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Dietary soy isoflavones differentially regulate expression of the lipid-metabolic genes in different white adipose tissues of the female Bama mini-pigs

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ABSTRACT

Soy isoflavones have been shown to affect lipid metabolism, however the underlying molecular mechanism(s) have not yet been fully understood. The present study, using female Bama mini-pig as a model, examined the effects of soy isoflavones on lipid metabolism and involved gene expression in different white adipose tissues. Female Bama Xiang mini-pigs of 35 days old were fed a basal diet (control, Con), or basal diet supplemented with increasing amounts of soy isoflavones (250, 500, or 1250 mg/kg diet) for 120 days. The results showed that soy isoflavones did not affect the body weight, but decreased the dorsal subcutaneous adipose tissue (DSA) mass and increased the mass of abdominal subcutaneous adipose tissue (ASA) and perirenal adipose tissue (PRA). Besides, soy isoflavones decreased the expression of lipogenic genes and increased the expression of lipolytic genes in DSA, while the opposite effects were observed in ASA and PRA. In addition, the expression of lipoprotein lipase was down regulated in DSA while up regulated in ASA and PRA by soy isoflavones. Moreover, the expression of estrogen receptors (ERs) was up regulated in DSA, and down regulated in ASA and PRA by soy isoflavones. Our results suggest that soy isoflavones affected the lipid metabolism in white adipose tissues of Bama mini-pigs in a site-specific manner, which might be mediated through PPARs and ERs regulated gene expression.

1. Introduction

Over the last decades, the prevalence of obesity and related diseases has increased rapidly worldwide [1,2]. Obesity is a disorder of energy balance and is associated with hyperinsulinemia, insulin resistance, and abnormalities in lipid metabolism [1]. Researchers are trying to develop new effective strategies in controlling obesity. Estrogen has been proved to be associated with a reduction in food intake, fat deposition, and body weight in both male and femaleadults [3]. Phytoestrogens (PEs) are naturally occurring plant components produced in a large range of plants, and can influence the lipid metabolism because of their structural similarity to that of estradiol [4,5]. Isoflavones, the major soy phytoestrogens, have beneficial effects by lowering liver or blood triglyceride, total and LDL cholesterol levels, enhancing HDL cholesterol and the ratio of HDL/LDL [1,6].

However, the effects of soy isoflavones on fat deposition, differentiation of adipocyte and obesity in both humans and animals are not fully understood and inconsistent. Genistein, one of the major soy isoflavones, decreased adipose deposition in mice [7]. Dietary relevant mixtures of isoflavones inhibited adipocyte differentiation in vitro [5]. However, the opposite effects have also been reported. For example, soy isoflavones favored fat deposition

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in male pigs [8]. The mechanisms involved in the modulation of lipid metabolism by soy isoflavones may be complex and multiple factors (such as gender, doses, and age) may be involved. It has been reported that genistein exposure during the early postnatal period favored the development of obesity in female, but not male rats [9]. So, in the present study, we use female Bama mini-pig as experimental model, to further investigate the effects of soy isoflavones on the lipid metabolism and fat deposition.

Adipose tissues, especially the white adipose tissue, play a central role in the regulation of lipid metabolism and maintenance of the energy balance in animals and humans [8]. The fat deposition in white adipose tissue, a key process of resulting host obesity, is regulated by the balance of lipogenesis and lipolysis. This process is mostly accomplished by key adipogenic transcription factors, such as peroxisome proliferator-activated receptor (PPAR), which mainly includes PPAR-α and PPAR-γ, lipogenic genes, such as fatty acid synthetase (FAS) and acetyl CoA carboxylase (ACC) and lipolytic genes (such as hormone-sensitive lipase (HSL) and adipose triglyceride lipase (ATGL)).

17β-Estradiol (E2), the most ubiquitous estrogen, is a major regulator of adipocyte development and adipocyte number in both females and males [10,11]. Soy isoflavones are nonsteroidal, diphenolic compounds with a structure similar to that of E2, and show certain biological activities in both animals and humans [1]. Binding of E2 to ERs inhibits lipogenesis primarily through decreasing activity of LPL, an enzyme that regulates lipid uptake by adipocytes [12]. Since isoflavones, particularly genistein and daidzein, can also bind to ERs [13], this suggests that ERs-mediated pathway may be also involved in the soy isoflavones regulated lipid metabolism in adipose tissues. Dieudonné et al. reported that the expression of ERs was different in human adipocytes from different adipose tissue depots after E2 treatment [14]. So, we thought that just like E2, maybe the soy isoflavones could differently regulate the expression of ERs in different adipose tissue.

The primary aim of this study is to elucidate the underlying mechanism involved in the effect of soy isoflavones on lipid metabolism through measuring the expressions of the lipogenic and lipolytic genes.

2. Materials and methods

2.1. Animals

A total of twenty four, 35-days old female weanling pigs (Bama Xiang mini-pigs) of average weight 2.80 ± 1.0 kg were obtained from a local pig herd in Bama County, a South-west county of China and used in this experiment. They were selected from six farrowing mothers amidst the herd. Four healthy female pigs each were selected from each mother and randomly assigned into four treatment groups. Thus, there were a total of six pigs in each treatment replicated six times with a pig each. This study was conducted according to the guidelines of the Animal Care and Use Committee of the Institute of Subtropical Agriculture, the Chinese Academy of Science.

2.2. Diets and feeding

A standard basal diet was formulated to meet the nutrient requirements and physiological needs of growing Xiang mini-pigs. Four diets were formulated to consist of basal diet (control) or basal diet supplemented with 250 mg/kg (low-dose, LSI), 500 mg/kg (moderate-dose, MSI), or 1250 mg/kg soy isoflavones (high-dose, HSI), respectively. The piglets were fed the respective diets for 120 days. The soy isoflavones were obtained commercially (Xi'An-Rongsheng Biotechnology Co. Ltd, Xi' an, P. R. China). Pigs were fed two times (at 08:00 and 17:00 GMT) on a daily basis at 3% of their body weight. Water was offered *ad libitum* to the animals. Pigs were housed individually with hard plastic slatted floor, containing separate feeder and water trough.

2.3. Sample collection

After 120 days of feeding, pigs were individually weighed immediately before feeding, and average body weight was calculated. Animals were electrically stunned, exsanguinated and eviscerated in a slaughter house. Dorsal subcutaneous adipose tissue (DSA), abdominal subcutaneous adipose tissue (ASA) and perirenal adipose tissue (PRA) were rapidly excised from the right side of the carcass and sampled and weighed. Adipose samples were then immediately frozen in liquid N2 and stored at −80 °C until further analyses.

2.4. Blood parameters

The plasma concentrations of glucose (Glu), triacylglycerols (TG) and low density lipoprotein (LDL) were determined using a Biochemical Analytical Instrument (Beckman CX4) and commercial kits (Sino-German Beijing Leadman Biotech Ltd., Beijing, China). Six samples of each group were measured.

2.5. RNA extraction and cDNA synthesis

Total RNA was isolated from DSA, ASA, and PRA using the TRIZOL reagent (Invitrogen, Carlsbad, CA, USA) and treated with DNase. The RNA quality was checked by 1% agarose gel electrophoresis, stained with 10 ug/ml ethidium bromide. The OD260:OD280 ratio of extracted RNA was between 1.8 and 2.0. Reverse transcription was performed using PrimeScript RT reagent Kit with gDNA eraser (Takara, Dalian, China).

2.6. Real-time PCR

The following primer sequences for the selected genes were used: for β-actin, 5′-TGCGGGACATCAAGGAGAAG-3′ and 5′-AGTTGAAGGTGGTCTCGTGG-3′ [8]; for FAS, 5′-CTACCTTGTGGATCACTGCATAGA-3′ and 5′-GGCGTCTCCTCCAAGTTCTG-3′ [8]; for 5′-ACC, GGAGACAAACAGGGACCATTACA-3′ and 5′-CAGGGACTGCCGAAACATC-3′ [8]; for PPARγ, 5′-CATTCGCATCTTTCAGGG-3′ and 5′-GGACGCCATACTTTAGGA-3′ [8]; for PPARα, 5′-CATCCTCGCGGGAAAGG-3′ and 5′-GGCCATACACAGTGTCTCCATGT-3′ [8]; for HSL, 5′-TCAGGTGTCTTTGCGGGTAT-3′ and 5′-CTTGTGCGGAAGAAGATGCT-3′ [8]; for ATGL, 5′-TCACCAACACCAGCATCCA-3′ and 5′-GCACATCTCTCGAAGCACCA-3′ [8]; for LPL, 5′-CAAACTTGTGGCTGCCCTAT-3′ and 5′-GTGGACATTGTTGGGAGGAT-3′ [15]; for ER-α, 5′-ATTGGTCTTGTCTGGCGCTCC-3′ and 5′-GGTCATAGAGGGGCACCACGT-3′ [16]; for ER-β, 5′-AGAGACATTGAAAAGGAAGG-3′ and 5′-GCCTTACATCCTTCACATGA-3′ [16]. Real-time PCR was performed using SYBR Green detection kit (Thermo Fisher Scientific, Inc., USA) in an ABI PRISM 7900HT Fast Real-time PCR system. An aliquot (2ul) of cDNA template solution was mixed with 5 ul SYBR Green mix, 0.2 ul ROX Reference Dye (50 ×), 0.2 ul each of forward and reverse primers, and 2.4 ul nucleic free H_2O. After a pre-denaturation program (7 min at 95 °C), 40 cycles of amplification were performed (94 °C for 15 s, 60 °C for 30 s), followed by a melting curve program (60–99 °C with a heating rate of 0.1 °C/s and fluorescence measurement), and the fluorescent signal was detected. A melting curve was generated for each sample at the end of each run to ensure the purity of the amplified products. The amplification of β-actin in each sample was used to normalize the expression of the selected genes. We calculated the relative expression ratio (R) of

mRNA by $R = 2^{-\Delta\Delta Ct(sample-control)}$, where $-\Delta\Delta Ct(sample - control) = (Ct\ gene\ of\ interest - Ct\beta actin)_{sample} - (Ct\ gene\ of\ interest - Ct\beta actin)_{control}$.

2.7. Western blot

Frozen samples were powdered under liquid nitrogen, and lysed in RIPA buffer (150 mMNaCl, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, 50 mMTris–HCl at pH 7.4), plus a protease inhibitor cocktail (Roche, Shanghai, China) and phosphatase inhibitors (Thermo Scientific, Bremen, Germany). Soluble proteins were subjected to SDS–PAGE, and transferred to PVDF membranes (Millipore, Billerica, MA), blocked with 5% nonfat milk in TBS-0.05% Tween-20 for 1 h and incubated overnight with estrogen receptor beta (ER-β; 1:500; AbDSerotec, UK), estrogen receptor alpha (ER-α; 1:250), lipoprotein lipase (LPL; 1:500) and β-actin (1:1000) (Santa Cruz, CA, USA) antibodies followed by horseradish peroxidase-linked secondary antibodies (Santa Cruz, CA, USA). The immunostaining signals were detected with enhanced chemiluminescence kit (Applygen Technologies Inc., Beijing, China). The density of the specific protein bands of interest was measured using AlphaImager 2200 (Alpha Innotech Corporation, CA, USA) software and normalized by that of β-actin.

2.8. Statistical analysis

Data were analyzed by one-way ANOVA (SAS 9.2, Cary, NC). Differences between individual means were determined by the New Duncan multiple comparison test. Results were expressed as mean ± SEM. A probability of $P < 0.05$ was considered to be statistically significant.

3. Results

3.1. Growth performance, adipose tissue mass rate and contents of Glu, TG and LDL

Diets supplemented with soy isoflavones have no effects on body weight and body fat mass rate of Bama mini-pig (Table 1). The pigs fed MSI and HIS diets had significantly lower percentage of DSA ($P < 0.05$), but higher percentage of PRA ($P < 0.05$) compared to those fed Con and LSI diets. The percentage of ASA was markedly increased ($P < 0.05$) by all soy isoflavones supplemented diets (Table 1). The concentration of plasma Glu was decreased ($P < 0.05$) by soy isoflavones supplemented diets, and the soy isoflavones have no significant effects on plasma TG and LDL concentrations (Table 2).

Table 2
The effects of soy isoflavones on plasma Glu, TG, and LDL contents of female Bama mini-pigs.

	Con	LSI	MSI	HSI
Glu (mmol/L)	4.80 ± 0.11[a]	4.25 ± 0.04[b]	4.01 ± 0.21[b]	3.37 ± 0.15[c]
TG (mmol/L)	0.33 ± 0.01	0.37 ± 0.03	0.41 ± 0.06	0.37 ± 0.03
LDL (mmol/L)	0.85 ± 0.08	0.67 ± 0.06	0.88 ± 0.07	0.74 ± 0.05

Data are expressed as means ± SEM, n = 6.
Values in a row with different superscript differ ($P < 0.05$).

3.2. The expression of genes related to lipid anabolism and catabolism in adipose tissues

The mRNA expression of genes involved in lipogenic (fatty acid synthetase (FAS), acetyl CoA carboxylase (ACC) and peroxisome proliferators-activated receptor-γ (PPAR-γ)) and lipolytic (hormone-sensitive lipase (HSL), adipose triglyceride lipase (ATGL) and peroxisome proliferators-activated receptor-α (PPAR-α)) metabolism was measured. As shown in Fig. 1, in DSA, the gene expression of PPAR-γ was reduced ($P < 0.05$) by soy isoflavones supplemented diets, FAS and ACC was reduced ($P < 0.05$) by MSI and HSI, while the gene expression of PPAR-α, HSL and ATGL were increased ($P < 0.05$) by MSI and HSI. The mRNA level of PPAR-γ, FAS and ACC in ASA, PPAR-γ and ACC in PRA were up regulated ($P < 0.05$) by MSI and HSI, and FAS in PRA was also up regulated ($P < 0.05$) by all soy isoflavones supplemented diets. The gene expression of HSL and ATGL in ASA, PPAR-α, HSL and ATGL in PRA were down regulated ($P < 0.05$) by MSI and HSI, and PPAR-α in ASA was also decreased by soy isoflavones supplemented diets.

3.3. Gene and protein expression of ERs and LPL in adipose tissues

Estrogen receptors (ERs, mainly including ER-α and ER-β) in DSA were up regulated ($P < 0.05$) by soy isoflavones supplemented diets (especially MSI and HSI) both in mRNA and protein levels, while in ASA and PRA, the gene and protein expression of ERs were down regulated ($P < 0.05$) by soy isoflavones supplemented diets (especially MSI and HSI) (Fig. 2); The gene and protein expression of LPL in DSA were decreased ($P < 0.05$) by soy isoflavones supplemented diets, but in ASA and PRA, the LPL was up regulated ($P < 0.05$) by MSI and HSI both in mRNA and protein levels (Fig. 2).

4. Discussion

The current study showed that soy isoflavones at the tested doses had no effects on the body weight and body fat rate. The pigs fed soy isoflavones supplemented diets possess less percentage of DSA while more percentage of ASA and PRA compared with the pigs

Table 1
The effects of soy isoflavones on growth performance and fat deposition of female Bama mini-pig.

	Con	LSI	MSI	HSI
Initial body weight (kg)	4.03 ± 0.18	3.77 ± 0.22	3.83 ± 0.14	4.05 ± 0.13
Final body weight (kg)	20.06 ± 0.43	19.85 ± 0.25	20.33 ± 0.44	20.00 ± 0.32
Weight gain (kg)	16.03 ± 0.38	16.07 ± 0.14	16.50 ± 0.51	15.95 ± 0.22
Body fat mass rate (%)[a]	16.92 ± 0.58	16.71 ± 0.69	17.16 ± 1.08	16.96 ± 1.07
Percentage of DSA (%)[b]	8.99 ± 0.49[A]	8.83 ± 0.50[A]	6.95 ± 0.44[B]	6.87 ± 0.37[B]
Percentage of ASA (%)[c]	6.24 ± 0.28[C]	7.07 ± 0.29[B]	7.82 ± 0.25[A]	7.49 ± 0.31[AB]
Percentage of PRA (%)[d]	2.13 ± 0.09[B]	2.16 ± 0.10[B]	3.05 ± 0.22[A]	3.14 ± 0.22[A]

Data are expressed as means ± SEM, n = 6.
Values in a row with different uppercase superscript alphabets differ ($P < 0.05$).
[a] Total body fat mass: carcass weight.
[b] Dorsal subcutaneous adipose (DSA) weight: carcass weight.
[c] Abdominal subcutaneous adipose (ASA) weight: carcass weight.
[d] Perirenal adipose (PRA) weight: carcass weight.

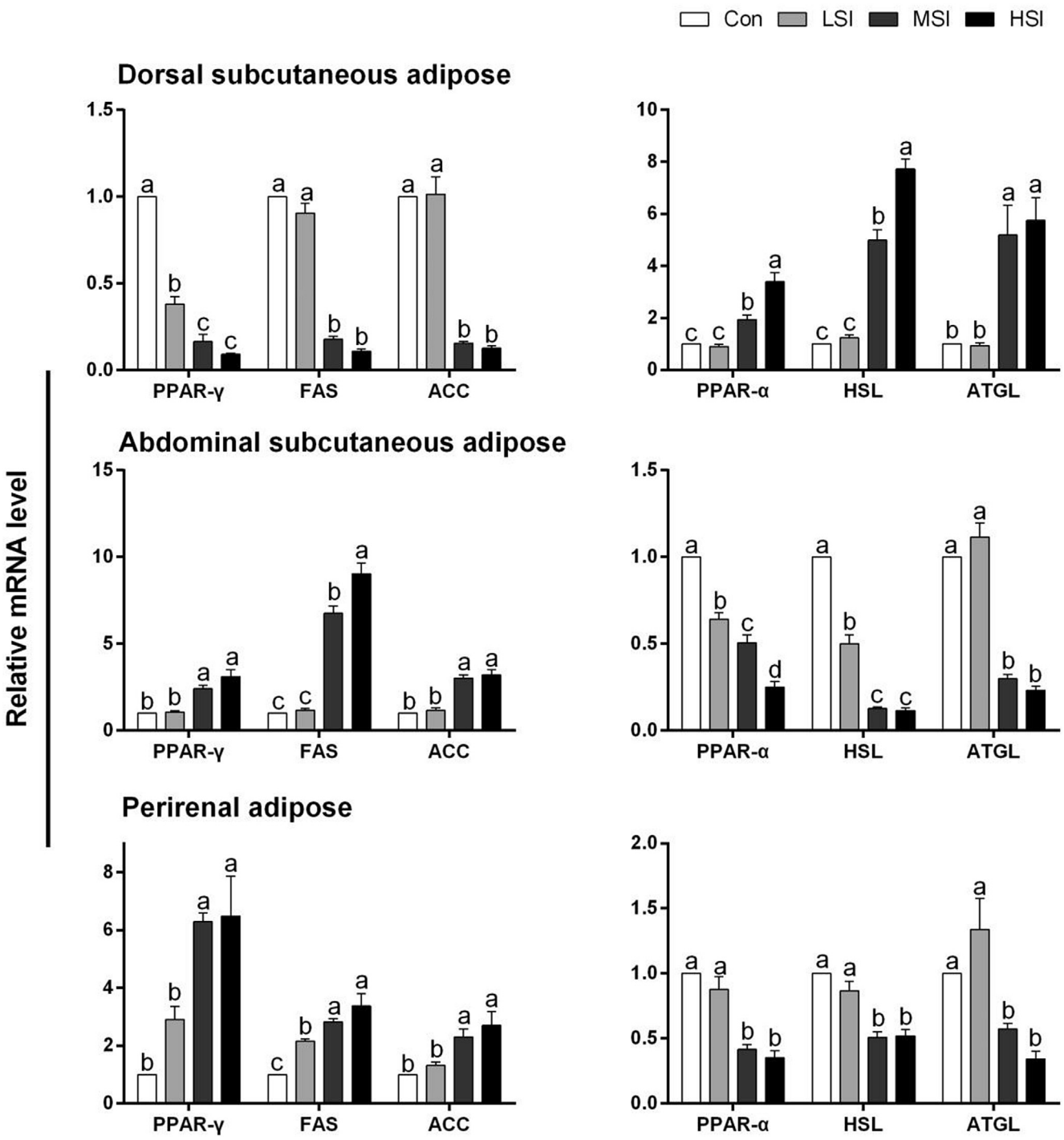

Fig. 1. The effects of soy isoflavones on expression of genes related to lipid anabolism and catabolism in different adipose tissues of female Bama mini-pig. The mRNA expression levels of target genes were normalized using β-actin. Data are expressed as mean ± SEM, n = 6. Values with different superscript differ ($P < 0.05$).

fed control diet. We also showed that soy isoflavones decreased the expression of genes (PPAR-γ, FAS and ACC) involved in lipogenic metabolism and increased the expression of genes (PPAR-α, HSL and ATGL) involved in lipolytic metabolism in DSA, while the opposite effects of soy isoflavones on these gene expression were observed in ASA and PRA. In addition, the gene and protein expression of LPL were down regulated in DSA while up regulated in ASA and PRA by soy isoflavones. Moreover, the gene and protein expression of ERs were up regulated in DSA while down regulated in ASA and PRA by soy isoflavones. These results suggest that soy isoflavones affect the lipid metabolism in white adipose tissues of female Bama mini-pigs in a site-specific manner, and the soy isoflavones most likely modulate the fat synthesis by regulating the expression of ERs in different white adipose tissues.

The effects of feeding soy isoflavones on body weight reported previously in different species and studies are inconsistent. For example, Ju, Y. H. et al. showed that dietary genistein, one of the major soy isoflavones, at 1000 mg/kg diet did not affect the body weight of athymic nude mice [17]. Davis, J et al. reported that dietary isoflavones significantly decreased the body weight of the

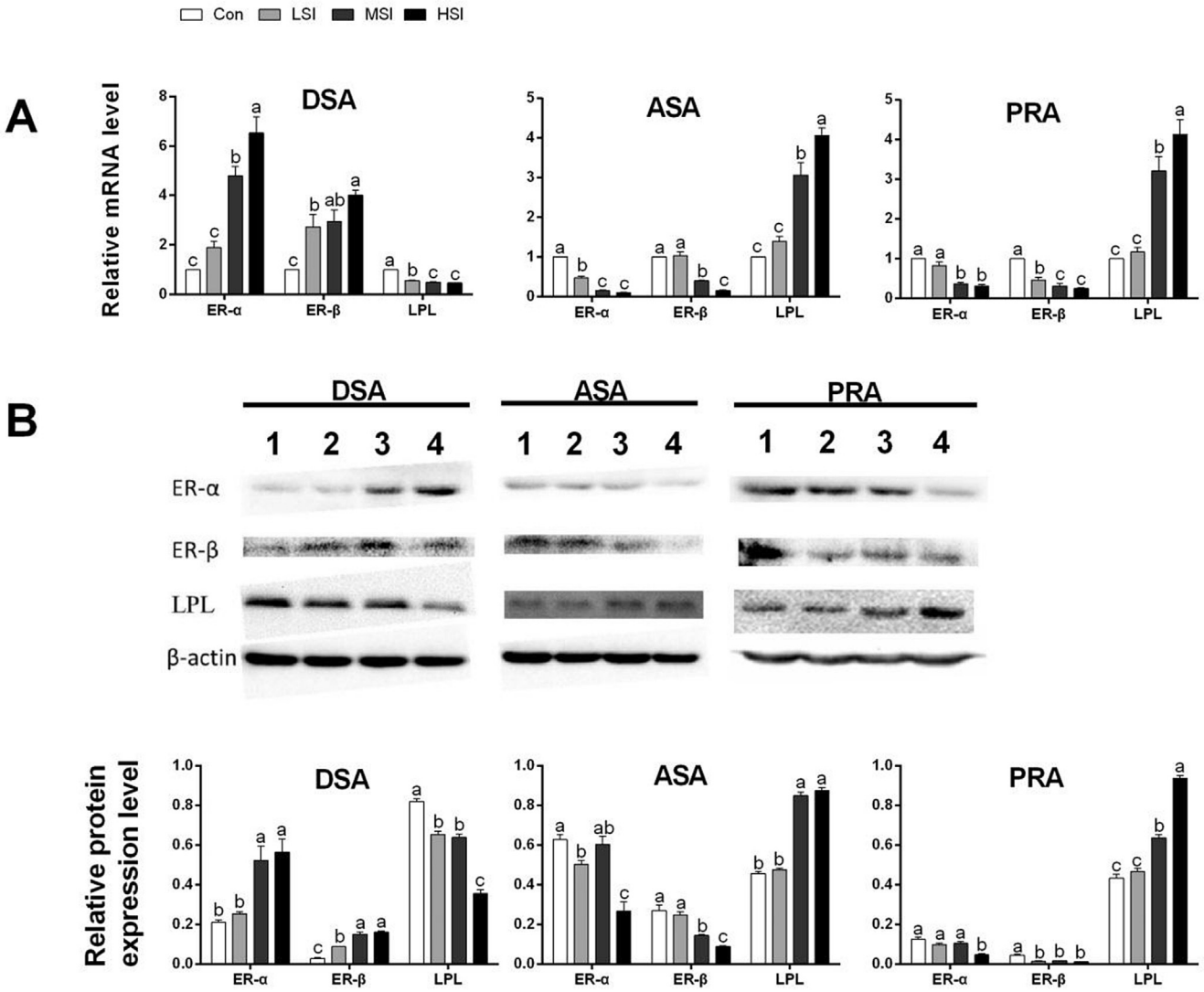

Fig. 2. Soy isoflavones regulate the gene (A) and protein (B) expression of ERs and LPL in different adipose tissues of female Bama mini-pig. The mRNA expression levels of target genes were normalized using β-actin. The protein expression levels of ERs and LPL were normalized using β -actin. DSA: dorsal subcutaneous adipose, ASA: abdominal subcutaneous adipose, PRA: perirenal adipose. [1]Con, [2]LSI, [3]MSI, [4]HSI. Data are expressed as mean ± SEM, n = 6. Values with different superscript differ ($P < 0.05$).

obese male rat [18]. Our previous study found that diets supplemented with soy isoflavones increased the body weight in male pigs [8]. The result of present study showed that soy isoflavones had no effect on the body weight of female Bama mini-pig. This discrepancy may be the results of sex or species-dependent differences. Michael et al. reported that isoflavones significantly reduced the rat adipose tissue mass [19]. Our results showed that soy isoflavones did not affect the body fat mass rate, but reduced the percentage of DSA and increased the percentage of ASA and PRA. Maybe the number of decreased mass of DSA just equal to the increased mass of ASA and PRA, thus the soy isoflavones seems exert no effect on the body fat mass rate.

The mass of adipose tissues are regulated by the balance of lipogenesis and lipolysis. Adipogenesis is regulated by the hormonally induced cooperative interaction between members of the CCAAT/enhancer binding protein (C/EBP) and peroxisome proliferator-activated receptor (PPAR) families. PPAR family which includes PPAR-α and PPAR-γ are the primary adipogenic transcription factors [1]. PPAR-α controls the transcription of the genes involved in lipid catabolism, whereas PPAR-γ controls the expression of genes involved in adipocyte differentiation and insulin sensation [1]. It has been shown that the major soy isoflavones, genistein and daidzein, could activate both PPAR-α and PPAR-γ in vitro [20–22]. FAS and ACC, down-stream target genes of PPAR-γ, play great role in lipogenic metabolism [8,23,24]. HSL and ATGL are two down-stream target genes of PPAR-α [8]. ATGL regulating the initial step in TG catabolism is an important component of the lipolytic process and the mobilization of lipid stores in mammals. HSL is a key enzyme responsible for the conversion of digylceride (DG) into monoglyceride (MG) and catabolizes adipose TGs [25]. Our results showed that dietary soy isoflavones suppressed the expression of lipogenic genes (PPAR-γ, FAS and ACC) and increased the expression of lipolytic genes (PPAR-α, HSL and ATGL) in DSA, while increased the expression of lipogenic genes and inhibited the expression of lipolytic genes in ASA and PRA. This may suggest that the lowered DSA in higher levels of soy isoflavones fed pigs could be a consequence of enhanced lipolysis and suppressed lipogenesis, whereas the elevated lipogenesis and decreased lipolysis in ASA and PRA by isoflavones may contribute to the increased ASA and PRA contents. The physiological significance of the differential regulation in the lipid metabolism by soy isoflavones in different white adipose tissues remains to be investigated.

As mentioned in introduction, binding of E2 to ERs inhibits the activity of LPL, So, the expressions of ERs maybe affect the lipid metabolism strongly in adipose tissues. Genistein has been shown to decrease the mRNA level of LPL in adipose tissue and the lipid filling of adipocytes concomitantly in mice and human primary bone marrow stromal cells [7,26]. The result in the present study showed that the expressions of ERs were up regulated in DSA, and down regulated in ASA and PRA by soy isoflavones. The expression of LPL was also regulated by soy isoflavones in different white adipose tissues. These results support our hypothesis that soy isoflavones modulate the lipid metabolism in white adipose tissues through regulating the expressions of ERs. Moreover, the result indicated that the soy isoflavones also affect the expressions of ERs in a site-specific manner.

Conflict of interest

There is no conflict of interest.

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Transparency document

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