The effects of intrauterine undernutrition on pancreas ghrelin and insulin expression in neonate rats

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Abstract

Ghrelin has a correlation with insulin secretion, β -cell development, and diabetes in crucial development period. The aim of this study was to compare the changes in plasma ghrelin, insulin, and glucose concentrations, and variation of ghrelin expression in the pancreas in response to intrauterine malnutrition in newborn rats. Pregnant rats at day 2 were randomly divided into two groups: nourished (fed *ad libitum*; NR) and undernourished rats (UR). The offspring of NR were defined as normal-birth-weight group (NBW, $n=79$) and those of UR were defined as low-birth-weight group (LBW, $n=74$). Plasma glucose, ghrelin, and serum insulin of both dams and their pups were analyzed at the first day after birth. The entire pancreas was collected for determination of ghrelin and insulin mRNAs, and quantification of pancreas ghrelin and insulin. Immunohistochemical double staining and confocal microscopy were performed on rat pancreas. Birth weight was 5.81 ± 0.64 and 4.76 ± 0.23 g in NBW group and LBW group respectively. Fasting plasma ghrelin concentrations in UR group (1382 (1287–1513) pg/ml) were higher than that of NR

group (1072 (974–1205) pg/ml). Plasma ghrelin concentrations in the LBW group (2176 (2031–2384) pg/ml) were significantly lower than that of the NBW group (2493 (2311–2675) pg/ml). Undernutrition caused a decrease in plasma insulin concentrations in both UR dams and LBW pups ($P<0.001$). Ghrelin mRNA and total ghrelin of pancreas were significantly affected by intrauterine nutrition state. Pancreas insulin concentrations were significantly affected by intrauterine nutrition ($P=0.007$). The majority of ghrelin-producing cells were present at the periphery of islets in the NBW group. Ghrelin was colocalized with insulin in β -cells in LBW group. The percentage of ghrelin-positive cells in the islets of LBW group was significantly higher than that of the NBW group ($P<0.01$). Intrauterine undernutrition may affect the birth weight, plasma insulin and ghrelin levels, islet ghrelin expression, and ghrelin cell distribution. It will be interesting to investigate intrauterine nutrition which is involved in islet ghrelin expression and ghrelin cell distribution.

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Introduction

Ghrelin is a 28-amino acid peptide secreted mainly by the gastrointestinal tract (stomach, duodenum, and jejunum). It is also present in the placenta, the hypothalamus, and the pancreas. Ghrelin may play a role in meal initiation in humans and rodents (Kojima *et al.* 1999, Shuto *et al.* 2001). Ghrelin secretion was nutritionally manipulated. The relationship between ghrelin, insulin, and glucose remains unclear. There is a reciprocal change in plasma ghrelin and insulin concentrations after a meal. Most human and animal studies have shown that hyperglycemia and insulin (without hypoglycemia) decrease plasma ghrelin. In human neonates, a large amount of ghrelin is present in umbilical cord plasma samples (Farquhar *et al.* 2003).

In the adult rat, ghrelin appears to affect insulin secretion by the pancreas. Both stimulatory and inhibitory effects of ghrelin on insulin secretion have been reported. The role of ghrelin in the fetus remains unclear. In *Nkx2.2* or *Pax4* gene knockout mouse models, *Nkx2.2* and *Pax4* are required to

specify or maintain differentiation of the cell fate; ghrelin cells replace insulin-producing β -cells in pancreas development. The expansion of ghrelin-producing cells is at the expense of cells (Prado *et al.* 2004). Interestingly, in humans, ghrelin is abundant in the fetal pancreas (but not the adult pancreas), where ghrelin-secreting cells represent up to 10% of the endocrine cells. Ghrelin is expressed in a novel endocrine cell type in developing rat islets and inhibits insulin secretion from INS-1 (832/13) cells (Wierup *et al.* 2004). Ghrelin-immunoreactivity cells were observed in 2.6 and 3.8% of the total cell population in the islet of Langerhans of normal and diabetic rats respectively (Adeghate & Ponery 2002a,b, Wierup *et al.* 2002). Ghrelin inhibits insulin and stimulates glucagon secretion from the pancreatic islets of the mouse and rat and is related to diabetes (Adeghate & Ponery 2002a,b, Katsuki *et al.* 2004).

The prevalence of type 2 diabetes is much higher in adults with a low weight at birth; intrauterine malnutrition is associated with impaired insulin secretion and increased susceptibility to diabetes (Barker 1993, Hofman *et al.* 2004).

The mechanism between intrauterine malnutrition and increased susceptibility to diabetes is unclear. The reduced insulin secretion in islets of low-protein pups may result from intrinsic alterations in the islets, such as a smaller size and/or a reduced volume of β -cells (Dahri *et al.* 1991). Maternal food restriction can result in the early developmental β -cell alteration, and this alteration further generates inadequacies in adult endocrine pancreas to adapt to situations of increased insulin demand (Garofano *et al.* 1998).

Because ghrelin has a correlation with insulin secretion, β -cell development, and diabetes in crucial development period, we speculated that pancreas ghrelin expression may be a key bridge to the association between low birth weight and increased susceptibility to diabetes. We hypothesized that intrauterine malnutrition may alter fetal circulating and tissue ghrelin, and that ghrelin distribution in pancreas may differ in normal-birth-weight (NBW) and low-birth-weight (LBW) rats. Meanwhile, we compared the changes in plasma ghrelin, insulin, and glucose concentrations, and in ghrelin gene expression in pancreas in response to intrauterine malnutrition in newborn rats.

Material and Methods

Animal model

All procedures used in this study were approved and performed in accordance with the guidelines established by the Animal Ethics Committee of the Zhejiang University School of Medicine. Twenty Sprague–Dawley (SD) pregnant rats (12 weeks old, 250–300 g, day 2 of pregnancy at the time of the experiment) obtained from Zhejiang Medical Science Academy (Hangzhou, China) were used to generate the offspring used in this study. The rats were housed in a $22 \pm 1^\circ\text{C}$ environment at 60% humidity and were maintained on a 12 h light:12 h darkness cycle.

Feeding protocol

The rats were housed individually in standard rat cages at day 2 of the pregnancy. Pregnant rats were randomly divided into two groups: nourished (fed *ad libitum*; NR) and under-nourished rats (UR). Rats in the NR group were fed a standard commercial rat diet, which contained (per 100 g) protein (22.5 g), carbohydrates (57.0 g), fat (3.9 g), cellulose (8.0 g), minerals (1 g), vitamins (mixed, 5.0 g), and water (2.5 g). Rats in the UR group were fed the same diet at 50% of the NR intake, as determined by the amount of food consumed by the control group, from day 2 of pregnancy until day 23 (parturition). Water was provided *ad libitum*. All rats were fed daily, in the morning, and the consumption was determined 24 h later. The body weight of dams was determined during pregnancy (on days 5, 10, 15, and 20), and the body weight of pups was determined at birth. To prevent variation in neonatal growth related to the

unavailability of milk, all the pups were killed within 2 h after birth without suckling. Therefore, UR pups differed from NR pups only in their prenatal dietary experience. After parturition, all the dams were fasted for 10 h and killed by anesthesia. Pentobarbital sodium (60 mg/kg, i.p.) was used before killing. The time between anesthesia induction and killing was no more than 10 min.

Effect of maternal undernutrition on plasma ghrelin, insulin, and glucose concentrations, and on pancreatic ghrelin and insulin gene expression

Twenty delivered female rats and their offspring were used. The offspring of NR were defined as NBW group ($n=79$); the pups of UR were defined as LBW group ($n=74$). Plasma glucose, ghrelin, and insulin of both dams and their pups were analyzed at the first day after birth. One plasma EDTA aliquot was collected from each dam. Fetal EDTA plasma was pooled from two to three pups ($n=30$ samples per group of pups). Plasma concentrations of total ghrelin and insulin were determined in all samples (plasma EDTA without additives). One microliter of dam or pup blood was used at the time of the experiment to determine the plasma glucose concentrations. All samples were kept on ice until centrifugation and stored thereafter at -80°C until the assay was performed. The entire pancreas was collected. All tissue samples were immediately frozen at -80°C for later determination of total ghrelin concentrations and ghrelin and insulin mRNAs, as appropriate.

Hormone determinations

Ghrelin circulates in two forms: a biologically active (octanoylated) and inactive (des-octanoylated) form. In these experiments, plasma total (active and inactive) ghrelin (RK-031-30, Phoenix, Belmont, CA, USA) and insulin (Boster, Wuhan, China) immunoreactivities were determined by RIA. All determinations in one experiment were performed in duplicate in the same assay. Glucose was determined by the glucose oxidase method using Hitachi System 717 (Roche Diagnostics, Basel, Switzerland).

Ghrelin and insulin RT-PCR

Ghrelin and insulin mRNAs were estimated by RT-PCR with slight modifications and the results normalized for β -actin. Total RNA was extracted using TRIzol reagent (Life Technologies) according to manufacturer's protocol. Two micrograms of total RNA were used for RT. cDNAs were synthesized using 200 U Moloney murine leukemia reverse transcriptase (Life Technologies), 6 μl dNTPs mix (10 mM of each dNTP), 6 μl first-strand buffer (250 mM Tris-HCl (pH 8.3), 375 mM KCl, 15 mM MgCl_2 (Life Technologies)), 2.75 μl random hexamer solution (200 ng/ μl ; Invitrogen Life Technologies), and 0.25 μl RNase Out (recombinant ribonuclease inhibitor, 40 U/ μl ; Invitrogen) in a total volume of 30 μl . Reaction mixtures were incubated at 37°C for 60 min and 42°C for 10 min.

The reaction was stopped by heating at 95 °C for 5 min and subsequently quickly chilled on ice. Three microliters of RT reaction were used for PCR amplification. The amplification conditions were as follows: 5 µl PCR buffer (200 mM Tris-HCl (pH 8.4) and 500 mM KCl (Life Technologies)), 1.5 µl of 50 mM MgCl₂, 4 µl dNTPs mix (10 mM of each dNTP), 2 µl of 10 µM solutions of upstream and downstream primers, and 2.0 U *Taq* DNA polymerase (Life Technologies) in a 50 µl reaction volume. We used the following primers: rat ghrelin, upstream primer 5'-TTGAGCCCAGAGCACCAGAAA-3' and downstream primer 5'-AGTTGCAGAGGAGGCA-GAAGCT-3' (length of the amplicon: 347 bp, accession no. AB029433, GenBank; Date *et al.* 2000); insulin, upstream primer 5'-GGGAACGTGGTTTCTTCTACACA-3' and downstream primer 5'-TCCAGTGCCAAGGTCTGAA-GAT-3' (length of the amplicon: 118 bp, accession no. gi 56487, GenBank). The amplification profile was denaturation at 94 °C for 30 s, annealing at 58 °C (ghrelin) or 62 °C (insulin) for 30 s, and extension at 72 °C for 1 min. Amplification was completed with an additional step at 72 °C for 10 min. The amplification was performed in an automatic thermal cycler. To normalize the results for differences in RNA sampling, the second half of the RT reaction was used to amplify a of the rat β-actin (upstream primer 5'-TCACCCACACTGTGCC-CATCTATGA-3' and downstream primer 5'-CATCG-GAACCGCTCATTGCCGATAG-3' (length of the amplicon: 280 bp, accession no. AC121985, GenBank).

To ensure that PCR was performed in the linear amplification range, samples of each tissue were taken every two to three cycles over a wide range, showing that the reaction was linear between 26 and 32 cycles for ghrelin and insulin. PCRs were run at 29 cycles. The number of cycles for β-actin was 28 when run with ghrelin or insulin. All reactions presented in a given figure were performed in the same assay. The amplified products of ghrelin were a 347 bp product that spans through the first and fourth exons, and the amplified products of insulin were a 118 bp product, which is confirmed by electrophoresis with DNA ladder marker. PCR products were separated on 1.8% agarose gel, stained with ethidium bromide, examined with u.v. light, and visualized with a Gel Doc 1000 documentation system (Bio-Rad Laboratories Inc). In addition, multiple negative controls were made by omitting the reverse transcriptase from the reactions, suggesting that the PCR product obtained was not amplified genomic DNA. Finally, both PCR products of ghrelin and insulin were sequenced and compared with the expected sequences, which confirmed the specificity of the PCR product.

Quantification of pancreas ghrelin and insulin

Pancreas ghrelin. Pancreas ghrelin was extracted as described by Toshinai *et al.* with slight modifications. Aliquots of pancreas were minced and boiled at 98 °C for 10 min in 1 ml water to inactivate the intrinsic proteases. After the samples were cooled to 4 °C for 2–3 min, acetic acid (CH₃COOH) and hydrochloric acid (HCl) were added to the respective final concentrations of 1 M and 20 mM. The tissues were then

homogenized in a polytron for 2 min, after which the homogenate was centrifuged at 13 000 *g* for 15 min at 4 °C. The supernatant was collected and lyophilized. The appropriate RIA measured total ghrelin after reconstitution in 10 mM phosphate buffer with 10 mM EDTA, 0.1% gelatin, and 0.08% sodium azide (pH 6.85).

Insulin secretion. Islets were isolated by collagenase digestion. Briefly, the pancreas was inflated with Hanks solution containing 0.7–0.9 g collagenase/l, excised, and then maintained at 37 °C for 20 min. The digested tissue was harvested and the islets were handpicked. Groups of five islets were first incubated for 45 min at 37 °C in 0.5 ml Krebs-bicarbonate buffer of the following composition (mmol/l): 115 NaCl, 5 KCl, 2.56 CaCl₂, 1 MgCl₂, 24 NaHCO₃, and 5.6 glucose, supplemented with 3 g/l BSA and equilibrated with a mixture of 95% O₂/5% CO₂ (pH 7.4). This medium was then replaced with fresh buffer and the islets incubated for 90 min in the presence of 16.7 mmol/l glucose. Insulin was measured by RIA in the medium at the end of the incubation period.

Immunofluorescence double staining, DAPI staining, and confocal microscopy

Ghrelin and insulin or glucagon double fluorescence immunostaining was performed on formalin-fixed and paraffin-embedded rat pancreas, using poly-L-lysine-coated slides. Each slide was about 4 mm, dextranated by dimethylbenzene, and dehydrated by gradient ethanol. A standard manual immunofluorescence procedure was used with antigen retrieval procedure. Antigen retrieval was done by modified high-temperature technique pioneered by Shi *et al.* (1997). The procedure was as follows: each section was deparaffinized twice, 10 min each in 100% xylene, and then hydrated with 100% ethanol for 5 min, twice, 95% ethanol for 3 min, and 80% ethanol for 5 min. After 2–5 min of soaking in distilled water, the slides were put into the pressure cooker filled with 1000 ml boiling sodium citrate buffer (pH 6.0) and heated under pressure. Two minutes after steaming, the pressure cooker was removed from the heating source and cooled down to room temperature with tap water. The slides were rinsed 2×3 min with PBS. Afterward, the primary antibody was applied.

First, in order to detect ghrelin-like immunoreactivity, the sections were incubated with a rabbit anti-rat ghrelin (amino acids 13–28; FG-G-031-30 Phoenix), diluted 1:500, and the sections were incubated with the ghrelin antibody for 1 h at 37 °C, then washed in PBS buffer for 3×5 min. Subsequently, the sections were then incubated with rhodamine B-labeled serum goat anti-rabbit antibody (1:400, ZF-0316 Zymed) for 40 min at 37 °C, and then washed in PBS buffer for 3×5 min. A subsequent washing step was performed in hot PBS (at 95 °C) to reduce the background and increase sensitivity.

Next, in order to demonstrate simultaneously the presence of insulin-like immunoreactivity or glucagon-like immunoreactivity on the same section as that in which ghrelin-like immunoreactivity was detected, a double

immunofluorescence technique was performed in sequence. Sections were incubated with a monoclonal mouse anti-rat insulin antibody (1:1500, Clone K36aC10, BM0080 Boster) or a monoclonal mouse anti-rat glucagon antibody (1:200, Clone K796B10, No.G2654; Sigma) for 1 h at 37 °C, followed by incubation with serum goat anti-mouse antibody labeled with fluorescein-5-isothiocyanate (FITC; 1:400, ZF-0312 Zymed) for 40 min at 37 °C. Control experiments were included in serial sections by omitting either primary antibody. In experimental conditions, no cross-reactivities were observed in the control tissues. The slides were then counterstained with the nuclear stain 4'-6-diamidino-2-phenylindole (DAPI; Sigma) to detect the number of islet cells and mounted with antifade reagent, and thus DAPI staining was preformed. Briefly, after quick wash with PBS, the slides were incubated for 5 min with DAPI at a concentration of 0.025 mg/ml. Excess staining was removed by rinsing in PBS and in distilled water for 3 × 5 min. The sections were enveloped with glycerin.

Double immunolabeling was detected by a Zeiss LSM510 confocal laser scanning microscope (Carl Zeiss Jena, Jena, Germany). This procedure relied on rhodamine-labeled secondary anti-rabbit antibody to reveal ghrelin immune complexes and FITC-labeled anti-mouse secondary antibody to reveal insulin- or glucagon-producing cells. The final tricolor images were compiled from overlaid images of the blue (nuclei), green (insulin or glucagon), and red (ghrelin) images. Point counting for double-positive cells was used for a quantitative analysis of the number of islet cells containing insulin or ghrelin at various sections. Five pancreata were subjected to quantitative analysis in each group. The number of islets of each pancreas section varied from 9 to 19 including large and small islets. By dividing the number of ghrelin-positive (ghrelin⁺) cells or insulin-positive (insulin⁺) cells by the number of DAPI-positive (DAPI⁺) cells in each islet, the percentage of ghrelin⁺ or insulin⁺ cells was determined, and the results were presented as mean percentage per islet per section (section number, $n=5$). In some sections, the percentage of ghrelin⁺ insulin⁺ cells or ghrelin⁺ glucagon⁺ cells was also determined as above.

Statistical analysis

Data are presented as mean (95% confidence interval; CI) or median (range) as appropriate. Statistical analysis was performed by independent-samples *t*-test as appropriate. A *P* value of <0.05 was regarded as significant. Means and 95% CIs were used to perform comparisons and define significance between individual subgroups.

Results

Effect of maternal undernutrition on plasma ghrelin and insulin concentrations

As mentioned earlier, NR and UN refer to the dams, and LBW and NBW refer to the pups during grouping. Body

weight of both dams and fetuses was significantly affected by feeding status ($P<0.001$). After parturition, mean (95% CI) body weight was 287 (265–326) g in NR rats and 234 (198–267) g in UR animals. In the NBW group (NR pups), body weight at birth was 5.81 ± 0.64 g, whereas in the LBW group (UR pups), it was 4.76 ± 0.23 g. Median (range) number of fetuses per dam was 7 (5–13) and was not affected by maternal feeding state.

Plasma total ghrelin concentrations were significantly affected by feeding state ($P<0.001$; Fig. 1A). After parturition, fasting plasma ghrelin concentrations in UR group (1382 (1287–1513) pg/ml) were higher than that of NR group (1072 (974–1205) pg/ml). Ghrelin concentrations were two to three times higher in pups when compared with their mothers. At the first day after birth, pups' plasma ghrelin concentrations in the LBW group (2176 (2031–2384) pg/ml) were significantly lower than that of NBW group (2493 (2311–2675) pg/ml), which was in contrast to what was observed in the dams. Plasma insulin concentrations were significantly affected by nutrition state ($P<0.001$). Under-nutrition caused a decrease in plasma insulin concentrations in both UR dams and LBW pups (Fig. 1B). Plasma glucose concentrations were also significantly affected by maternal nutrition state ($P<0.001$). Fasting caused a significant decrease in plasma glucose concentrations in the dams as well as in their fetuses (Fig. 1C).

Effect of intrauterine undernutrition on pups' pancreas ghrelin and insulin gene expression

To determine whether the differences in plasma ghrelin and insulin concentrations observed in the LBW group when compared with the NBW group could be explained by the differences in tissue gene expression, we determined insulin and ghrelin mRNAs and protein in the pancreas of rat pups. Ghrelin mRNA and concentrations of total ghrelin in the pancreas were significantly affected by intrauterine nutrition state. Pancreas ghrelin/ β -actin ratio (expressed as percentage) was 372% (307–513%) in the LBW group, which was significantly higher when compared with that in the NBW group (124% (57–198%); Fig. 2A). Accordingly, pancreas total ghrelin content was 714 (632–759) ng/g tissue in the LBW group, while it was 479 (381–732) ng/g tissue in the NBW group, hence there was a difference in the pancreas total ghrelin content between the two groups ($P<0.01$; Fig. 2C). Thus, ghrelin content of pancreas in the pups is influenced by the intrauterine nutrition. To test whether intrauterine nutrition plays a role in pancreas insulin expression, we investigated pancreas insulin mRNA expression and insulin content expressed in the pups. Pancreas insulin/ β -actin ratio (expressed as percentage) was 112% (67–194%) in the LBW group, while it was 183% (157–302%) in the NBW group (Fig. 2B). Insulin expression in pup pancreas was affected by maternal nutrition. The results show that insulin content was 782.1 (522–986) pmol/islet-90 min and 2448.9 (1987–2954) pmol/islet-90 min in LBW and NBW

Figure 1 Plasma ghrelin (A), insulin (B), and glucose (C) concentrations in dam rats (UR and NR; $n=10$ per group) and their fetuses (LBW and NBW, $n=30$ per group; mean). Plasma ghrelin, insulin, and glucose concentrations were significantly affected by feeding state ($P<0.001$). (#Represents $P<0.01$ versus NR group. *Represents $P<0.01$ versus NBW group.

groups respectively (Fig. 2D). Pancreas insulin concentrations of newborn rats were significantly affected by intrauterine nutrition ($P=0.007$).

Ghrelin expression in pancreatic islets

We further investigate the cell type(s) expressing ghrelin in the pancreatic islet by means of double immunofluorescence stains and counterstained with DAPI (Fig. 3).

Pancreatic islet of NBW group showed a strong immunoreactivity for insulin in many β -cells at the center of islets

(Fig. 3A, green), and most of the ghrelin-immunoreactive cells distributed at the periphery of the islets (Fig. 3B, red), which was confirmed by overlapping the two fluorescence reactions (Fig. 3C). A double immunofluorescence for insulin and ghrelin in pancreatic islet of LBW group showed that most of insulin (Fig. 3D, green) and ghrelin (Fig. 3E, red) were colocalized in the center of islet, and majority of insulin-producing cells (or β -cells) coexpressed ghrelin, as also confirmed by the yellow color obtained by overlapping the two fluorescence reactions (Fig. 3F, yellow). Ghrelin had a preferential central distribution within the endocrine islet of the LBW group. The percentage of ghrelin⁺ insulin⁺ cells in the LBW group was 33.7% (95% CI: 16.2–41.1%). Glucagon⁺ cells (Fig. 3G, green), ghrelin⁺ cells (Fig. 3H, red), or cells coexpressing both hormones (glucagon⁺ ghrelin⁺ cells; Fig. 3I, yellow) in the pancreatic islet of the NBW group were detected. Glucagon⁺ cells distributed at the periphery of the islets of the NBW group, and many glucagon⁺ cells coexpressed ghrelin. The percentage of ghrelin⁺ glucagon⁺ cells was 5.3% (95% CI: 3.9–9.7%) in the NBW group. Two populations of cells in the islet of the LBW group were detectable: cells uniquely expressing glucagon (Fig. 3J, green) at the periphery of the islets or cells uniquely expressing ghrelin at the center of the islets (Fig. 3K, red). No glucagon⁺ ghrelin⁺ cells were found (Fig. 3L). Relative proportions of insulin- and ghrelin-producing cells were compared between the LBW and NBW groups. Further quantitative analysis showed that the percentage of ghrelin⁺ cells and insulin⁺ cells was 83.1% (95% CI: 76.2–90.1%) in the islets of the LBW group and 90.1% (95% CI: 85.7–95.1%) in the NBW group respectively (Fig. 3M). The percentage of ghrelin⁺ cells in the islets of the LBW group (48.7% (95% CI: 33.2–56.7%)) was significantly higher than that of the NBW group (15.3% (95% CI: 7.2–20.1%); Fig. 3N, $P<0.01$).

Discussion

Barker (1993) suggested that low birth weight was associated with increased risk of metabolic syndrome in adulthood. The restriction of dietary protein or a global restriction in nutrients during pregnancy gives rise to the offspring with glucose intolerance and insulin resistance (Langley *et al.* 1994, Vuguin *et al.* 2004), which supports the above concept. Insulin resistance is recognized as a common underlying feature of metabolic syndrome, but the mechanisms at the cellular and biochemical levels are not completely understood. Some studies have demonstrated a link between poor nutrition in early life and insulin resistance and diabetes in later life. However, the mechanism is unclear. On the basis of the relationship between ghrelin and insulin, we hypothesized that the interactions between ghrelin and insulin in early life might be a possible link between poor nutrition and insulin resistance.

When maternal diet is restricted, the availability of nutrients for transplacental transport is decreased, reducing

Figure 2 RT-PCR analysis of ghrelin and insulin expression in the pancreas of rat pups. Ghrelin/ β -actin mRNA ratios are expressed as percentage in (A). Insulin/ β -actin mRNA ratios are shown in (B). Ghrelin mRNA and total ghrelin concentrations were significantly affected by intrauterine nutrition state ($P < 0.001$). Insulin mRNA expression was significantly affected by intrauterine nutrition state ($P = 0.009$) too. Tissue total ghrelin content was determined in pancreas by RIA after extraction and was expressed in nanograms per gram of tissue (figure C, $P < 0.01$). Glucose stimulation (16.7 mmol glucose/l) of insulin secretion in islets of pup rats were shown in (figure D); the columns represent the cumulative 90-min insulin secretion and are the means; $n = 10$ independent experiments ($P < 0.01$). *Represents $P < 0.01$ versus NBW group.

the nutrient supply and thereby limiting the growth of the fetus (Holemans *et al.* 2003). In the present study, we demonstrated that restriction of maternal nutrition in pregnant SD rats resulted in severe maternal weight loss and in a significant reduction in the birth weight of the offspring. Plasma insulin concentrations in pups were in accordance with their dams; undernutrition caused a decrease in plasma insulin concentrations in both UR dams and LBW pups. Alteration in the maternal metabolic milieu during pregnancy influences the development and functional maturation of β -cells in fetal pancreas (Sparre *et al.* 2003). The reduced insulin secretion in islets of LBW pups may result from intrinsic alterations in islets such as a smaller size, a reduced number of β -cells, a diminution in the proliferation of islet cells, and a marked decrease in islet vascularization (Snoeck *et al.* 1990, Boujendar *et al.* 2003).

Ghrelin and insulin content of pancreas in the pups is influenced by the intrauterine nutrition, which was supported by insulin and ghrelin mRNAs and protein in the pancreas of rat pups. Fasting plasma ghrelin concentrations in UR group were higher than that of NR group, which further supported that ghrelin expression is upregulated under conditions of negative energy balance (Toshinai *et al.* 2001). Plasma total ghrelin concentrations were markedly higher in pups when compared with their dams, suggesting that the pup plasma ghrelin may originate from different source(s) when compared with their mothers.

Circulating ghrelin of fetuses could originate from the placenta (Gualillo *et al.* 2001), the stomach (Date *et al.* 2000, Kojima *et al.* 1999), or other tissues, such as the pancreas or the lung (Volante *et al.* 2002a,b) that are known to synthesize ghrelin during early fetal life. Ghrelin is also produced in the hypothalamus (Mozid *et al.* 2003). In rodents, ghrelin mRNA is present in the stomach by embryonic day 17 of gestation and increases markedly during the first postnatal weeks (Chanoine *et al.* 2006). The increase in plasma ghrelin concentrations in response to fasting is observed in rats by the end of the first postnatal week (Hayashida *et al.* 2002, Chanoine & Wong 2004). The pups in this study were killed within 2 h after birth without suckling, so the ghrelin concentration in our newborn rats may reflect the ghrelin levels as fetuses. In this study, plasma ghrelin concentrations in the LBW pups were significantly lower than that of the NBW group. However, ghrelin contents in the pancreas of the LBW group were significantly higher than that of NBW group. It seems that there was a discrepancy among these results, that resulted in the lower plasma ghrelin concentrations in the LBW pups. First, placenta ghrelin does not contribute to circulating levels (Fuglsang *et al.* 2006). Though cord ghrelin concentration was 40% higher in small for gestational age neonates (SGAs) when compared with appropriate (AGAs) and large (LGAs) for gestational age neonates (Farquhar *et al.* 2003), circulating fetal ghrelin appears to be derived from a fetal source (Kitamura *et al.* 2003, Fuglsang *et al.* 2006). Secondly, ghrelin contents (ng/g) in the pancreas of our LBW group were higher. Maternal food restriction induced a

Figure 3 A double immunofluorescence for ghrelin (red) and insulin (green) or glucagon (green) and counterstained with DAPI in pancreatic islet, which was detected by laser confocal fluorescence microscopy. NBW group (ghrelin and insulin double staining: A, B, and C; ghrelin and glucagon: G, H, and I); LBW group (ghrelin and insulin double staining: D, E, and F; ghrelin and glucagon: J, K, and L). Pancreatic islet of the NBW group showed a strong immunoreactivity for insulin in many β -cells (A, green), most of the ghrelin-immunoreactive cells distributed at the periphery of the pancreatic islets (B, red), which was confirmed by overlapping the two fluorescence reactions (C). Glucagon-positive cells (G, green), ghrelin-positive cells (H, red), and cells coexpressing both hormones (I, yellow) in the pancreatic islet of the NBW group were detected. Glucagon cells distributed at the periphery of the islets of the NBW group and many glucagon-positive cells coexpressed ghrelin. Some of the central cells of islets from the NBW group also showed a weak immunoreactivity for ghrelin. A double immunofluorescence for insulin and ghrelin in pancreatic islets of the LBW group showed that most of insulin (D, green) and ghrelin (E, red) were colocalized in the center of islet, and some β -cells coexpressed ghrelin and insulin, as also confirmed by the yellow color obtained by overlapping the two fluorescence reactions (F, yellow). Two populations of cells in the islets of the LBW group were detectable: cells uniquely expressing glucagon (J, green) at the periphery of the islets or cells uniquely expressing ghrelin at the center of the islets (K, red), and no cells producing both hormones (L). Relative proportions of insulin- and ghrelin-producing cells were compared between the LBW and NBW groups (M and N). Numbers were provided as the percentage of insulin- and ghrelin-producing cells in relation to the total population of DAPI-immunoreactive cells per islet. The percentage of ghrelin-positive cells in the islets of the LBW group was significantly higher than that of the NBW group ($P < 0.01$) (N). Arrows represent ghrelin⁺ insulin⁺ or ghrelin⁺ glucagon⁺ double-stained cells. *Represents $P < 0.01$ versus the NBW group.

reduction in pancreas weight in the offspring at day 1 after birth (Garofano *et al.* 1997), which may reduce pancreas whole ghrelin (ghrelin contents (ng/g) $\times$ pancreas weight) of LBW pups. Finally, maternal undernutrition not only affected the overall growth of the offspring, but also caused a change in body composition and function, such that the organs were selectively affected. Weight of organs such as liver, muscle, spleen, etc., tended to be proportionally lower than the body weight (Desai *et al.* 1996). The origin of the high concentration of ghrelin in neonates may need further investigation (Fuglsang *et al.* 2006); these affected organs should be taken into account. Poor maternal nutrition may result in the decrease of ghrelin cells in these tissues. The decreased ghrelin cells could lead to the decline of ghrelin secretion, which may contribute to the lower plasma ghrelin levels in the LBW group. Further study should be carried out on ghrelin assay to investigate ghrelin expression in these organs and clarify the mechanism.

Ghrelin has powerful effects on islet cells, suggesting that endogenous ghrelin may contribute to the physiological control of insulin and glucagon release (Prado *et al.* 2004). In the pancreatic endocrine islets, numerous cells expressed ghrelin in the cytoplasm, as indicated by a moderate to intense staining; however, which kind of pancreatic islet cells express ghrelin is still in dispute. In adult rat islets, most of the ghrelin-immunoreactive cells were found at the periphery of the pancreatic islets as α -cells, ghrelin-immunoreactive cells colocalized with glucagon in immunofluorescence double staining (Heller *et al.* 2005). Recently, the expression of the peptide hormone ghrelin was also detected in ϵ -cells of the islets, a newly discovered endocrine cell type (Wierup *et al.* 2002, 2004). In the present study, we provide further evidence that ghrelin coexpressed in the α -cells of NBW rat islets, which was in accordance with the previous result (Heller *et al.* 2005); but in the LBW group, most of the ghrelin-immunoreactive cells distributed in the center of islet cells, and some β -cells coexpressed ghrelin and insulin, and the percentage of ghrelin-positive cells in the LBW group was significantly higher than that of the NBW group. It indicated that intrauterine undernutrition might affect ghrelin expression abundance and the distribution of ghrelin-producing cells in newborn rat pancreatic islets. Prado *et al.* (2004) found that insulin and ghrelin cells share a common progenitor, and that Nkx2.2 and Pax4 are required to specify or maintain differentiation of the β -cell fate. We speculated that intrauterine nutrition may be a regulating component in β -cell differentiation in prenatal life, which is the key period of β -cell development; intrauterine undernutrition of LBW newborn rats may affect the differentiation of progenitor cell to ghrelin cell. Ghrelin cells are most numerous pre- and neonatally and decrease gradually after birth (Wierup & Sundler 2005). Ghrelin may play a role in meal initiation in human and rodents. Poor maternal nutrition may also permanently alter glucose/insulin metabolism directly through the availability of nutrients for transfer to the fetus (Oliver *et al.* 2005). In the condition of intrauterine

undernutrition, fetus needs higher ghrelin level to take more nutrients. In order to adapt to the undernutrition state, ghrelin cells secreted more ghrelin, which may lead to the delayed disappearance of ghrelin cells in the pancreas of LBW newborn rats.

Numerous experimental studies have shown that nutritional deficiencies during critical periods of life result in a range of fetal adaptations and developmental changes (Barker 1993). Although these adaptations may be beneficial for short-term survival, they can lead to permanent alterations in body structure and metabolism, and thereby to cardiovascular and metabolic diseases in adult life (Arantes *et al.* 2002). There is a genetic component underlying the balance between insulin and ghrelin in regulating glucose metabolism. Maternal diet could affect gene expression in offspring (Park *et al.* 2003). Ghrelin may play a role in the pathological procedure of diabetes. Taken together, though it remains unclear how ghrelin is related in lineage to diabetes, here, we supposed that islet ghrelin cell alterations caused by intrauterine undernutrition may be a key linked to the correlation of low birth weight and adulthood diabetes.

In conclusion, the present study showed that intrauterine undernutrition may affect birth weight, plasma insulin level, ghrelin level, islet ghrelin expression, and ghrelin-positive cell distribution. Thus, it will be interesting to investigate the intrauterine nutrition that is involved in islet ghrelin expression and ghrelin cell distribution. Further studies will be required to elucidate the mechanisms by which malnutrition in early life causes permanent changes in later life.

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