Exp. Anim. 66(4), 387–395, 2017

—Original—

A high sucrose and high fat diet induced the development of insulin resistance in the skeletal muscle of Bama miniature pigs through the Akt/GLUT4 pathway

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Abstract: A high sucrose and high fat (HSHF) diet induces insulin resistance (IR) and increased susceptibility to type 2 diabetes mellitus (T2DM), but the underlying mechanisms are poorly characterized. This study aimed to investigate the molecular mechanisms by which the HSHF diet impairs insulin sensitivity in Bama miniature pigs (*sus scrofa domesticus*). Twelve Bama miniature pigs were randomly assigned to the control diet (CD) group (n=6) or the HSHF group (n=6) for 6 months. Biochemical parameters were measured. Western blot, RT-qPCR and immunohistochemistry were used to profile the changes of protein expression, mRNA expression and glucose transporter 4 (GLUT4) expression in skeletal muscle tissues, respectively. In comparison to the CD group, the homeostasis model assessment-insulin resistance (HOMA-IR) index of the HSHF group demonstrated a 2.9-fold increase, and the insulin sensitivity showed a 24.8% decrease. Compared with the CD group, p-Akt S473 decreased by approximately 59% and GLUT4 decreased by 43.8% in the skeletal muscle of the HSHF group. However, the expression of p-mTOR S2448 between the 2 groups was not significantly different (*P*=0.309). This study demonstrates that a 6-month HSHF diet caused IR, decreased insulin sensitivity, and reduced the expression of p-Akt S473 and GLUT4 in the skeletal muscle of Bama miniature pigs.

Key words: Akt, GLUT4, insulin resistance, mTOR.

Introduction

It is estimated that over 400 million people will be affected by type 2 diabetes mellitus (T2DM) worldwide by the year 2030 [27]. Insulin resistance (IR) is a characteristic feature of T2DM, and a significant factor in the development of the disease. It is triggered by excess nutrient intake and impaired insulin secretion caused by pancreatic β-cell dysfunction, and is characterized by reduced sensitivity and response to insulin in insulin-sensitive organs [12].

A high sucrose and high fat (HSHF) diet decreases insulin sensitivity and promotes hyperinsulinemia, which can induce the development of IR and glucose intoler-

(Received 23 January 2017 / Accepted 1 June 2017 / Published online in J-STAGE 3 July 2017)
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Supplementary Figure and Table: refer to J-STAGE: https://www.jstage.jst.go.jp/browse/expanim

ance [32]. Skeletal muscle cells, which have the highest level of insulin-stimulated glucose uptake, are responsible for 70 to 80% of insulin-stimulated glucose uptake [1], and are the main site of IR [8]. A serine threonine protein kinase, Akt, is activated in response to insulin or insulin growth factor signaling, and it exists in three isoforms, Akt-1, 2, and 3. Additionally, Akt Ser473 phosphorylation (p-Akt S473) is needed for glucose uptake, and is a key player in promoting glucose transporter 4 (GLUT4) translocation by phosphorylation of Akt substrate of 160KDa (As 160) [16, 20]. The protein GLUT4 is specifically expressed in skeletal muscle and adipose tissue and mainly localized in intracellular storage vesicles, and it is particularly important for maintaining glucose metabolism homeostasis and insulin sensitivity. The GLUT4 storage vesicles translocate to the plasma membrane in response to various stimuli and take up glucose to reduce postprandial hyperglycemia [3]. The greater the GLUT4 protein concentration is at the cell surface, the higher is the insulin sensitivity and glucose clearance activity [10].

The mammalian target of rapamycin (mTOR), a serine threonine kinase, has been implicated in several specific human pathologies, including tumor, obesity, and T2DM [17]. It is the catalytic subunit for 2 functionally and structurally distinct mTOR complexes (mTORC1 and mTORC2) [11]; mTORC1 is a major regulator of protein synthesis and cell growth, and mTORC2 is a major upstream kinase for the p-Akt S473 residue [25]. It has been proposed that mTOR phosphorylation and its downstream target Akt could contribute to the reduction in skeletal muscle development observed caused by a high fat diet [22] .

Bama miniature pigs (*sus scrofa domesticus*) are genetically stable, highly inbred, small, and easier to handle than domestic pigs [33]. They are relatively susceptible to HSHF-diet-induced diabetes [4], and these HSHF-diet-induced Bama minipigs have been used for IR and diabetes research in the past [18, 19, 23]. The aim of the present study was to determine the effects of an HSHF diet on the impairment of skeletal muscle insulin sensitivity and glucose intolerance in Bama miniature pigs, and then to elucidate the mechanisms involved.

Material and Methods

This study was approved by the Animal Care and Use Committee of the Germplasm Resource Center of Chinese Experimental Minipigs and conducted in accordance with the criteria Guideline in the PLAGH for the Care and Use of Laboratory Animals.

Table 1. Nutrition content of the feed

Components		CD	HSHF
Corn	g%	48	26.4
Wheat middling	g%	20	11
Soybean cake	g%	15	8.25
Rice bran	g%	12	6.6
Salt	g%	0.38	0.21
Fish powder	g%	3	1.65
Phosphate calcium	g%	1	0.55
Trace elements	g%	0.08	0.04
Multivitamin	g%	0.02	0.01
Premix feed	g%	0.52	0.29
Cattle fat	g%	0	10
Sucrose	g%	0	35
Protein	g%	16.11	7.81
Fat	g%	5.36	13.64
Carbohydrate	g%	78.53	78.55
Protein	kcal%	15.1	6.7
Fat	kcal%	11.3	26.2
Carbohydrate	kcal%	73.6	67.1
Total energy content	kcal/100 g	357.7	425.6

Animals

Twelve Bama miniature pigs, obtained from the Beijing Shichuang minipig breeding base were divided into 2 groups at the age of 8 months, and then they were given 6 months of treatment. Half of the minipigs in each group were female, and half were male. The control diet (CD) group was fed with a basic feed (Tianjin jingqishen animal feeds Ltd., co., Tianjin), and the HSHF group was fed with a HSHF diet (55% basic feed, 35% sucrose, and 10% beef tallow) (Table 1) [4]. The pigs were fed twice daily, and each minipig received a quantity of feed equivalent to 3% of its body weight, with the amount of feed adjusted after monthly weighing. During the experiment, the animals were separately fed in pens kept at 18 to 22°C with the relative air humidity at 30% to 70%, and water was freely available. At the end of the experiment, all the animals were killed by lethal injection of ketamine and xylazine after a fast of 12 to 16 h. Tissues were sampled and preserved at −80°C.

Serology assay and homeostasis model assessment -IR

Blood was collected from the posterior branch of the lateral saphenous vein of the pig and centrifuged (1,500 g for 10 min at 4°C) to separate the serum. Glucose, levels of serum triglycerides, total cholesterol, high-

density lipoprotein cholesterol (HDL-C), low-density lipoprotein cholesterol (LDL-C), ALT, AST, total protein, serum albumin, serum creatinine, uric acid (UA), and urea were assessed using a HITACHI-8000 automatic biochemical analyzer and cobas kits (Roche, Basel, Switzerland).

Insulin concentrations were detected by a radioimmunoassay kit (China Institute of Atomic Research, Beijing, China), and IR was assessed by the homeostasis model assessment (HOMA), using the equation HOMA-IR=[fasting insulin (FINS) × fasting glucose (FPG)] / 22.5 [21].

Insulin sensitivity was calculated as follows [13]:

1 / [(log (fasting insulin concentration (μU/ml)) + log (fasting glucose concentration (mg/dL))]

Intravenous glucose tolerance test

Pigs were fasted for 8 h before intravenous glucose tolerance tests (IVGTTs) were performed under a non-narcotic state in a sling [4]. Basal blood samples were drawn at 10 and 5 min before the start of the experiment. Pigs then received 1g of glucose in 0.9% NaCl/kg body weight through a Saphenous Vein catheter. Blood was collected from the posterior branch of the lateral saphenous vein at the indicated time points after glucose administration[4].

Real-time quantitative PCR (RT-qPCR)

Total RNA was extracted from frozen skeletal muscle (longissimus dorsi) specimens using a Trizol kit (Invitrogen, Beijing, China). The RNA was reverse transcribed to first-strand cDNA by using a kit (Promega, Madison, USA). Relative mRNA levels were calculated by using the Ct values and normalized using *GAPDH* expression, based on the $2^{-\Delta\Delta CT}$ method. Triplicate samples were analyzed to ensure the statistical significance.

All primers have been referred to in past reports [6]. The primer sequences (5′ to 3′) were as follows: *GAPDH*: GGT CGG AGT GAA CGG ATT TG (forward) and CCT TGA CTG TGC CGT GGA AT (reverse); *Akt*: ACA ACC AGG ACC ACG AGA AG (forward) and GAA ACG GTG CTG CAT GAT CT (reverse); *mTOR*: GGT TTG ATT ATG GTC ACT GG (forward) and TGC AAT GAG CTG AGG TAT AA (reverse). All primers were purchased from Invitrogen (Shanghai, China).

Western blot

Skeletal muscle (longissimus dorsi) tissues were homogenized in ice-cold RIPA buffer containing phosphatase and protease inhibitors. Lysate protein content was determined with the bicinchoninic acid (BCA) method using BSA standards (Pierce), and all lysates were diluted to the same protein concentration. Total protein and phosphorylation levels of indicated proteins were determined by standard immunoblotting techniques and loading equal amounts of protein (30 μg). After the electrophoresis was complete, the samples were transferred to polyvinylidene fluoride (PVDF) membranes (Millipore, Billerica, MA). Proteins were detected with specific primary antibodies purchased from Santa Cruz Biotechnology (USA): p-Akt1/2/3 (Ser473): sc-33437; p-mTOR (Ser2448): sc-101738; Akt1 (C-20): sc-1618; mTOR (H-266): sc-8319, and HRP-conjugated secondary antibodies using an ECL kit. Protein content was detected using an ECL kit (Amersham Biosciences). The signals from the Western blotting were quantified with the Image J program (NIH).

Histological analysis

The 4% paraformaldehyde-fixed and paraffin-embedded longissimus dorsi tissue sections were dewaxed in xylene, followed by rehydration in decreasing ethanol concentrations. After antigen retrieval by high-temperature heating in citrate buffer (pH=6.0) and blocking in inhibitor buffer (3% H_2O_2), the slides were incubated with anti-glucose transporter GLUT4 primary antibody (Abcam, Cambridge, UK , Cat. ab654) overnight at 4°C. Signals were visualized by 3,3′-diaminobenzidine (DAB); brown staining represents GLUT4, and blue staining represents nuclei. Average optical density values of every field were measured using Image-Pro Plus 6.0 software.

Statistical analysis

Data were analyzed using the IBM SPSS Statistics 19 program (SPSS Inc., Chicago, IL, USA). The error bars represent SEM. Student's *t*-test was used to compare basic characteristics of subjects in the HSHF and CD groups. Data were presented as mean ± SEM with $P<0.05$ as the limit for statistical significance.

Results

Comparison of biochemical criteria of Bama miniature pigs in the CD group and HSHF group

The initial body weights, blood glucose, and serum

Table 2. Baseline body weight,blood glucose, and serum insulin of the Bama miniature pigs in the CD (n=6) group and HSHF (n=6) group

	CD	HSHF	*P* Value
Body weight (kg)	22.25 ± 0.7	22.67 ± 0.60	*P*=0.66
Blood glucose (mmol/L)	4.30 ± 0.38	4.36 ± 0.22	*P*=0.89
Serum insulin (μIU/mL)	4.84 ± 0.32	5.01 ± 0.28	*P*=0.69

The values are the mean ± SEM.

Table 3. Body weight and serum biochemistry of the Bama miniature pigs in the CD (n=6) group and HSHF (n=6) group

	CD	HSHF
Body weight(kg)	34.54 ± 0.77	58 ± 2.07**
Blood glucose (mmol/L)	4.1 ± 0.42	5.54 ± 0.78
Insulin (μIU/mL)	9.88 ± 1.08	24.13 ± 4.5**
Serological indicators		
Total cholesterol (mmol/L)	1.98 ± 0.16	3.74 ± 0.19**
HDL cholesterol (mmol/L)	0.84 ± 0.15	2.26 ± 0.15**
LDL cholesterol (mmol/L)	1.14 ± 0.16	1.77 ± 0.17*
Triglycerides (mmol/L)	0.25 ± 0.06	0.79 ± 0.21**
Kidney function		
Total protein (g/L)	73.74 ± 1.13	72.96 ± 3.12
Serum albumin (g/L)	45.94 ± 1.1	52.46 ± 0.78*
serum creatinine (μmol/L)	117.02 ± 12.2	79.22 ± 5.43*
Uric acid (mg/dL)	1.28 ± 0.35	2.53 ± 0.64**
Urea (mg/dL)	2.79 ± 0.23	1.17 ± 0.19**

The values are the mean ± SEM. *P<0.05, **P<0.01compared with the CD group.

insulin were not significantly different between the CD (n=6) group and HSHF (n=6) group (Table 2). Pigs in the HSHF group developed obesity and hyperinsulinemia, and the concentration of blood lipid also increased after 6 months of the HSHF diet (Table 3). Body weight and serum biochemistry of female and male Bama miniature pigs in the CD group and HSHF group was shown in supplementary Table 1. Compared with the CD group, pigs in HSHF group demonstrated a 2.44-fold increase in serum insulin (P<0.01). The level of total blood cholesterol in the HSHF group was 1.88 times higher than that in the CD group (HDL-C was increased 2.69-fold, LDL-C 1.55-fold, triglyceride level 3.2-fold, and uric acid 1.97-fold). The body weight was 1.65 times higher in the HSHF group compared with the CD group. However, levels of serum creatinine decreased to 67%, and urea decreased to 42% in the HSHF group compared with the CD group. There was no significant difference in the fasting glucose between the 2 groups.

HSHF diet induced glucose intolerance and IR in Bama miniature pigs

As shown in Fig. 1 A, the baseline IVGTT in the HSHF group was comparable to that in the CD group. After a 6-month HSHF diet, the glucose levels at 60 min, 90 min, and 120 min were significantly increased in the HSHF group (Fig. 1B), and the insulin levels at 10 min and 30 min were significantly increased in the HSHF group (Fig. 1C). The HOMA-IR index of the HSHF group, compared with the CD group, was 2.9 times higher (P<0.01), which indicated that pigs in the HSHF group had developed IR in peripheral tissues (Fig. 1D). Moreover, the insulin sensitivity of the HSHF group was decreased by 24.8% compared with the CD group (Fig. 1E). These results indicated that a 6-month HSHF diet induced IR and glucose intolerance in Bama miniature pigs.

The mRNA expression of Akt and mTOR in skeletal muscle tissues

The relative *Akt* mRNA expression in skeletal muscles of HSHF group compared with the CD group was 0.529 ± 0.063. The relative mRNA expression of *mTOR* in skeletal muscle of HSHF group compared with the CD group was 0.978 ± 0.019. The mRNA expression of *Akt* in the HSHF group decreased 47% compared with the CD group (P<0.05). However, the mRNA expression of

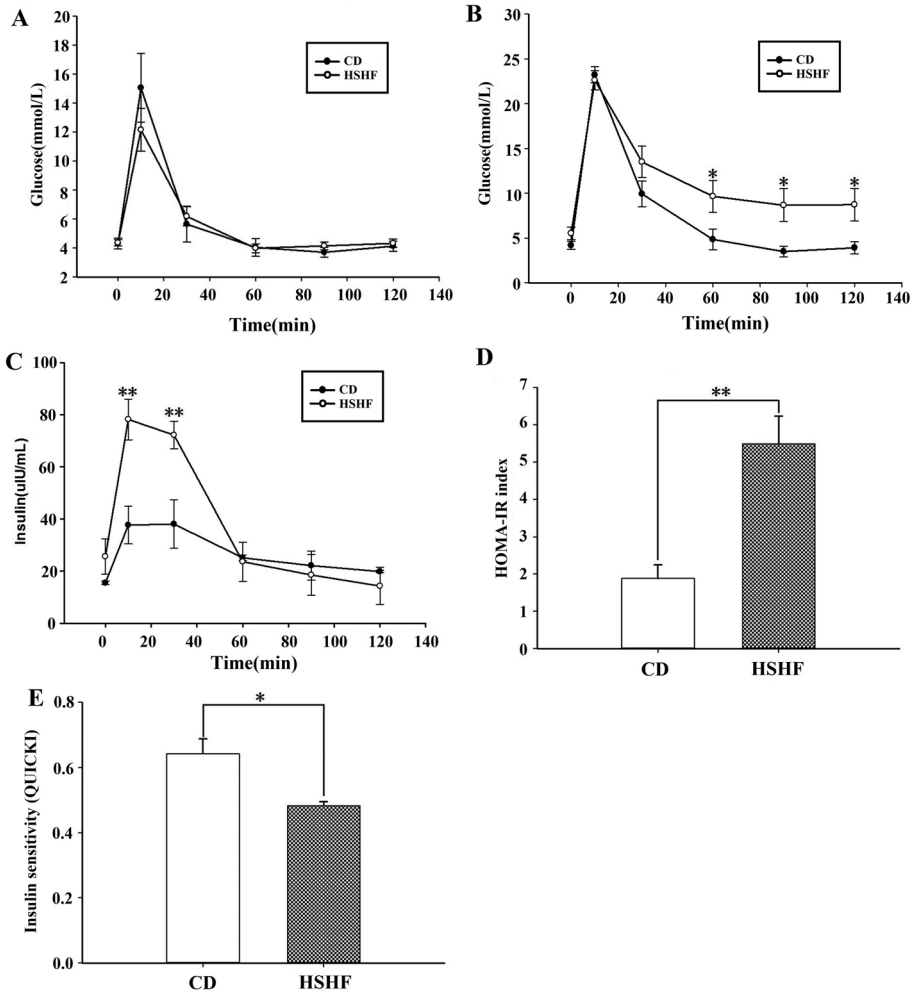

Fig. 1. Glucose intolerance and IR induced by HSHF diet. (A) The baseline IVGTT of the CD (n=6) group and HSHF (n=6) group. (B) The IVGTT of the CD (n=6) group and HSHF (n=6) group fed for 6 months with the 2 different diets. (C) Insulin in IVGTT of the CD (n=3) group and HSHF (n=3) group fed for 6 months with the 2 different diets. The homeostasis model assessment-insulin resistance (HOMA-IR) (D) and insulin sensitivity (E) of the CD (n=6) group and HSHF (n=6) group fed for 6 months with the 2 different diets. **P*<0.05,** *P*<0.01; (*t*-test).

mTOR was not significantly different between the 2 groups (*P*=0.476) (Fig. 2).

Protein expression of p-Akt S473 and p-mTOR S2448 in skeletal muscle tissues

The relative values of p-Akt S473 in HSHF group compared with the CD group was 0.409 ± 0.092. The relative values of p-mTOR S2448 in HSHF group compared with the CD group was 0.83 ± 0.138. The level of p-Akt S473 in the skeletal muscle in the HSHF group decreased 59% compared with the CD group (*P*<0.05). However, analysis of p-mTOR S2448 indicated that there was no significant difference between the 2 groups (*P*=0.309) (Fig. 3). All samples' western blot figures was provided in supplementary Fig. 1.

GLUT4 immunocytochemistry

The relative average optical densities of GLUT4 of skeletal muscle in HSHF group compared with the CD group was 0.561 ± 0.200. The average optical density of GLUT4 in HSHF group decreased 43.8% compared with the CD group (*P*<0.05) (Fig. 4).

Discussion

Miniature pig models were designed to bridge the gap

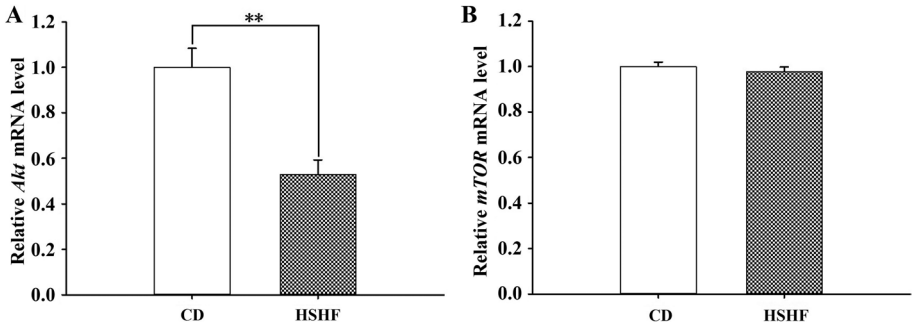

Fig. 2. Effect of HSHF diet on mRNA expression of Akt and mTOR in skeletal muscle tissues. Evaluation of the relative mRNA expression of Akt (A) and mTOR (B) of the CD (n=6) group and HSHF (n=6) group by qRT-PCR. ** $P<0.01$; (*t*-test).

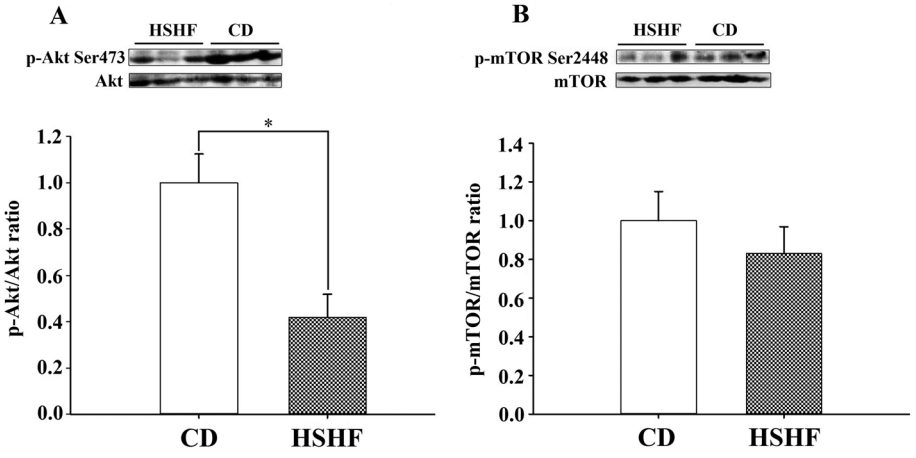

Fig. 3. Effect of HSHF diet on protein expression of p-Akt S473 and p-mTOR S2448 in skeletal muscle tissues. (A) Evaluation of the relative protein expression of p-Akt S473 of the CD (n=3) group and HSHF (n=3) group by Western blot. (B) Evaluation of the relative protein expression of p-mTOR S2448 of the CD (n=6) group and HSHF (n=6) group by Western blot. The results of the relative quantification of p-Akt S473 and p-mTOR S2448 expression are provided below. *$P<0.05$; (*t*-test).

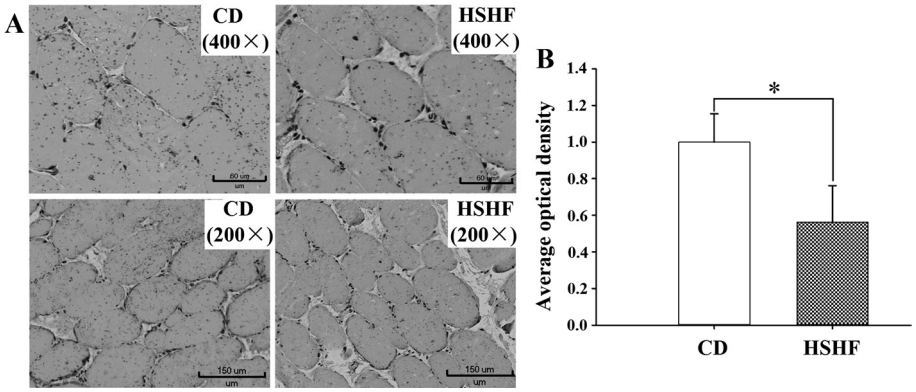

Fig. 4. Immunohistochemical localization of GLUT4 in skeletal muscle of the CD (n=6) group and HSHF (n=6) group (400×, 200×). (A) Signals were visualized by DAB; brown staining represents GLUT4 and blue staining represents nuclei. (B) The results of the relative quantification of GLUT4 average optical density in the CD (n=6) group and HSHF (n=6) group. *$P<0.05$; (*t*-test).

between mouse and human in translational research for diabetes and other metabolic diseases. Previous reports have demonstrated that short or long term effects of the HSHF diet on the body composition of swine could lead to obesity and metabolic disturbances [15]. In the present study, we evaluated whether a 6-month HSHF diet could modify the metabolic status of Bama miniature pigs from 8 months of age, and determined the effects of the HSHF diet on the impairment of insulin sensitivity of skeletal muscle and the relative molecular mechanisms. Our study indicated that a 6-month HSHF diet for Bama miniature pigs increased the plasma triglyceride levels, total cholesterol levels, and promoted the development of IR. Disregulation of Akt plays an important role in obesity and IR induced by a high fat diet, and Akt kinase also appears to be a common regulator of insulin metabolism in several physiologically important target tissues [26]. It is known that GLUT4 is the major subtype form of glucose transporter present in skeletal muscle, heart, and adipose tissues [7], and regulates glucose homeostasis through translocation and activation. Our results indicated that a 6-month HSHF diet decreases GLUT4 expression in the skeletal muscle in Bama miniature pigs.

As the mTOR pathway is highly regulated by physiological status and improves protein synthesis and anabolism in skeletal muscle [9], we examined whether the HSHF diet impairs insulin-stimulated glucose disposal via mTOR/Akt phosphorylation and subsequently inhibits insulin signaling in skeletal muscle. Accordingly, we observed the mRNA expression and protein levels of p-mTOR S2448 and p-Akt S473 in the skeletal muscle in the 2 groups. There was a decrease in p-Akt S473 in the HSHF group, but the level of p-mTOR S2448 was similar between the 2 groups. These results indicated that the HSHF diet could be an important regulator of glucose homeostasis, but its adverse effect on IR is not mediated by p-mTOR S2448 signaling in skeletal muscle.

Several studies in cultured myotubes isolated from swine and rodent skeletal muscles have generated inconsistent results. For example, Saha *et al.* [24] reported that incubation of rat extensor digitorum longus muscle (EDL) with 100 or 200 μM leucine for 1 h significantly increased p-mTOR S2448, p70S6K T389, and protein synthesis. Leucine incubation also caused IR in rat EDL, as demonstrated by a decrease in insulin-stimulated p-Akt S473. In contrast, Smith *et al.* [28] reported that protein ingestion induced muscle IR independent of leucine-mediated mTOR activation. Gao *et al.* [6] reported that low doses of insulin growth factor-I increased the size of skeletal muscle satellite cells via the Akt/S6K signaling pathway, and did not affect p-mTOR S2448 and p-mTOR S2481 in Landrace pigs. The mechanisms responsible for the effect of the HSHF diet on p-mTOR in IR minipigs are unclear. The results from studies conducted in cultured rat and mice muscle have suggested that mTOR inhibition induced muscle IR [5, 14]. However, Stanimirovic *et al.* [30] reported that the phosphorylation of mTOR was not significantly decreased in female rats with induced IR after 10 weeks of a high fat diet. Our results confirmed that a 6-month HSHF diet decreased muscle p-Akt S473 and GLUT4, without altering p-mTOR S2448. This disconnect between p-Akt and p-mTOR signaling is consistent with several previous studies in humans [2, 34], and suggests that other mechanisms (such as those involving PI3K, FoxO, AMPK, PDK1, and Nox2) [29, 31] might be responsible for the decrease in muscle p-Akt S473 and GLUT4 in our study.

Our study has 2 limitations that need to be considered. First, we studied only 8-month-old Bama miniature pigs. Second, it is possible that our study did not contain an adequate number of pigs

In summary, this study demonstrated a realistic, translational model of diet-induced IR and glucose intolerance, which showed a decrease of p-Akt S473 and GLUT4, independent of p-mTOR S2448 in the skeletal muscle of Bama miniature pigs. Therefore, the HSHF diet could be an important precipitating factor of IR and glucose intolerance, which is consistent with previous studies. Skeletal muscle is a major contributor to glucose tolerance through regulation of glucose uptake by glucose transporters. Therefore, identifying the precise mechanisms responsible for the regulation of insulin sensitivity in skeletal muscle could lead to the identification of novel therapeutic targets for T2DM. In conclusion, this study provides basic information for further studies regarding the use of Bama miniature pigs to study IR and T2DM using an HSHF diet.

Acknowledgments

This study was supported by grants from the National Natural Science Foundation of China (No. 31472057).

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