

Breed-specific patterns of hepatic gluconeogenesis and glucocorticoid action in pigs

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Abstract

In the present study, Erhualian and Pietrain pigs were employed to investigate the breed-specific patterns of hepatic gluconeogenesis by detecting the related enzyme mRNA expression, and to analyse the relationship with the hepatic glucocorticoid receptors and 11 β -hydroxysteroid dehydrogenase type 1 (11 β -HSD1) expression. Furthermore, hepatic DNA methyltransferase 1 (DNMT1) expression was determined to detect DNA methyltransferase state difference between the two breeds. The results demonstrated that the Erhualian pigs exhibited significantly lower plasma lactate acid concentration, but higher liver lactate acid content than the Pietrain pigs. A significantly higher expression of pyruvate carboxylase (PC), fructose-1, 6-bisphosphatase (FBP) and the mitochondria phosphoenolpyruvate carboxykinase (PCK2) mRNA were observed in Erhualian pigs. The Erhualian pigs demonstrated a significantly higher expression of glucocorticoid receptor and 11 β -HSD1 mRNA than the Pietrain pigs, though there was no difference in the hepatic cortisol content between the two breeds. The hepatic DNMT1 mRNA expression in the Erhualian pigs was significantly lower, and the DNMT1 protein content in Erhualian pigs tended to be decreased compared with Pietrain pigs ($P=0.066$). The results suggest that the Erhualian pigs demonstrated higher hepatic gluconeogenesis capacity in comparison to the Pietrain pigs. The up-regulation of hepatic glucocorticoid receptor and 11 β -HSD1 expression may be involved in the enhanced hepatic gluconeogenesis in Erhualian pigs. Moreover, the down-regulation of DNMT1 in Erhualian pigs implies possible involvement of DNA methylation in a breed-specific pattern of hepatic gluconeogenesis.

Keywords: pigs, gluconeogenesis, liver, glucocorticoid receptor, DNA methyltransferases 1

Introduction

Gluconeogenesis, a key metabolic process, involves the formation of glucose and glycogen from non-glucose precursors. Gluconeogenesis provides the glucose required during prolonged fasting and stress, and the process primarily occurs in the liver. Most studies on gluconeogenesis in pigs focused on the newborn piglets (Robinson *et al.* 1981, Pégrier *et al.* 1984, Lepine *et al.* 1993). In fact during pig growth and slaughter process, pigs are exposed to considerable stress. One of stress consequences is the increasing blood lactate acid resulting

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in detrimental effects on skeletal muscle (Von Borell 2000, Manteuffel *et al.* 2002, Hambrecht *et al.* 2004). While gluconeogenesis provides a solution to reduce the blood lactate acid concentration.

Glucocorticoids are essential for diverse physiological functions, including cell differentiation, carbohydrate, protein and lipid metabolism, and stress responses. *In vitro* studies demonstrated that dexamethasone significantly elevated the rates of hepatic gluconeogenesis (Allan *et al.* 1984). Additionally, the absence of glucocorticoid receptors in hepatocytes limits the development of hyperglycaemia in mice with streptozotocin-induced diabetes mellitus, which is likely due to impaired induction of gluconeogenesis (Opherke *et al.* 2004).

Erhualian pigs are a typical indigenous Chinese breed, belonging to the same family as the Meishan pigs, which are characterised by high fertility and stress resistance (Li *et al.* 2008a). In contrast, Pietrain pigs are known for their fast lean mass deposition, but higher stress sensitivity. Furthermore the big difference of plasma cortisol content was confirmed in previous studies between the two breeds (Désautés *et al.* 1999, Li *et al.* 2008b). The question arises whether a big difference in the gluconeogenesis capacity exists between the two breeds and whether the different gluconeogenesis capacity is related to the basal plasma glucocorticoid level.

Recent studies have shown that gene expression is largely regulated by epigenetic modifications of DNA and histones in chromatin. Among these epigenetic modifications, DNA methylation plays an important role in transcriptional regulation, and it is essential for the development of mammals (Jones & Takai 2001). In general, DNA methylation is associated with gene silencing, and it is controlled by the DNA methyltransferase (DNMT) family (Szyf 2005).

Therefore, in the present study Erhualian and Pietrain pigs were employed to investigate the breed specific gluconeogenesis pattern by detecting the related enzymes mRNA expression in liver, and to analyse the relationship with the hepatic glucocorticoid receptor and 11 β -hydroxysteroid dehydrogenase type 1 (11 β -HSD1) expression. Additionally, liver DNMT1 expression was measured to show the difference of the DNA methylation state between the two breeds.

Materials and methods

Animals and sample collection

Castrated male Erhualian and Pietrain (n=6) piglets, weighing 20 kg in average, were randomly selected from a pig breeding farm in Wuxi, Jiangsu Province, China. The Erhualian pigs were 50-60 days of age and the Pietrain pigs were 30-40 days of age. The piglets were fed twice daily at 06.00 and 17.30 and were allowed free access to water. The piglets were sacrificed via an intravenous injection with an overdose of 3 % sodium pentobarbitone. Blood samples were collected to heparinized tubes and immediately placed on ice. Plasma was separated by centrifugation at 1 300 \times g at 4 °C for 15 min. Plasma samples were stored at -20 °C until assay. Small portions of liver were excised, immediately frozen in liquid N₂ and then stored at -80 °C until further analysis. The animal handling and sampling procedures were performed following the regional Animal Ethics Committee guidelines.

Lactic acid and glucose content

Lactic acid concentrations were measured using a commercial lactic acid kit (Nanjing Jiancheng Bioengineering Institute, Nanjing, China) and a UV-visible spectrophotometer Type-752 (Shanghai Jinghua Technology Instrument Ltd. Co., Shanghai, China) at 530 nm according to the manufacturer's instructions. Glucose content was measured using a commercial glucose kit (Nanjing Jiancheng Bioengineering Institute, Nanjing, China) according to the manufacturer's instructions.

Frozen liver sample (100 mg) was homogenized in 1 mL of cold HClO_4 (0.5 mmol/L). The homogenates were centrifuged at $15\,000\times g$ for 20 min to obtain clear lysates after 10 min standing. The 3 mmol/L K_2CO_3 was added into the supernatant equal mol to HClO_4 . After keeping it for 2 h in 0°C , it was centrifuged at $15\,000\times g$ for 10 min to obtain the supernatant. Commercial Bradford assay kit (Nanjing Jiancheng Bioengineer Institute, Nanjing, China) was used to determine the total protein concentration. Lactic acid and glucose content were determined per mg total protein.

Radioimmunoassay for cortisol content

Cortisol concentrations were measured using commercial multispecies radioimmunoassay (RIA) kits purchased from the Beijing North Institute of Biotechnology (Beijing, China) according to the manufacturer's instructions. The RIA detection limit was 2 ng/mL. The intra- and interassay coefficients of variations were 7.6 % and 8.7 %, respectively.

RNA extraction and mRNA quantification

Total RNA was extracted from homogenized liver tissues using a TRIzol Total RNA Kit (Invitrogen Life Technologies, Carlsbad, CA, USA) and was subsequently purified with the RNase-Free DNase Set (Promega, Fitchburg, WI, USA), according to the manufacturer's instructions. The total RNA concentration was then quantified by measuring the absorbance at 260 nm with a photometer (Eppendorf AG, Hamburg, Germany). The 260/280 nm absorption ratios of all the preparations were between 1.9 and 2.1. Two μg of total RNA were reversely transcribed in a final volume of 25 μL with M-MLV reverse transcriptase (Promega, Fitchburg, WI, USA) and random hexamer primers (SunShine, China) in a Thermal Cycler PTC0200 (Bio-Rad, Hercules, CA, USA).

Real-time PCR was performed in a Mx3000P machine (Agilent Technologies, Inc., Santa Clara, CA, USA) with specific primers. All of the primers were designed and synthesized by Takara Biotechnology (China). All samples were normalized with the housekeeping gene 18S rRNA. The sequences of the PCR primers are shown in Table 1. Different controls were used to monitor genomic and environmental DNA contamination during reverse transcription (RT) and PCR. Samples were pooled by mixing equal quantities of cDNA from all samples to optimize the PCR conditions and generate standard curves for each target gene. The specificities of the reactions were checked using melting curve analyses for each gene.

The $2^{-\Delta\Delta\text{Ct}}$ method (Livak & Schmittgen 2001) was used to analyse the real-time PCR data. The average value of the Pietrain pigs was set as 1.

Table 1
Primer sequences of the target genes

Target genes	GenBank accession	PCR products (bp)	Primer sequences
PC	AY225510	130	F: 5'-CCG CAA GAT GGG AGA CA-3' R: 5'-GAA GCC GTA GGT GTT GGA3-3'
PCK1	AY855075	123	F: 5'-CGG GAT TTC GTG GAG A-3' R: 5'-CCT CTT GAT GAC ACC CTC T-3'
PCK2	AY855076	323	F: 5'-CAT TCA GCA TGG GTC C-3' R: 5'-GAT GCG AAG GGC AAA G-3'
FBP	NM_213979	106	F: 5'-ACC CTA ACC CGC TTC GTC-3' R: 5'-CGG TGG AGA TGG CTT TGA-3'
G6P	NM_213979	264	F: 5'-CAA TCA GTG CCA AGT C-3' R: 5'-GCT ACC CAG AGG AGT T-3'
GR	NM_001008481	382	F: 5'-ACG CTA AGT TGT TTA TCT CG-3' R: 5'-CCC ATC ACT TTA GTT TCG-3'
11 β -HSD1	AY225510	115	F: 5'-CCA TGC TGA AGC AGA GCA AC-3' R: 5'-AAG AAC CCG TCC AGA GCA AA-3'
DNMT1	DQ060156	133	F: 5'-GCG GGA CCT ACC AAA CAT-3' R: 5'-TTC CAC GCA GGA GCA GAC-3'
18S	AY265350	122	F: 5'-CCC ACG GAA TCG AGA AAG AG-3' R: 5'-TTG ACG GAA GGG CAC CA-3'

GR: glucocorticoid receptor

Determination of protein expression

Frozen liver sample (100 mg) was homogenized in 1 mL of cold homogenization buffer (500 mM Tris-HCl pH 6.8, 10 % [v/v] glycerol, 10 % [w/v] sodium dodecyl sulfate [SDS], 10 % [v/v] 2-mercaptoethanol). The homogenates were centrifuged at 5000×g for 20 min to obtain clear lysates. Protein concentrations were measured using Bradford assays. Protein extract (30 µg) was mixed with loading buffer and denatured by boiling for 5 min before being loaded on a 12 % SDS-PAGE gel. Following electrophoresis, proteins were transferred to a nitrocellulose membrane and then blocked with a solution of 5 % fat-free milk in Tween-Tris-buffer saline (TTBS) for 2 h at room temperature. After repeated washing with TTBS, the membrane was incubated with a monoclonal antibody to DNMT1 (Imgenex Corp., San Diego, CA, USA; dilution 1:2000) and a monoclonal antibody to β -actin (Abcam, Cambridge, UK; dilution 1:2000) for 2 h at room temperature. Then the membrane was washed five times (15 min for the first wash and 5 min wash thereafter) to remove unbound antibodies. Anti-rabbit IgG conjugated with horseradish peroxidase (Jingmei Biological Engineering Company, Wuhan, China; 1:5000 dilution) was used as the secondary antibody. Finally, the membrane was washed five times (as indicated above) and the specific signals were detected by chemiluminescence using the LumiGlo substrate (Pierce Super Signal West Pico Trial Kit, Thermo Fisher Scientific Inc., Waltham, MA, USA). Enhanced chemiluminescence signals were recorded on X-ray film. The enhanced chemiluminescence signals were scanned and analysed with a Kodak 1D Electrophoresis Documentation and Analysis System 120 (Kodak Photo Film Co. Ltd., Rochester, NY, USA).

Statistic analysis

All data are presented as the mean \pm SEM. Statistical differences were tested using t-test for independent samples with SPSS13.0 for Windows (SPSS Inc., Chicago, IL, USA). The mRNA and protein levels were expressed as the fold change relative to the mean value of the Pietrain pigs. A *P*-value<0.05 was considered significant.

Results

Differences of lactate acid and glucose levels in plasma and liver between the Erhualian and Pietrain pigs

As shown in Table 2, the Erhualian pigs exhibited significantly lower plasma lactate acid concentration but higher hepatic lactate acid content than the Pietrain pigs (*P*<0.05). The glucose content in plasma and liver were not significantly different between the two pig breeds.

Table 2
Lactate acid contents in plasma and liver of Erhualian and Pietrain pigs

Indices	Erhualian	Pietrain
Plasma glucose, mmol/L	4.16 \pm 0.10	4.33 \pm 0.12
Hepatic glucose, mmol/g protein	27.72 \pm 3.66	32.51 \pm 3.80
Plasma lactate acid, mmol/L	1.52 \pm 0.07*	1.81 \pm 0.06
Hepatic lactate acid, mmol/g protein	0.45 \pm 0.02**	0.39 \pm 0.01

P*<0.05, *P*<0.01, represent significant differences between the two breeds

Differences of hepatic gluconeogenesis key enzymes expression between the two breeds

The Erhualian pigs exhibited a significantly higher expression of pyruvate carboxylase (PC) mRNA (*P*<0.05) than the Pietrain pigs (Figure 1a). No difference was observed in cytosolic phosphoenolpyruvate carboxykinase (PCK1) mRNA expression, whereas mitochondrial phosphoenolpyruvate carboxykinase (PCK2) mRNA expression was higher in the Erhualian pigs (*P*<0.05) than in the Pietrain pigs (Figure 1b, 1c). Fructose-1, 6-bisphosphatase (FBP) mRNA was significantly up-regulated in Erhualian pigs (*P*<0.05) compared with the Pietrain pigs (Figure 1d). There was no difference in glucose-6-phosphatase (G6P) mRNA between the two pig breeds (Figure 1e).

Differences of cortisol content and the glucocorticoid receptor, 11 β -HSD1 expression between the two breeds

The cortisol concentrations in serum of Erhualian and Pietrain were 290.6 \pm 26.0 ng/ml and 156.1 \pm 30.1 ng/ml, and the cortisol contents in liver were 51.3 \pm 7.8 ng/ml and 47.1 \pm 4.6 ng/ml, respectively. There was a significant difference in serum cortisol content, while no difference was observed in hepatic cortisol concentration between the two breeds.

The Erhualian pigs demonstrated significantly higher hepatic expression of glucocorticoid receptor and 11 β -HSD1 mRNA (*P*<0.05) than the Pietrain pigs (Figure 2).

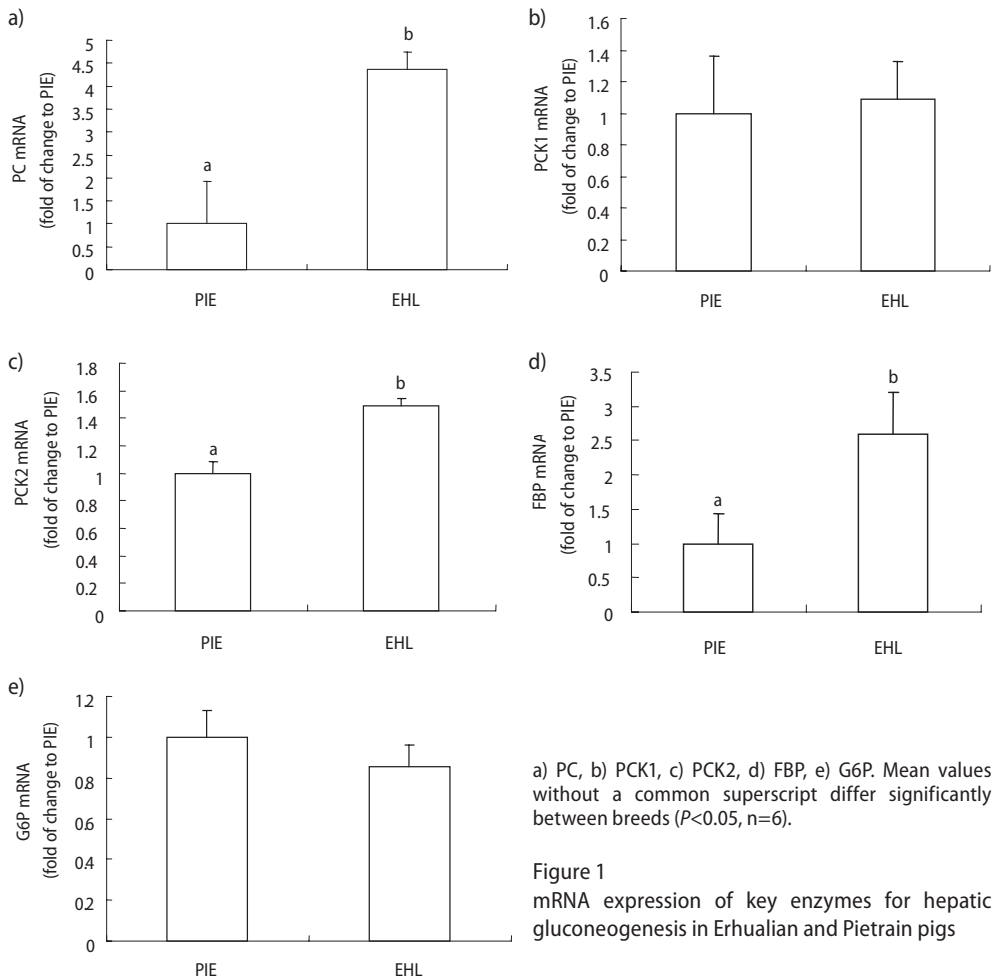
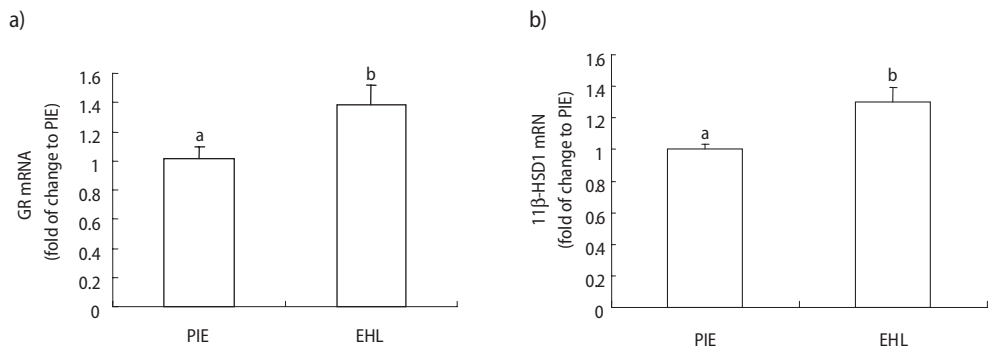


Figure 1
mRNA expression of key enzymes for hepatic gluconeogenesis in Erhualian and Pietrain pigs

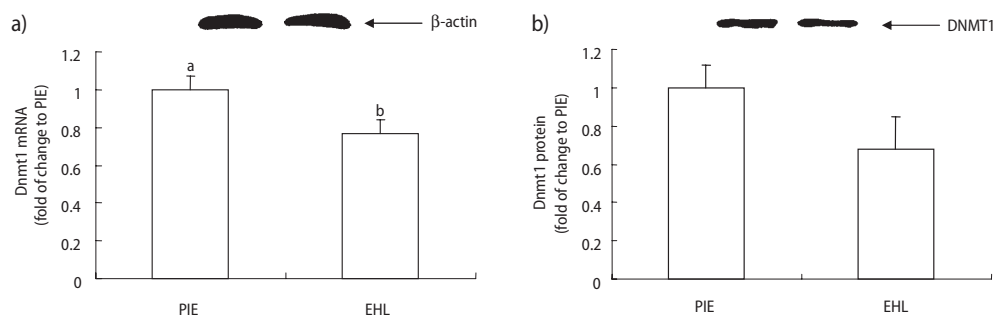


a) glucocorticoid receptor, b) 11β-HSD1. Mean values without a common superscript differ significantly between breeds ($P < 0.05$, $n = 6$).

Figure 2
Hepatic glucocorticoid receptor and 11β-HSD1 mRNA expression in Erhualian and Pietrain pigs

Differences of the hepatic DNMT1 expression between the two breeds

The hepatic DNMT1 mRNA expression (Figure 3a) in the Erhualian pigs was significantly lower than in the Pietrain pigs ($P < 0.05$). The hepatic DNMT1 protein content (Figure 3b) in the Erhualian pigs tended to be lower than in the Pietrain pigs ($P = 0.066$).



a) mRNA expression, b) top: protein expression, immunoreactive bands; bottom: statistical results. Mean values without a common superscript differ significantly between breeds ($P < 0.05$, $n = 6$).

Figure 3

Expression of liver DNMT1 mRNA and protein in Erhualian and Pietrain pigs.

Discussion

Most of earlier studies on the gluconeogenesis in pigs focused at the newborn stage (Robinson *et al.* 1981, Pégorier *et al.* 1984, Lepine *et al.* 1993), yet in the present study we first demonstrated the breed difference of hepatic gluconeogenesis between the pure Chinese pigs and Pietrain pigs after weaning. In previously studies it has been confirmed that there is different stress response between the two breeds (Désautés *et al.* 1999, Li *et al.* 2008a). The present study implied the different capacity of gluconeogenesis may be partly responsible for the distinct stress reaction.

The mutation of the type 1 ryanodine receptor gene was detected in the present study and the results showed that all the Pietrain pigs were homozygous halothane-positive (Hal^{nn}) pigs, whereas Erhualian pigs are homozygous halothane-negative (Hal^{NN}) pigs; and it has been confirmed that Hal^{nn} pigs showed a drastic reduction in the level of glycogen to cope with stress (Scholz *et al.* 2003). Then amount of lactate is generated in muscle tissue. One of important way to removal of excess lactate is by gluconeogenesis in the liver (Brooks 2009). The present study showed the lactate acid concentration in plasma and liver showed distinct pattern between two breeds. Plasma lactate acid concentration in the Erhualian pigs was significantly lower than in Pietrain pigs, whereas the reverse condition was observed for the hepatic lactate acid content. The higher plasma lactate acid is consistent with the characteristic of stress susceptible in Pietrain pigs. The different lactate acid metabolic pattern between the two breeds has never been reported before as far as we know. It indicates that Erhualian pigs have a stronger ability to transport the lactate acid to the liver for metabolism and to decrease the hurt of lactate acid to the body. The glucose content in plasma and liver showed no significant difference between the two breeds.

In the liver, gluconeogenesis can be controlled by a number of key enzymes including PC, PCK, FBP, and G6P (Pilkis & Claus 1991). In mammals, PC plays a crucial role in gluconeogenesis by catalysing the ATP-dependent carboxylation of pyruvate to oxaloacetate (Jitrapakdee & Wallace 1999). In the present study, the mRNA expression of PC in Erhualian pigs was significantly higher than in Pietrain pigs. The next rate-limiting step in gluconeogenesis is the conversion of oxaloacetate to phosphoenolpyruvate (PEP) by PCK, which is considered the major regulative step in gluconeogenesis (Hanson & Garber 1972). Previous research has demonstrated that there are cytosolic and mitochondrial forms of the PCK enzyme: PCK1 and PCK2 (Cornell *et al.* 1986). The intracellular distribution of these isoenzymes varies widely with species and organs (Suzuki *et al.* 2004). In pigs, it has been reported that starvation increased PCK1 activity without changing the mitochondrial activity (Hamada & Matsumoto 1984). Our results demonstrated that in PCK2 expression existed significant breed differences, while there were no significant differences in PCK1 expression. FBP also plays an important role in gluconeogenesis (Tashima *et al.* 1983), and the FBP mRNA expression in the Erhualian pigs was significantly higher than in the Pietrain pigs as expected. G6P was reported to contribute significantly to the glucose supply during long-term fasting (Mithieux 1996). However, in the present study, there was no significant difference in G6P expression between the two breeds. Taken together, the higher expression of PC, FBP, and PCK2 genes may contribute to the higher hepatic gluconeogenesis capacity in the Erhualian pigs in comparison to the Pietrain pigs. Therefore, the higher capacity to utilize blood lactate acid for gluconeogenesis may be one reason for higher stress resistance of Erhualian than Pietrain pigs.

In previous studies, dexamethasone treatment was reported to affect gluconeogenesis directly by increasing PC, PCK and FBP mRNA expression (Allan *et al.* 1984, Tashima *et al.* 1984, Hammon *et al.* 2005). Although there was large difference in the blood cortisol levels between the two breeds, there was no significant difference in the hepatic cortisol concentration in the present study. The glucocorticoid receptor mediates glucocorticoid action (Payne & Adcock 2001). Opherke (2004) reported that glucocorticoid receptor play the essential role in liver glucose metabolism for the mice showed hypoglycaemia after prolonged starvation due to reduced expression of genes involved in gluconeogenesis. In the present study glucocorticoid receptor mRNA expression was shown to be significantly higher in Erhualian pigs than in Pietrain pigs. We selected PC as an example to analyse the relationship with glucocorticoid receptor, and the data showed that glucocorticoid receptor mRNA expression was correlated with the PC mRNA expression ($r=0.57, P<0.05$). 11 β -HSD1, which converts inert 11-dehydrocortisone to active cortisol, influences intracellular glucocorticoid concentrations (Altuna *et al.* 2006). It has been reported that 11 β -HSD1 knockout mice showed attenuated activation of the key hepatic gluconeogenesis enzymes (Kotelevtsev *et al.* 1997). In the present study, 11 β -HSD1 mRNA expression was significantly higher in Erhualian pigs than in Pietrain pigs. It can be inferred that it is hepatic glucocorticoid receptor and 11 β -HSD1, but not hepatic cortisol content, mediated the breed difference of glucocorticoid action in the liver.

DNA methylation is supposed to play a crucial role in repressing gene expression possibly by blocking the promoters at which activating transcription factors bind (Wang *et al.* 2007, Collas 2009). DNMTs catalyse the addition of methyl groups, which are derived from the methyl donor S-adenosyl methionine, onto DNA strands (Rice *et al.* 2007). Based on structural

differences in their regulatory N-terminal domains, three distinct families of DNMTs have been classified, namely DNMT1, DNMT2 and DNMT3 (Okano *et al.* 1998, Snykers *et al.* 2009). Among them, DNMT1 is the major DNA methyltransferase in the liver (Saito *et al.* 2003).

Both *in vivo* and *in vitro* studies show that maternal behaviour increases glucocorticoid receptor expression in offspring by increasing DNA demethylation (Weaver 2007). In maternal protein-restriction experiments, glucocorticoid receptor gene methylation was 22.8 % lower ($P < 0.05$) in restricted protein pups (Lillicrop *et al.* 2005) and DNMT1 expression was 17 % lower ($P < 0.05$) than in control pups (Lillicrop *et al.* 2007). In the present study, the DNMT1 mRNA expression in liver of Erhualian pigs was significantly lower than in Pietrain pigs. Furthermore, the DNMT1 protein content in Erhualian pigs tended to be lower in Pietrain pigs. These data indicate possible involvement of DNA methylation in the breed-specific pattern of hepatic gluconeogenesis. Clearly, further studies are necessary to clarify the specific genes and methylation status, and give the new insights into the underlying mechanisms of the breed gluconeogenesis difference.

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