [44].

Our data showed a significant increase in fasting insulin level in the Gestation group and HCh + Gestation group compared with Control, but there were no obvious changes in fasting insulin level between the HCh and Control groups. This suggested that it was pregnancy leading to the increase of fasting insulin level in rats with normal or high-cholesterol diet. Such changes were contrary to the changes of GnRH level in pancreas. The report about the direct

effect of insulin on the GnRH is from the diabetes model research. Insulin treatment and physiological levels of central insulin supplementation are able to modulate LH (GnRH) pulse frequency in diabetic models [45, 46]. These results support the notion that central insulin plays a role in regulating pulsatile GnRH secretion. It was proved in in vitro experiments that GnRH could inhibit insulin release from islet cell mass [47]. Clinical research indicated that GnRH agonists could decrease insulin sensitivity and increase FIN level [24]. Together with these earlier results, we speculated that in the normal physiological state of pregnancy, with the increase of pregnancy IR and fasting insulin levels, glucose metabolism could be modulated at normal levels. However, in pregnancy with hyperlipidemia, as GnRH level in pancreas increased inappropriately, it is possible to play a physiological function through autocrine and paracrine effects, contributing to failure of further compensatory secretion of insulin and further deterioration of IR. The mechanism is not clear.

Recently, Weksler-Zangen et al. [48] have reported that impaired glucose-stimulated insulin secretion is coupled with exocrine pancreatic lesions in the Cohen diabetic rat. They indicated the changes in the islet microenvironment are the culprit in the insulin secretory malfunction. However, the study did not point out whether pancreatic exocrine function modulated the pancreatic endocrine function by paracrine effect, and whether there were molecules regulating pancreatic exocrine and endocrine function. Our results indicated that GnRH may be the regulatory factor linking pancreatic exocrine function with endocrine function. Its changes may affect the function of islet cells, including the effects of glucagon and insulin secretion. Our further mission is to explore how GnRH affects islet cells function at the molecular level.

Material and methods

Animals and diets

Thirty-two female Sprague–Dawley (SD) rats (4 weeks old, weighing 80–90 g) were obtained from the Laboratory Animal Center of The Fourth Military Medical University. The rats were maintained at a stable temperature (22 ± 2°C) and humidity (55 ± 5%), with a 12-h light/dark cycle and were given free access to water. After feeding the rats on standard laboratory chow (Laboratory Animal Centre, The Fourth Military Medical University) for 7 days, all rats were randomly divided into four groups. The first and the second groups (Control and Gestation) of rats were given a standard laboratory chow, and the third and the forth groups (HCh and HCh + Gestation) of the rats were given a high-cholesterol diet. The high-cholesterol diet contained 1% cholesterol, 10% lard, 10% yolk, and 74% standard chow. The rats in the four groups were fed the respective diets for 16 weeks. Then, rats in the Gestation and Hch + Gestation groups were mated with normal male SD rats. After mating, the first day of gestation was estimated by the presence of spermatozoa in vaginal smears. Pregnant rats were housed individually in wood-chip bedded plastic cages. The experimental procedure used in the present study met the guidelines of the National Institutes of Health and the regulations of The Fourth Military Medical University.

Biochemical analysis

Blood samples were collected from the rats before and 14 days (starting the experimental diets for 18 weeks) after gestation. Blood was drawn from the tail vein into disinfection centrifugal tube after overnight (12 h) fasting. The blood was centrifuged at $3000\times g$ for 10 min. Serum cholesterol (CH) and triglyceride (TG) were determined by biochemistry analyzer (Cobas Integra 400 Plus, Roche). Serum GnRH and LH were measured by ELISA using KIT (Adlitteram Diagnostic Laboratories, Inc.).

Oral glucose tolerance test (OGTT) and insulin releasing test (IRT)

OGTT were performed on the rats at 0 and 18 weeks (14 days after gestation) after feeding the respective diets. The rats were orally administered with a glucose solution (2 g/kg body weight) after overnight (12 h) fasting, and blood samples were collected at fasting (0 min) and 15, 30, 60, and 120 min from the tail vein after glucose administration [30, 31]. To determine the blood glucose and insulin levels, approximately 250 μl of blood was taken from the tail vein at each time indicated above. Blood glucose concentrations were determined by a blood glucose meter (SureStep, LifeScan). The blood was centrifuged at $3000\times g$ for 10 min, and the serum was stored at −20°C, and used for determining the insulin level [49, 50].

The insulin level was determined by human insulin-specific RIA kit. Glucose responses during the glucose tolerance test were evaluated by estimation of the total area under the curve (AUC), using the trapezoidal method [51]. The homeostasis model assessment for insulin resistance (HOMA IR) was also used to calculate insulin resistance from fasting plasma insulin and glucose concentrations, with HOMA IR = [fasting insulin (μIU/m) × fasting plasma glucose (mmol/l)/22.5] [52]. The whole-body ISI was calculated from fasting plasma insulin and glucose concentrations and mean plasma insulin and glucose concentrations during the OGTT, with ISI = $10{,}000/\sqrt{}$(fasting plasma glucose × fasting plasma insulin) × (mean OGTT glucose × mean OGTT insulin) [53].

Determinations of GnRH peptide in pancreas

Pancreatic tissues were homogenized in boiled PBS and centrifuged as described by Koch [54]. Total proteins in the resulting supernatant were determined by BCA Protein Assay Kit (Pierce BCA Protein Assay Kit, Pierce Biotechnology, USA), and the GnRH concentration in the resulting supernatant was determined by ELISA using KIT (Adlitteram Diagnostic Laboratories, Inc.).

Isolation of mRNA and degradation of genomic DNA

The rats were anesthetized with sodium pentobarbital on 15 days after gestation. Fresh pancreatic tissues were obtained from sacrificed rats and immediately frozen in liquid nitrogen and stored at −80°C. Tissue samples were homogenized in Trizol-Reagen (Invitrogen Corporation, Carlsbad, CA, USA), shaken rapidly, and kept at 4°C for 5 min.

In total, 200 ml chloroform and 500 μl isopropyl alcohol were added to the homogenate, which were then centrifuged at 7500×*g* at 4°C for 5 min. The pellets were washed with 75% ethanol, air-dried and dissolved in RNase-free water. The sample was diluted by diethylpyrocarbonate (DEPC)-treated water. The RNA samples were quantified using a DU 800 UV/Visible Spectrophotometer (Beckmen Coulter, CA, USA), and its purity was assessed on the same machine using 260:280 nM ratios. Integrity of RNA was determined by fractionation on agarose gel and staining with ethidium bromide. DNA contamination was removed by treatment with DNase I (TaKaRa Bio Inc., Japan). In total, 20–50 μg total RNA in a reaction volume of 50 μl, containing 10XDNase I Buffer (supplied) 5 μl, DNase I (RNase-free) 2μl (10 units), ribonuclease inhibitor 20 units, DEPC-treated water up to 50 μl, was incubated for 20–30 min at 37–40°C. The procedures for degradation of genomic DNA were carried out following the instructions of the manufacturer. The degradation of the genomic DNA was confirmed by electrophoresis and the RNA concentration was measured again. Its concentration was adjusted to 0.2 μg/μl using RNase-free water.

Relative real-time polymerase chain reaction (PCR)

RNA samples were reverse transcribed into cDNA using the RT–PCR kit (SYBR PrimeScript, TaKaRa Bio Inc., Japan) according to the manufacturer's recommendations. In brief, the 1 μg total RNA was dissolved in RNase-free water and 5× PrimeScript Buffer 2 μl, PrimeScript RT Enzyme MixI0.5 μl, Oligo dt Primer 0.5 μl, Random 6 mers 0.5 μl, giving a final volume of 10 μl. The sample was incubated at 37°C (15 min) and 85°C (5 s) using the BIO-RAD IQ5 Multicolor Real-Time PCR Detection System (Bio-Rad Laboratories, Inc., USA). The primers were designed (Primer Express Software, TaKaRa Bio Inc., Japan) for rat GnRH, GnRHR, GLP-1R and *β*-actin and produced on an Expedite Nucleic Acid Synthesis System (TaKaRa Bio Inc., Japan) according to the manufacturer's protocol. The primer sequences are listed in Table 2. The *β*-actin gene served as the endogenous reference gene. SYBR Green real-time PCR was carried out in the BIO-RAD IQ5 Multicolor Real-Time PCR Detection System (Bio-Rad Laboratories, Inc., USA). For each gene, real-time PCR was conducted in duplicate with a 25 μl reaction volume, containing 2 μl of cDNA, 12.5 μl SYBR Premix EX Taq, 0.5 μl PCR Forward Primer, 0.5 μl PCR Reverse Primer, 9.5 μl dH20. The PCR was carried out as follows: 10 s at 95°C, 40 cycles of 95°C for 5 s, and 30 s at 62°C.

Statistical analysis and results presentation

Each value is presented as mean ± SE. Significances of differences among groups were evaluated using a one-way ANOVA. Correlation was assessed by Pearson's method. Regressions among variables were assessed by stepwise method. All statistical analyses were performed using SPSS 11.0 software. A probability value of <0.05 was considered to be statistically significant.

Table 2 Primer sequences and RT–PCR conditions

Gene (accession no.)	Primer sequence 5′–3′	Product size (bp)	Annealing *T* (°C)
GnRH (nm-012767)	5′-TGGTATCCCTTTGGCTTTCA-3′	190	85
	5′-TCCTCCTCCTTGCCCATCTC-3′		
GnRHR (nm-031038)	5′-TTGTTGATGGCTGAGCAGTGA-3′	76	81.5
	5′-AAGCCCGTCCTTGGAGAAAT-3′		
PG (nm-012707)	5′-GGGACCTTTACCAGTGATGTGAGT-3′	70	81
	5′-CCAGCCAAGCAATGAATTCC-3′		
GLP-1R (nm-012728)	5′-CATCCACCTGAACCTGTTTGC-3′	68	81
	5′-GGGCAGCGTCTTTGATGAAG-3′		
β-Actin (nm-031144)	D3701, TaKaRa, Inc.	132	83.5

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