Maternal Protein Restriction Increases Autophagy in the Pancreas of Newborn Rats

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Summary  A maternal low-protein diet increases the susceptibility of offspring to type 2 diabetes by inducing alterations in β cell mass and function. However, the mechanism of this pancreas injury remains poorly understood. The present study aimed to assess whether autophagy is altered in the pancreas of intrauterine growth restriction (IUGR). In addition, the autophagy associated mammalian target of rapamycin complex 1 (mTORC1) signaling and endoplasmic reticulum (ER) stress were further evaluated in the pancreas. The maternal protein restriction IUGR rat model was established as the IUGR group, and assessed alongside normal newborn rats (CON group). Then, the levels of autophagy markers were assessed by transmission electron microscopy, immunofluorescence, quantitative real-time PCR (qRT-PCR) and Western blot, respectively. In addition, mTORC1 signaling effectors were evaluated by Western blot; ER stress was quantitated by immunohistochemistry, qRT-PCR and Western blotting. Compared with the control group, the IUGR group showed increased levels of the autophagy markers LC3II and Beclin1, with decreased mTORC1 signaling activity. In addition, ER stress was confirmed in β cells of the IUGR group. These findings provided evidence that maternal protein restriction enhances autophagy in newborn pancreas, where ER stress was also induced in β cells, which might affect the pancreas development.

Key Words  intrauterine growth restriction, autophagy, mTORC1, endoplasmic reticulum stress, maternal nutrition, pancreas

The fetal nutrient environment has been proposed as a component that might affect the risk for developing type 2 diabetes mellitus (T2DM) later in life (1). Indeed, increasing evidence suggests that intrauterine growth restriction (IUGR) is closely associated with the development of T2DM, obesity and hypertension in adulthood (2–4). However, the mechanism by which IUGR causes metabolic syndrome remains poorly understood. Reduced insulin sensitivity is the pathological basis explaining why children with IUGR become susceptible to T2DM and cardiovascular disease in the future, in line with the so called the “Barker hypothesis” or “Fetal Origins of Adult Disease” (FOAD) (5, 6). Further studies (7, 8) have suggested that IUGR can result in impaired pancreatic development, decreased pancreatic area, and insulin hyposensitivity. However, the underlying mechanism remains unclear; although a few studies have proposed the involvement of insulin/FoxO1/Pdx1/MafA signaling, as well as insulin-like (IGFs) and fibroblast (FGFs) growth factors in IUGR associated pancreatic development impairment (3, 9, 10). Recent studies have demonstrated that autophagy plays an important role in β cell function (11–14). Indeed, low autophagy levels constitute an important cause of insulin dysfunction in obese patients (15). However, Fujimoto et al. (16) found that elevated autophagy may cause injury or even kill pancreatic β cells, decreasing the pancreatic area. The above findings indicated that the role of autophagy in cell function remains controversial.

Several signaling pathways and small molecules regulating autophagy have been identified in recent years (17), and the mammalian target of rapamycin (mTOR) pathway is by far the most studied. The mTOR protein is the mammalian ortholog of the yeast protein kinase TOR that negatively regulates autophagy (18). The mTOR signaling in IUGR has recently attracted increasing attention. For instance, Alejandro and colleagues (19) revealed that newborns of dams exposed to low-protein diets throughout pregnancy exhibit reduced mTOR signaling in the pancreas. Meanwhile, Wang et al. (20) reported decreased mTOR protein amounts and enhanced ULK1 and Beclin1 mRNA expression levels in the small intestine of IUGR rats. However, evidence supporting decreased mTOR signaling in the pancreas is limited.

Autophagy and endoplasmic reticulum (ER) stress are essential for cellular function in case of pathological stimuli, including nutrient deprivation and hypoxia. ER stress is clearly linked to autophagy, and both processes co-occur in multiple human diseases such as respiratory diseases, inflammatory diseases, cardiovascular diseases, neurodegenerative disorders, cancer, and diabetes (21). This indicated that ER stress may constitute an important factor in autophagy alteration in pancreatic β cells after IUGR. Recent studies related to autophagy and IUGR have mainly focused on altered autophagy in IUGR-associated placental tissue (22), not...
taking into account the pancreas. Therefore, the present study aimed to assess whether the levels of autophagy markers are altered in the pancreas of offspring after maternal protein restriction. In addition, ER stress markers in pancreatic β cells of newborn rats were assessed. We found that maternal protein restriction enhances autophagy in newborn pancreas, inducing ER stress in β cells.

MATERIALS AND METHODS

Animals. Healthy specific pathogen free (SPF) Wistar rats (license number: SCXK [J] 2014-008) and rodent chow were provided by the Beijing Huaafukang Laboratory Animal Center. The animals included 60 female virgin and 15 healthy adult male rats, with body weights ranging from 250 and 300 g. Female and male rats were randomly housed together overnight at a 4 : 1 ratio. The next day, vaginal smears were obtained; a vaginal smear showing sperm under the microscope indicated conception (day 0). Fertilized maternal rats were randomly divided into low-protein diet (calories, 1,558 kJ/100 g; protein, 23%) and control (calories, 1,558 kJ/100 g; protein, 23%). Daily diet uptake of pregnant rats in the conception (day 0). Fertilized maternal rats were randomly divided into low-protein diet (calories, 1,558 kJ/100 g; protein, 23%) and control (calories, 1,558 kJ/100 g; protein, 23%). Daily diet uptake of pregnant rats in the conception (day 0). Fertilized maternal rats were randomly divided into low-protein diet (calories, 1,558 kJ/100 g; protein, 23%) and control (calories, 1,558 kJ/100 g; protein, 23%). Daily diet uptake of pregnant rats in the conception (day 0). Fertilized maternal rats were randomly divided into low-protein diet (calories, 1,558 kJ/100 g; protein, 23%) and control (calories, 1,558 kJ/100 g; protein, 23%). Daily diet uptake of pregnant rats in the conception (day 0). Fertilized maternal rats were randomly divided into low-protein diet (calories, 1,558 kJ/100 g; protein, 23%) and control (calories, 1,558 kJ/100 g; protein, 23%) and observed under an Eclipse 801 microscope (Nikon, Tokyo, Japan).

Immunohistochemistry. After fixation of pancreatic tissue samples (n=8 for each group) with 4% paraformaldehyde for 24 h at 4°C and paraffin embedding, the sections (4 μm) were deparaffinized and rehydrated. Then, the sections were incubated in methanol/H2O2 for 30 min at room temperature to inhibit endogenous peroxidase activity. After antigen retrieval and blocking, the sections were hybridized overnight at 4°C with primary anti-BIP antibodies (1 : 400; Abcam). Then, the slides were incubated with secondary antibodies at 37°C for 40 min. The peroxidase reaction was achieved with DAB and stopped with water after 5 min. Hematoxylin staining was followed by cover slide mounting with Histomount.

Quantitative real-time PCR (qRT-PCR) Total RNA was isolated from islet tissues (n=6 for each group) using TRizol reagent (Takara Biotechnology Co., Ltd., Dalian, China). First-strand cDNA was synthesized using a PrimeScript RT reagent kit (Takara Biotechnology Co., Ltd., Dalian, China). All amplifications were performed on an ABI 7500 realtime PCR system (Life Technologies, Carlsbad, CA) in 20 μL reactions consisting of 1 μL of each primer, 2 μL of cDNA, 10 μL of 2×SYBR Green/ROX qPCR Master Mix (Takara Biotechnology Co., Ltd.) and water. The cycling conditions were: 37°C for 2 min and 85°C for 5 s melting curve analysis and agarose gel electrophoresis were conducted to verify the specificity of the qRT-PCR products obtained. β-Actin was used as an internal control. Relative gene expression was analyzed by the comparative Ct method. PCR primers are listed in Table 1.

Western blot. Pancreas samples (n=3 for each group) were homogenized with RIPA lysis buffer (Beyotime Institute of Biotechnology), and protein concentrations were measured with a BCA protein assay kit (Beyotime Institute of Biotechnology). Lysates containing equal amounts of protein were fractionated on different concentrations of SDS-polyacrylamide gels (Beyotime Institute of Biotechnology) according to the manufacturer’s instructions, and electroblotted onto PVDF membranes.
After blocking with 5% nonfat milk for 90 min at room temperature, the membranes were incubated with primary antibodies against LC3II (Abcam), mTOR (Cell Signaling Technology, Danvers, MA), phospho-ULK1 (Ser757) (Cell Signaling Technology), phospho-p70 S6 kinase (Thr 389) (Cell Signaling Technology), BIP (Abcam) and CHOP (Cell Signaling Technology) overnight at 4˚C with gentle agitation, at 1,000 dilutions; anti-β-actin antibodies (1 : 10,000; Sigma-Aldrich) were used for normalization. After three washes with 0.1% TBST, the membranes were incubated at room temperature for 120 min with horseradish peroxidase-conjugated secondary antibodies (goat anti-rabbit and anti-rat IgG; 1 : 2,000 dilution; Santa Cruz Biotechnology, Inc.). The protein bands were visualized with enhanced chemiluminescence reagents (Fisher Scientific, Shanghai, China). Signal quantitation was performed with the ImageJ software (version 1.47; National Institutes of Health, Bethesda, MD, USA).

**Statistical analysis.** All data are mean±SD. Data from samples were less than five were assessed by Mann-Whitney tests and others were assessed by independent-sample t-test. Statistical analyses were performed with the SPSS 17.0 software (SPSS, Chicago, IL), and *p*, *0.05 was considered statistically significant.

**RESULTS**

Newborns of dams fed a low-protein diet (IUGR rats) show reduced body and pancreatic weights

The incidence rate of IUGR was significantly increased by approximately 60% in the IUGR group compared with control animals. As shown in Fig. 1A, maternal body weights were lower in the IUGR group compared with control values. In addition, body weights of new-

| Genes | Primer sequences (5′→3′) | Annealing temperatures (˚C) |
|-------|--------------------------|-----------------------------|
| β-Actin | F: GGA GAT TAC TG CCC'TGG CTC CTA  
| | R: GAC TCA TCG TAC TCC TGC'TTG CTG |
| LC3II | F: AGC TCT GAA GGC AAC AGC AAC A  
| | R: GCT CCA TGC AGG TAG CAG GAA |
| Beclin1 | F: GAA ACT GGA CAC GAG CTT CAA GA  
| | R: ACC ATC CTG GCG AGT TCC AAF A |
| BIP | F: GTG CTG CTT GAT GTG TGT TGT CCT CTT  
| | R: ATT GTC TTT TGT CAG GGG TCG T |
| CHOP | F: CAG CGA CAG AGC AAA AT AAC A  
| | R: CAA AGG CGA AGG CAG AGA |
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born rats were markedly decreased compared with normal controls (4.61 ± 0.17 g vs. 6.63 ± 0.41 g, p < 0.001) (Fig. 1B), indicating a successful establishment of the IUGR model. Pancreas weights were also reduced (9.5 ± 2.06 mg vs. 16.5 ± 2.42 mg, p < 0.001) (Fig. 1C), thus reducing the pancreas to body weight ratios (2.06 ± 0.18 vs. 2.48 ± 0.23, p < 0.001) (Fig. 1D).

**Autophagosomes and ER dilation are present in pancreatic β cells of the IUGR group**

Autophagosomes without obvious aggregation were found in islet β cells of the IUGR group by TEM. Meanwhile, increased ER dilation (triangles in Fig. 2A) was detected in the IUGR group compared with the CON (Fig. 2B).

Fig. 2. Ultrastructure of autophagic vacuoles (arrows) and dilated ER (triangles) in pancreatic β-cells of IUGR rats. (A) IUGR group; (B) normal group.

![Image](image_url)

Fig. 3. Autophagy is increased in newborn rats after IUGR. (A) Immunofluorescence staining of LC3II (green) and insulin (red) in neonatal islets (original magnification, ×400) in the IUGR and CON group. (B, C) LC3II protein levels in newborn rats in the IUGR and CON groups. (D, E) Beclin1 protein levels in newborn rats of the IUGR and CON groups. (F) Relative mRNA levels of LC3II determined by real-time PCR in the IUGR and CON groups. (G) Relative mRNA levels of Beclin1 determined by real-time PCR in the IUGR and CON groups. Data are mean ± standard deviation, * p < 0.05 (n = 3 per group). LC3II, microtubule-associated protein light-chain 3 II.
Autophagy is increased in the pancreas of IUGR animals

To further examine autophagy in islet cells after IUGR, we performed immunostaining for LC3II and insulin detection in newborn pancreatic tissue samples. As shown in Fig. 3A, increased protein levels of LC3II were found in β-cells of the IUGR group. Compared with the CON group, the IUGR group also showed increased LC3II and Beclin1 protein expression and mRNA levels, as assessed by Western blot and qRT-PCR (Fig. 3B–G). These findings indicated that maternal low-protein exposure increased autophagy in the pancreas of IUGR offspring.

Maternal low-protein diet decreases mammalian target of rapamycin complex 1 (mTORC1) signaling in the pancreas of neonatal rats

To explore the mechanisms of increased autophagy, mTORC1 signaling activity was assessed. Western blot analysis indicated that the protein expression levels of mTOR (Fig. 4A and D) and P70S6K1 (Fig. 4B and E) were significantly decreased, while P-ULK1 (Ser757) amounts were increased (Fig. 4C and F) in the pancreas of the IUGR group compared with control values, suggesting that maternal protein restriction resulted in reduced mTORC1 activity in islets at birth.

ER stress was also found in the pancreas of newborn rats of IUGR

As ER dilation was observed in β-cells of the IUGR group by TEM, we assessed BIP and CHOP expression levels to determine ER stress occurrence. Immunohistochemistry revealed higher BIP protein expression in pancreas samples from the IUGR group compared with the CON group (Fig. 5A and B); similar findings were obtained at the gene expression level by qRT-PCR (Fig. 5C). The IUGR group also showed higher CHOP mRNA and protein expression levels compared with the CON group (Fig. 5D–F). The increased BIP and CHOP amounts in islets of the IUGR group indicated ER stress occurrence.

DISCUSSION

This study demonstrated that maternal low-protein diet increased autophagy in pancreatic β cells of newborns with altering mTORC1 signaling effectors, which are major targets of nutrients and growth factors. In addition, ER stress was detected in pancreatic β cells, as another potential inducer of autophagy.

An IUGR rat model was successfully established as reflected by reduced maternal and newborn body weights, as well as absolute and relative pancreatic weights. These models also exhibited decreased numbers and volumes of islet β cells (Dan Zhang, unpublished observations). Others (25) have reported approximately 35–40% less islet β cells in newborn IUGR rats compared with normal newborn animals, indicating
that the intrauterine environment in protein deficiency results in significantly impaired pancreatic development.

TEM is considered the gold standard for autophagosome detection. As shown above, autophagosomes in pancreatic β cells of the IUGR group had no overt aggregation. Autophagy markers, including LC3I, LC3II and Beclin1, in pancreas samples from the IUGR group were upregulated compared with control values, both at the protein and mRNA levels. Similarly, increased placental autophagy level was reported in pregnant women delivering babies with IUGR compared with those giving birth to normal babies (26), suggesting that increased autophagy plays an important role in IUGR occurrence. Fujimoto et al. (16) revealed an important role for autophagy in regulating the cellular response to reduced pancreas duodenal homeobox 1 (Pdx1) expression, with autophagy inhibition prolonging cell survival. Previous studies have reported decreased Pdx1 levels in offspring after maternal protein restriction (27); therefore, it is possible that autophagy induction may lead to increased cell death and affect β-cell mass and volume. This hypothesis should be tested in further investigation.

This study found reduced mTOR levels and increased ULK1 phosphorylation in the pancreas of newborn rats in the IUGR group compared with control values; these are upstream molecules involved in autophagy. mTOR, a conserved serine/threonine protein kinase (28), constitutes the catalytic unit of two distinct complexes (mTORC1 and mTORC2) and is essential for cell growth and proliferation (29). These complexes regulate different sets of substrates (30). For example, mTORC1 integrates inputs from various stimuli (e.g., growth factors, stress, energy, oxygen and amino acids) to control fundamental pathways of cell growth and metabolism (17) via phosphorylation of 4E-BPs and S6 kinases (S6Ks). S6Ks, including S6K1 and S6K2, regulate cell growth, protein translation and cell proliferation. Indeed, S6K1 importance in regulating glucose homeostasis and cell growth has been assessed in genetically modified mice (31). Therefore, the decreased mTOR signaling observed in the IUGR group might also play an important role in regulating β-cell growth.

As shown above, the IUGR group exhibited increased levels of ER stress markers in islet β-cells. Indeed, ER dilation was found by TEM; meanwhile, increasing evidence (21) demonstrates that autophagy can be induced by ER stress in multiple ways. Thus, we hypothesized that increased ER stress occurs in islet β cells in the current model of IUGR. As expected, increased BIP and CHOP expression levels were found in the IUGR group.

Fig. 5. ER stress in newborns β-cells after IUGR. (A) Immunohistochemical staining of BIP in neonatal islets (original magnification, A1 and A3, ×200; A2 and A4, ×400). (B) IOD of immunohistochemical staining of BIP in newborn rats. (C) Relative mRNA levels of BIP determined by qRT-PCR in the IUGR and CON groups. (D, E) Relative protein levels of CHOP in the IUGR and CON groups (n = 3 per group). (F) Relative mRNA expression levels of CHOP determined by qRT-PCR in the IUGR and CON groups. Data are mean±standard deviation, *p<0.05, **p<0.01, ***p<0.001 (n = 6–8 per group). BIP, binding immunoglobulin protein; IOD, integral optical density; CHOP, C/BEP homologous protein.
in comparison with control values, confirming ER stress occurrence. ER stress results from disturbances such as oxidative stress, nutrient deprivation, ER calcium depletion, and DNA damage (21). The molecular mechanism linking ER stress and autophagy remains unclear; however, LC3I conversion to LC3II is mediated by ER stress-induced protein kinase (PERK/eIF2α) phosphorylation (32). The AKT-TSC-mTOR and c-Jun N-terminal kinase (JNK) pathways are also involved in the cross-talk between autophagy and ER stress (32, 33). While autophagy is activated as a novel signaling pathway in response to ER stress, the precise signaling pathway responsible for ER stress associated autophagy activation in IUGR rats remains to be determined.

This study had a few limitations. First, we were unable to clearly define the pathway(s) linking ER stress occurrence and autophagy induction in IUGR animals. In addition, we only studied the pancreas of new-born rats, it is difficult to assess whether our findings are transitory and very specific to early postnatal life, or maintained in later life. Finally, this study was performed in rats, which are different from humans in terms of physiological responses. Therefore, more in depth studies are necessary to further unveil the above mechanisms, which should be confirmed in clinical trials.

In conclusion, maternal low-protein diet increases autophagy in the pancreas of newborn rats, with the involvement of mTORC1 signaling and ER stress. Therefore, autophagy should be assessed in studies of pancreatic development in IUGR and diabetes, as a target for novel therapeutic interventions.

Disclosure of state of COI

The authors declare no conflicts of interest.

Acknowledgments

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