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The protein level of isoenergetic formulae does not modulate postprandial insulin secretion in piglets and has no consequences on later glucose tolerance

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Abstract

Early postnatal nutrition is involved in metabolic programming, an excess of protein being suspected to enhance early growth and the propensity to later develop insulin resistance and type 2 diabetes mellitus. The aim of the present study was to test the hypothesis that excessive protein intake during the suckling period would overstimulate the endocrine pancreas in the short term and alter durably its maturation, contributing to the later disruption of glucose homeostasis. Normal-birth-weight and low-birth-weight piglets were fed isoenergetic formulae providing an adequate-protein (AP, equivalent to sow milk) or a high-protein (HP, + 48 %) supply between 7 and 28 d of age and were fed a standard diet until 70 d of age. During the formula-feeding period, the HP formula did not modify postprandial insulin secretion but transiently increased fasting insulin and the homeostasis model assessment-insulin resistance index (HOMA-IR, $P<0{\cdot}05$). Fasting insulin and HOMA-IR were restored to AP piglets' values 1 month after weaning. The structure of the endocrine pancreas was not affected by the protein content of the formula. The weight at birth had no major effect on the studied parameters. We concluded that a high-protein supply during the suckling period does not interfere with insulin secretion and endocrine pancreas maturation in the short term. It has no consequences either on glucose tolerance 1 month after weaning. The present study demonstrated that up-regulation of postprandial insulin secretion is not involved in higher growth observed in piglets fed a HP formula.

Key words: Endocrine pancreas: Intestinal insulinotropic hormones: Nutritional programming: Pigs

Observational studies have highlighted the protective effect of breast-feeding compared with formula feeding against childhood obesity[1–4]. The underlying mechanisms are not elucidated yet and are certainly multiple, including differences in nutrient and bioactive factor content and in ingested quantities between breast milk and formulae. Formulae usually contain more protein than human milk to counteract the difference in essential amino acid profile of cow *v.* human milk proteins, resulting in a higher protein:energy ratio[5]. Formula-fed babies grow faster from 3 to 9 months[6,7] and are fatter at 1 year of age[8] than breast-fed babies. Some authors have proposed that the greater weight gain in formula-fed infants is caused at least in part by the higher intake of metabolisable protein[9]. They hypothesised that protein intake, in excess of metabolic requirements, may enhance the secretion of insulin and insulin-like growth factor I (IGF-I), and consequently growth. This faster neonatal growth could predispose to later insulin resistance, as suggested in rodents[10] and human subjects[11]. The early growth of low-birth-weight (LBW) babies is promoted by special formulae with an even greater protein: energy ratio, possibly enhancing later metabolic drawback.

Proteins are known to stimulate insulin secretion but the extent of this stimulation differs between food proteins: milk and cheese meals display higher postprandial insulin secretion than a gluten meal, for instance[12]. Individual amino acids can

Abbreviations: AP, adequate-protein; AUC, area under the curve; CCK, cholecystokinin; EHC, euglycaemic–hyperinsulinaemic clamp; G6Pase, glucose-6-phosphatase; GIP, glucose-dependent insulinotropic peptide; GLP-1, glucagon-like polypeptide-1; HOMA-IR, homeostasis model assessment-insulin resistance; HP, high-protein; IVGTT, intravenous glucose tolerance test; LBW, low-birth-weight; NBW, normal-birth-weight; pAT, perirenal adipose tissue; PEPCK, phosphoenol pyruvate carboxykinase; scAT, subcutaneous adipose tissue.

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also enhance insulin secretion, such as the branched amino acids leucine, valine and isoleucine[13,14] or arginine[14,15]. The insulinotropic properties of protein might be related to their propensity to rapidly release such amino acids. Proteins and amino acids also stimulate the secretion of the insulinotropic hormones cholecystokinin (CCK), glucose-dependent insulinotropic peptide (GIP) and glucagon-like polypeptide-1 (GLP-1) from the enteroendocrine cells of the gut[16,17]. Furthermore, some amino acids such as leucine and taurine are known to up-regulate β-cell growth and proliferation, and to decrease apoptosis[18,19].

The impact of a high-protein (HP) diet on insulin sensitivity is more controversial. Long-term high dietary protein intake induced insulin resistance in human subjects[20] but a HP diet along with fruits and vegetables conferred higher insulin sensitivity than a low-protein, cereal-based diet in domestic pigs[21]. The nature of the proteins and associated macronutrients is of importance since high intake of milk but not meat increased insulin resistance in 8-year-old boys[22], but a high-whey protein diet increased insulin sensitivity compared with a high-red meat protein diet in rats[23].

Changes in the postprandial stimulation of insulin secretion under a HP formula as well as a higher supply of leucine and taurine may affect the maturation of the endocrine pancreas still going on during the suckling period[24] and lead to metabolic disorders.

Testing this hypothesis requires numerous blood and tissue samplings, which are not accessible in human infants for ethical reasons, and necessitates the use of an animal model. The piglet provides an interesting model since it allows artificial rearing, enabling modulation and control of food intake during the neonatal period. Furthermore, the naturally occurring LBW piglets among littermates in normally fed sows display the same metabolic disorders as LBW babies later in life[25,26].

The present study was therefore designed to analyse the endocrine pancreatic function (glycaemia and insulinaemia) and structure, and gut insulinotropic hormone secretion in normal-birth-weight (NBW) and LBW piglets fed formulae differing in protein:energy ratio. Glucose tolerance was further investigated 1 month after weaning. Expression of genes related to insulin sensitivity was also examined in liver, skeletal muscle and adipose tissue.

Materials and methods

Animals, diets and experimental design

The experiment was conducted in accordance with the guidelines of the French Agriculture and Fishing Ministry for use and care of animals in research (authorisation to experiment on living animals no. 3562). It was part of a larger study whose results on growth and the somatotropic axis have already been published[27,28]. Piglets from thirty litters were born in the experimental herd of INRA (St-Gilles, France). All piglets were weighed at birth. Litters were selected to be within the mean litter size and weight of the INRA herd[29]. Pairs of piglets with similar birth-weight range and sex were defined within litters. Piglets with a weight near the mean weight of the litter were defined as NBW (1·37 (SE 0·02) kg, n 52) and those with a 30 % lower weight were defined as LBW (0·99 (SE 0·02) kg, n 52)[27]. The range of birth weights was 1·28–1·56 kg in the NBW group and 0·76–1·1 kg in the LBW group. Depending on availability, one or two pairs of piglets were selected within litters. There was most often one pair of LBW piglets and one pair of NBW piglets selected per litter. Additional piglets remained with the sow to equalise the litters to eight piglets, so the access to sow milk was identical between litters, and those other piglets were chosen to keep as constant as possible the mean birth weight of the litter. Therefore, all the piglets selected in pairs of LBW and NBW piglets were suckling their own dam. Piglets were allowed to suckle the dam until 7 d of age, a stage at which piglets are similar to newborn babies regarding their fat mass and digestive physiological maturation[30–32]. At this stage, four pairs of NBW and LBW piglets (n 8 per group) were slaughtered as initial controls. Within the remaining pairs, piglets were randomly assigned to one of the two dietary groups. They were separated from their dam and individually housed in stainless-steel cages in a temperature-controlled room (30 ± 0·5°C) up to 28 d of age. They were fed milk replacers formulated to provide an adequate-protein (AP) or a HP supply with an automatic formula feeder as described previously[27,28]. The AP diet was formulated to match the protein, amino acid, fat and carbohydrate composition as well as the ratio of casein:soluble whey protein (46:54) of sow's milk (Table 1)[33]. The HP diet was formulated to provide 48 % more protein per unit of net energy than the AP diet but the same proportion of each amino acid in proteins. The protein and amino acid supplements were incorporated in partial substitution for non-protein ingredients, keeping constant both the casein:soluble whey protein and the fat:carbohydrate ratios. A first group of animals (n 9 per dietary and birth-weight group) was used at 21 d of age to investigate postprandial insulin secretion and killed at 28 d to examine pancreas maturation and peripheral tissue insulin sensitivity. Power calculation was used to determine the adequate group size: for basal glucose, taking a significance level of 5 %, a power of 90 % ($\beta = 10$), a smallest worthwhile difference of 1 mmol/l (0·18 g/l) for a standard deviation of the variable of 0·6, the group size was eight[34]. A second subset of animals was used to investigate the subsequent effects of formula feeding. After weaning at 28 d of age, animals of both dietary groups (n 13 per dietary and birth-weight group) were fed *ad libitum* the same standard commercial diets until 70 d of age[35] (Table 2). At 48–54 d of age, an intravenous glucose tolerance test (IVGTT) and a euglycaemic–hyperinsulinaemic clamp (EHC) were performed. Pigs were then slaughtered at 70 d of age (Fig. 1). The group size was also determined by power calculation: for glucose rate of the EHC, taking a significance level of 5 %, a power of 90 % ($\beta = 10$), a smallest worthwhile difference of 8 mg/kg per min – i.e. 50 % augmentation of glucose rate – for a standard deviation of the variable of 5·4, the adequate group size was twelve[34].

Table 1. Composition of the adequate-protein (AP) and high-protein (HP) formulae

Diet	Sow milk	AP	HP
Ingredients (100 g powder)			
Whey protein	–	13·27	18·47
Casein	–	8·31	11·56
Skimmed milk powder	–	8·90	12·39
Lactose	–	17·47	11·50
Milk fat	–	14·07	11·60
Palm olein	–	17·85	15·27
Vegetable oil*	–	8·33	6·88
L-Arg	–	0·56	0·79
L-Cystine	–	0·09	0·12
L-Glu	–	0·85	1·18
Gly	–	0·43	0·60
L-His	–	0·13	0·18
L-Pro	–	1·33	1·85
L-Val	–	0·24	0·33
Vitamin mix†	–	0·30	0·30
Choline bitartrate	–	0·17	0·17
Calcium phosphate, dibasic	–	2·64	2·28
Calcium citrate, tribasic	–	0·80	1·02
Sodium citrate, tribasic	–	0·56	0·54
Potassium citrate, tribasic	–	0·27	0·03
Sodium chloride	–	0·15	0·08
Magnesium chloride	–	0·10	0·09
Mineral mix‡	–	0·30	0·30
Water	–	2·90	2·50
Composition			
Powder (g/l)	–	195·8	208·1
Protein (g/l)	50	50·5	74·7
Arg	–	2·43	3·58
Ile	–	2·39	3·54
Leu	–	5·27	7·76
Val	–	3·05	4·41
Lipid (g/l)	80	80	73
Lactose (g/l)	51	51	46
NE (MJ/l)	3·580	3·559	3·559
Protein:NE (g/100 kJ)	1·41	1·42	2·10

NE, net energy.
* Mix of maize, rapeseed and sunflower oils.
† Retinol 442·5 μg as retinyl acetate, cholecalciferol 10·5 μg, *all-racemic* α-tocopherol acetate 0·77 mg, phylloquinone 0·28 mg, ascorbic acid 75 mg, thiamin mononitrate 0·56 mg, nicotinamide 6 mg, riboflavin 1·10 mg, pyridoxine 1·10 mg, folic acid 0·21 mg, pantothenic acid 2·65 mg, cyanocobalamin 2·30 μg, biotin 15·00 μg, iodine 100 μg as potassium iodide.
‡ Fe 11·9 mg as ferrous sulphate, Cu 2·00 mg as copper sulphate, Zn 11·60 mg as zinc sulphate, Mn 2·99 mg as manganese sulphate, Se 20 μg as sodium selenate.

Meal test

At 14 d of age, a catheter was inserted, under general anaesthesia, into one external jugular vein of the animals to be slaughtered at 28 d (*n* 36). At 1 week after surgery, after an overnight fast, the meal test was performed: two basal samples were taken 30 and 15 min before the formula meal and blood samplings (0·8 ml) were performed at times 0 (beginning of the meal), 1, 5, 10, 15, 30, 45, 60, 90, 120 and 180 min. Blood was collected in tubes containing EDTA and aprotinin (0·5 IU/ml blood, Iniprol; Choay laboratories, Choay, France) except for GLP-1 for which aprotinin was replaced with a dipeptidylpeptidase-IV (DPP-IV) inhibitor (10 μl/ml blood, catalogue no. DPP4; Millipore, Billerica, MA, USA). After centrifugation, plasma samples were stored at −20°C for later analyses. Insulin, C-peptide and glucose assays were performed on all plasma samples. Intestinal insulinotropic hormone, GIP, GLP1 and CCK, assays were performed on five (at −30, 1, 30, 60 and 120 min; NBW piglets) or four (at −30, 1, 45 and 120 min; LBW piglets) blood samples due to scarce blood availability. The homeostasis model assessment-insulin resistance index (HOMA-IR) was calculated (basal glucose × basal insulin/22·5). Incremental area under the curve (AUC) was calculated for postprandial glucose, insulin and insulinotropic hormone concentrations. The insulinogenic index of β-cell function was calculated as the ratio of the increment in insulin concentration over the first 30 min of the meal test to the increment in glucose concentrations over the same time period ($\Delta I_{0-30}/\Delta G_{0-30}$). The Matsuda index, reflecting the overall insulin sensitivity, was calculated as described previously: Matsuda index $= 10\,000/\sqrt{[(gly_0 \times ins_0)(gly_{moy} \times ins_{moy})]}$[36]. The disposition index (insulin secretion/insulin resistance index) was calculated as the product of the insulinogenic index and the insulin sensitivity index obtained from the Matsuda index.

Intravenous glucose tolerance test and euglycaemic–hyperinsulinaemic clamp

At 42 d of age, fifty-two pigs had a catheter inserted into one external jugular vein and into one carotid artery under general anaesthesia. At 1 week later, the IVGTT and the EHC were performed at 5 d intervals after an overnight fast.

The IVGTT consisted of multiple blood sampling before ($t = -10$, 0 min) and after ($t = 2$, 3, 4, 5, 6, 8, 10, 12, 15, 18, 20, 25, 30, 35, 40, 60, 70, 80, 100, 120, 140, 160, 180 and 240 min) an intravenous injection of glucose (0·3 g/kg) to assay glucose and insulin concentrations. The incremental AUC was calculated over 240 and 60 min, which corresponded to the time at which concentrations returned to basal values. The insulinogenic indexes ($AUC_{insulin}/AUC_{glucose}$) was calculated at 240 and 60 min, as well as the acute insulin response (acute insulin response = mean insulin concentration above basal values for the first 5 min). The glucose and insulin responses were also integrated using the minimal model described by Bergman *et al.*[37] to obtain the insulin sensitivity index S_I as well as the glucose efficiency index S_G.

The EHC was performed over 120 min as described by DeFronzo *et al.*[38]. Briefly, insulin (Actrapid; Novo Nordisk, Bagsvaerd, Denmark) was injected in the venous port as a prime dose (20 mIU/kg) followed by a constant (2 mIU/kg per min) infusion to induce hyperinsulinaemia. Glucose

Table 2. Composition (% DM) of adequate-protein (AP) and high-protein (HP) formula powders as well as standard diets*

	AP (7–28 d)	HP (7–28 d)	Starter diet (28–42 d)	Piglet diet (42–70 d)
Protein (% DM)	26·6	36·8	19·5	18·5
Lipids (% DM)	42·1	36·0	6·6	2·5
Starch (% DM)	–	–	25·4	42·9
Lactose (% DM)	26·2	22·7	–	–
Minerals (% DM)	0·31	0·31	7·1	5·5
Crude fibres (% DM)	–	–	3·0	3·7
NE (MJ/kg)	18·7	17·5	10·6	9·6

NE, net energy.
* For details of the ingredients in the piglet diet, see Huguet *et al.*[35].

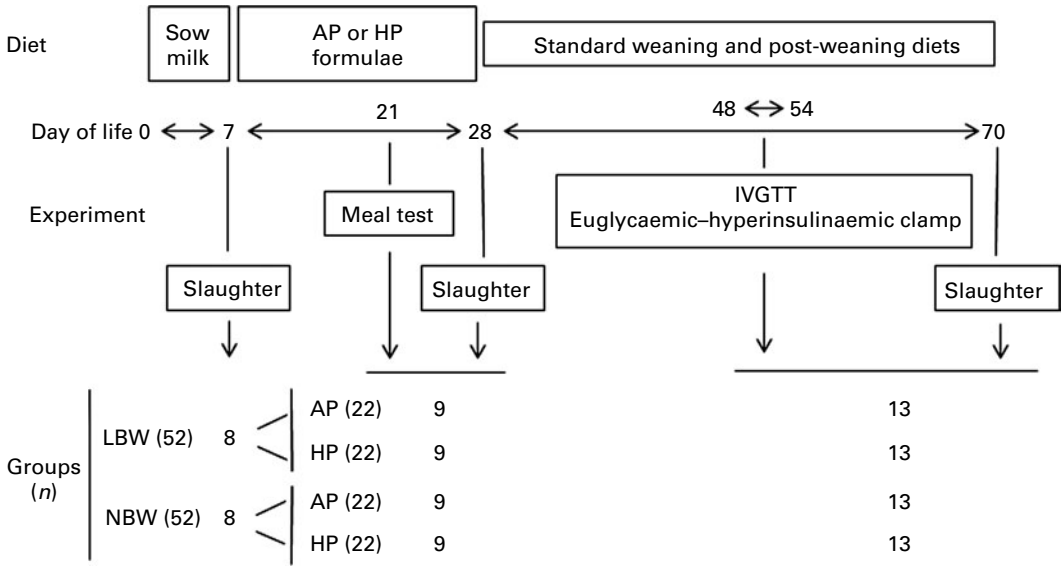

Fig. 1. Overview of the study design. Normal-birth-weight (NBW, near the litter mean birth weight) and low-birth-weight (LBW, 30 % less than the mean birth weight) piglets were allowed to suckle the dam until 7 d of age. At this stage, eight NBW and eight LBW piglets were slaughtered as initial controls. Piglets were then separated from their dam and fed milk replacers formulated to provide an adequate-protein (AP) or a high-protein (HP, + 48 %) supply with an automatic formula feeder until 28 d of age. A first group of animals (*n 36*) was used at 21 d of age to investigate postprandial insulin secretion and killed at 28 d to examine pancreas maturation and peripheral tissue insulin sensitivity. A second subset of animals (*n 52*) was weaned and fed *ad libitum* the same standard commercial diets until 70 d of age. At 48–54 d of age, an intravenous glucose tolerance test (IVGTT) and a euglycaemic–hyperinsulinaemic clamp were performed. Pigs were then slaughtered at 70 d of age to analyse pancreas and insulin sensitive tissues.

(30 % solution) was infused simultaneously to counteract hyperinsulinaemia-induced hypoglycaemia, at a variable rate readjusted every 5 min after the arterial blood glucose assay (One-Touch II; Life Scan, Milpitas, CA, USA). The first 90 min of the clamp represented the period required to reach the steady-state glucose infusion rate allowing euglycaemia. The last 30 min of the clamp represented the steady state during which three arterial blood samples were withdrawn at 10 min intervals to measure insulin concentration. The *M* value of the clamp (glucose metabolic clearance, mg/kg per min) was calculated as the average of glucose infusion rates over the period of 90–120 min from the start of the clamp. The disposition index was calculated as the ratio of the *M* value to the average plasma insulin concentration during the same period of time.

Tissue sample collection

At 28 and 70 d of age, these piglets were slaughtered in the experimental slaughterhouse by electrical stunning and exsanguination. Samples of liver and dorsal longissimus muscle (at 28 and 70 d) and perirenal adipose tissue (pAT) and dorsal subcutaneous adipose tissue (scAT) (at 70 d) were frozen in liquid N_2 and stored at −80°C. Then, two samples of 1 cm^3 were dissected in the body of the pancreas and fixed in 4 % paraformaldehyde for immunohistological analyses.

Hormone and glucose assays

Insulin concentrations were measured using a modified validated RIA[39] that used iodinated porcine insulin (INSULIN-CT; Cis Bio International, Gif-sur-Yvette, France) as a tracer and porcine monocomponent insulin (Novo Research Institute, Copenhagen, Denmark) for standard curves. Antiserum (Valbiotech, AbCys SA, Paris, France) was used at a final dilution of 1:1000. The quantification limit was 1 μIU/ml and the intra-assay CV was less than 5 % at 70 μUI/ml. C-peptide, GIP and CCK were measured using RIA kits: porcine C-peptide RIA kit (catalogue no. PCP-22K; Millipore), porcine GIP RIA kit (S-2131; Peninsula Laboratories Inc., San Carlos, CA, USA) and EURIA-CCK RIA kit (RB 302-1204; Euro-Diagnostica AB, Malmö, Sweden), respectively. For CCK and GIP, samples were extracted and rehydrated in specific buffers before being assayed[40]. GLP-1 was measured using a GLP-1 (active) ELISA kit (catalogue no. EGLP-35K; Millipore). Plasma glucose was measured in duplicate by an automated spectrophotometric method (Cobas Mira; Roche, Basel, Switzerland) using the glucose RTU kit (Biomérieux, Marcy L'Etoile, France). The inter-assay CV was less than 5 %.

Immunohistochemical analyses

After fixation for 24 h in 4 % paraformaldehyde, samples of the pancreas were cryoprotected overnight at 4°C in PBS containing 30 % sucrose, embedded in the OCT™ compound (TissueTek; Sakura Finetek Europe B. V., Zoeterwoude, The Netherlands), frozen in isopentane and sectioned (10 μm) using a cryostat-microtome. Sections of the pancreas were incubated with 4 % normal horse serum and Triton X-100 (0·5 %) in PBS for 1 h. Sections were then exposed overnight to a mouse anti-insulin antibody (1:50; Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA). After washing with PBS, they were incubated with a donkey anti-mouse antibody coupled to fluorescein isothiocyanate (1:200; Jackson ImmunoResearch Laboratories Inc., West Grove, PA, USA) for 3 h. Finally, sections were washed again with PBS and were coverslipped with Vectashield™ (Vector Labs, Burlingame, CA, USA). Control sections were

Table 3. Primers used for the analysis of gene expression by real-time RT-PCR

Gene	Accession no.	Forward primer (5′ → 3′)	Reverse primer (5′ → 3′)	Length (bp)
GLUT2	EF140874	TTGTTAGTCAGATCATAGGCCTCG	ATAGCTCATGATTGCCCAGGA	51
GLUT4	NM_001128433	GGCAGCCCCTCATCATTG	TCGAAGATGCTGGTTGAATAGTAGAA	91
Insulin R	AF102858	TTCTTCGAACCCCGAGTACCT	CGATGTCCCTGGCGTTTC	158
PEPCK	BX670047	TCGAGAAAGCCTTCAATGCC	GCGTGCGACCCTTCATG	51
G6Pase	AK232989	CGGCTTTCGGTGCTTGAA	CTGCACAGTCCAGAATCCCA	52

R, receptor; *PEPCK*, phosphoenol pyruvate carboxykinase; *G6Pase*, glucose-6-phosphatase.

incubated with normal goat serum instead of primary antibodies. Sections were examined by using a fluorescence microscope (Eclipse E400; Nikon Instruments France, Champigny-Sur-Marne, France) attached to a digital camera (Digital Still DXM 1200; Nikon Instruments France). The whole section of the tissue was scanned using a motorised stage (Proscan H29; Prior Scientific Instruments Limited, Cambridge, UK). Recorded files were analysed using the software JMicrovision (N Roduit, J Microvision, version 1.2.2; http://www.jmicrovision.com) for automatic determination of the number and area of islets within the section. The diameter (d) of the islets was extrapolated from their area ($d = 2\sqrt{(\text{area}/\pi)}$). The total area of the pancreatic tissue was also measured. For each section, the number of islets was normalised to 0·47 cm^2, corresponding to the mean section area. The percentage of endocrine tissue was calculated in each section as the ratio of insulin-positive area to the total tissue area of the section.

Real-time RT-PCR

Total RNA was extracted from liver, adipose tissues and skeletal muscle using TRIzol reagent (Invitrogen, Cergy-Pontoise, France). The porcine-specific primers were designed using Primer Express Software (Applied Biosystems, Courtaboeuf, France; Table 3). Real-time RT-PCR was carried out on an ABI PRISM 7000 SDS thermal cycler (Applied Biosystems) as described previously[27,41]. In brief, forty cycles of PCR consisting of denaturation at 95°C for 15 s and annealing and extension at 60°C for 1 min were performed. Specificity of the amplification products was checked by dissociation curve analysis.

Statistical analysis

Data were analysed using the General Linear Model procedure of SAS (SAS Institute, Cary, NC, USA). The model allowed testing of diet, birth weight and diet × birth-weight interaction effects against the residual mean square error. The effect of sex and interactions of sex with diet and birth weight was tested, and as it was not significant, it was not considered in the results. All data are presented as means with their standard errors. Differences were considered significant at $P \leq 0{\cdot}05$.

Results

Growth

During the suckling period, piglets fed the HP formula had a higher daily weight gain (either absolute – g/d, $P<0{\cdot}05$ – or relative to the mean body weight over the suckling period – g/d per kg, $P<0{\cdot}01$) than piglets fed the AP formula (Table 4). LBW piglets tended to have a higher relative daily growth rate than NBW piglets over this period ($P<0{\cdot}1$; Table 4).

The protein content of the formula given during the suckling period had no effect on the post-weaning absolute or relative daily weight gain (Table 4). LBW piglets gained less weight daily than NBW piglets during the post-weaning period ($P<0{\cdot}01$), but the difference disappeared when the

Table 4. Body weight and growth of pigs fed the adequate-protein (AP) or high-protein (HP) formula between 7 and 28 d of age and fed a standard diet thereafter

(Mean values with their standard errors)

	NBW				LBW				Effects		
	AP (*n* 13)		HP (*n* 13)		AP (*n* 13)		HP (*n* 13)				
	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Diet	BW	I
Birth weight (kg)	1·44	0·01	1·43	0·02	0·98	0·03	0·99	0·02	NS	***	NS
Weight at 7 d (kg)	2·93	0·15	2·89	0·07	2·21	0·14	2·05	0·10	NS	***	NS
Weight at 28 d (kg)	6·89	0·39	7·53	0·28	5·77	0·34	5·85	0·33	NS	***	NS
Weight at 70 d (kg)	27·91	1·40	29·89	1·04	21·18	1·48	22·04	1·63	NS	***	NS
Growth of piglets between 7 and 28 d of age											
Absolute growth rate (g/d)	189	14	220	14	170	13	181	12	*	†	NS
Relative growth rate (g/d per kg MBW)	38·0	1·6	41·9	1·7	42·4	2·0	45·4	1·3	**	†	NS
Growth of piglets between 28 and 70 d of age											
Absolute growth rate (g/d)	524	29	559	28	389	34	400	36	NS	**	NS
Relative growth rate (g/d per kg MBW)	30·0	0·7	29·7	1·0	28·6	1·1	28·1	1·0	NS	NS	NS

NBW, normal birth weight; LBW, low birth weight; BW, birth weight; I, interaction (diet × BW); NS, $P>0{\cdot}1$; MBW, mean body weight during the experimental period.
Mean values were significantly different: * $P<0{\cdot}05$, ** $P<0{\cdot}01$, *** $P<0{\cdot}001$.
† Mean values tended to be significantly different ($P<0{\cdot}1$).

relative weight gain (over the mean body weight during the post-weaning period) was considered (Table 4).

Glucose homeostasis during the suckling and post-weaning periods

Before any formula was provided, LBW 7-d-old piglets had a lower insulin concentration 1 h after the last sow milk feed than NBW piglets (7·58 (SE 1·63) *v.* 12·94 (SE 1·16) μIU/ml for LBW *v.* NBW, $P=0·03$), but glycaemia was identical in the two groups (8·37 (SE 0·25) *v.* 8·64 (SE 0·37) mmol/l for LBW *v.* NBW, $P=0·56$), illustrating a higher insulin sensitivity in LBW piglets compared with NBW piglets at 7 d of age.

At day 21 of the suckling period, the size of the meal test (ml/kg body weight) as well as its energy content (kJ/kg body weight) were not different between the groups (Table 5). Postprandial glucose, insulin and C-peptide AUC were not significantly different between piglets fed the AP or HP formula. There was no effect of birth weight on glucose, insulin or C-peptide AUC. The insulinogenic index, as well as the Matsuda index, calculated over the first 30 min post-meal was not different between the groups. Finally, the disposition index accounting for the global postprandial glucose tolerance was not affected by the protein content of the diet or the birth weight (Table 5). Postprandial GIP, GLP-1 and CCK AUC were not significantly different in piglets fed the AP and HP formulae (Table 5). The body weight at birth had no effect on postprandial gut insulinotropic hormone secretion (Table 5).

Fasting insulin concentration was higher ($P=0·04$) in piglets fed the HP formula than in piglets fed the AP formula, whereas fasting glucose and C-peptide concentrations were not different between the two groups (Table 5). HOMA-IR was also increased ($P=0·04$) in HP formula-fed piglets compared with AP formula-fed piglets (Table 5). There was no effect of birth weight on fasting concentrations of glucose, insulin, C-peptide or HOMA-IR (Table 5).

At 50 d of age, no difference was observed between the groups in fasting glucose, fasting insulin and HOMA-IR. During the IVGTT, glucose and insulin AUC over 240 and 60 min were identical between the groups. The acute insulin response to the intravenous bolus of glucose was not different between the groups, neither were the indices of glucose efficiency and insulin sensitivity calculated by the minimal model (Table 6), showing an identical glucose tolerance between the groups. The glucose infusion rate and insulin concentration at the steady state of the EHC, as well as the insulin sensitivity index, were not affected by the protein content of the formula during the suckling period or by the birth weight (Table 7).

Maturation of the pancreas during the suckling and post-weaning periods

The structure of endocrine pancreatic tissue (percentage of endocrine tissue, number and mean diameter of the islets of Langerhans; Table 8) was not modified by the protein level of the formula at the end of the suckling period (in 28-d-old piglets) nor 1 month later (in 70-d-old pigs). The body weight at birth had no effect on endocrine pancreas maturation either.

Peripheral tissue sensitivity to insulin during the suckling and post-weaning periods

Sensitivity to insulin was investigated by determining the expression of genes related to insulin action, insulin receptor and GLUT (GLUT2 or GLUT4), in liver, skeletal muscle and adipose tissue. Expression of genes encoding gluconeogenic

Table 5. Metabolic outcomes of the meal test performed on day 21 during the suckling period†
(Mean values with their standard errors)

	NBW				LBW						
	AP (*n* 9)		HP (*n* 9)		AP (*n* 9)		HP (*n* 9)		Effects		
	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Diet	BW	I
Meal size (ml/kg body weight)	31·4	2·5	29·2	0·6	30·4	0·8	26·4	3·3	NS	NS	NS
Energy (kJ/kg body weight)	115·6	9·2	105·9	2·1	111·8	2·9	95·5	12·1	NS	NS	NS
Protein (g/kg body weight)	1·59	0·13	2·21	0·05	1·54	0·04	1·99	0·25	***	NS	NS
Fasting glucose (mmol/l)	5·39	0·23	5·72	0·12	5·51	0·19	5·52	0·12	NS	NS	NS
Fasting insulin (μIU/ml)	2·29	0·29	3·73	0·74	2·51	0·28	3·05	0·46	*	NS	NS
Fasting C-peptide (ng/l)	0·12	0·02	0·11	0·01	0·10	0·009	0·15	0·02	NS	NS	NS
HOMA-IR	0·56	0·08	0·96	0·19	0·62	0·08	0·76	0·13	*	NS	NS
Glucose AUC (180 min × mmol/l)	61·0	21·7	50·2	19·6	109·8	30·4	69·3	15·7	NS	NS	NS
Insulin AUC (180 min × μIU/ml)	1862·8	137·1	2092·6	93·3	1759·0	294·2	1670·3	413·2	NS	NS	NS
Peptide-C AUC (180 min × ng/l)	53·1	5·8	72·7	7·9	840·8	241·2	788·1	158·2	NS	NS	NS
GLP-1 AUC (120 min × pmol/l)	374·7	109·7	992·4	313·2	49·2	7·5	65·8	19·1	NS	NS	NS
GIP AUC (120 min × pmol/l)	9730	1818	13 213	3100	9704	1031	9506	1741	NS	NS	NS
CCK AUC (120 min × pmol/l)	252·4	95·8	302·5	71·5	257·9	69·9	205·5	69·1	NS	NS	NS
Insulinogenic index ($\Delta ins_{0-30}/\Delta glu_{0-30}$)	2·21	0·50	2·69	0·68	2·18	0·71	2·69	1·07	NS	NS	NS
Matsuda index	17·0	5·1	13·1	1·9	17·2	2·2	16·6	2·8	NS	NS	NS
Disposition index	38·4	12·6	33·5	11·6	31·0	8·4	42·8	17·2	NS	NS	NS

NBW, normal birth weight; LBW, low birth weight; AP, adequate-protein; HP, high-protein; BW, birth weight; I, interaction between diet and BW; NS, $P>0·1$; HOMA-IR, homeostasis model assessment-insulin resistance index; AUC, area under the curve; GLP-1, glucagon-like polypeptide-1; GIP, glucose-dependent insulinotropic peptide; CCK, cholecystokinin.
Mean values were significantly different: * $P<0·05$; *** $P<0·001$.
† The meal consisted of the AP or HP formula.

Table 6. Metabolic outcomes of the intravenous glucose tolerance test in 50-d-old piglets fed the adequate-protein (AP) or high-protein (HP) formula between 7 and 28 d of age and a standard diet thereafter

(Means values with their standard errors)

	NBW				LBW						
	AP (*n* 11)		HP (*n* 13)		AP (*n* 13)		HP (*n* 13)		Effects		
	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Diet	BW	I
Fasting glucose (mmol/l)	4·71	0·12	4·60	0·17	4·64	0·17	4·65	0·18	NS	NS	NS
Fasting insulin (μIU/ml)	8·40	0·86	9·91	1·06	9·21	0·77	8·51	0·80	NS	NS	NS
HOMA-IR	1·77	0·20	2·07	0·27	1·90	0·18	1·78	0·20	NS	NS	NS
Glucose $AUC_{240\ min}$ (240 min × mmol/l)	66·1	33·4	54·2	23·0	81·2	27·9	42·4	25·6	NS	NS	NS
Insulin $AUC_{240\ min}$ (240 min × μIU/ml)	630·6	164·6	1313·6	689·8	471·4	156·8	718·4	312·8	NS	NS	NS
Glucose $AUC_{60\ min}$ (60 min × mmol/l)	92·7	13·7	90·1	12·7	103·1	11·7	97·6	12·0	NS	NS	NS
Insulin $AUC_{60\ min}$ (60 min × μIU/ml)	897·0	106·7	1181·5	246·5	814·2	122·2	1071·1	223·4	NS	NS	NS
AIR (μIU/ml)	32·4	4·7	38·8	4·2	29·2	4·1	34·5	3·8	NS	NS	NS
S_I ($\times 10^{-3}$)	3·82	2·31	5·20	1·86	3·35	1·64	8·02	4·80	NS	NS	NS
S_G ($\times 10^{-2}$)	4·17	0·73	4·47	0·76	4·30	0·65	4·85	0·82	NS	NS	NS

NBW, normal birth weight; LBW, low birth weight; BW, birth weight; I, interaction between diet and BW; NS, $P>0·1$; HOMA-IR, homeostasis model assessment-insulin resistance index; AUC, area under the curve; AIR, acute insulin response (mean insulin concentration above basal values for the first 5 min); S_I, insulin sensitivity index; S_G, glucose efficiency index.

enzymes, phosphoenol pyruvate carboxykinase (PEPCK) and glucose-6-phosphatase (G6Pase), were also examined in liver.

In liver (Fig. 2), the expression of the *GLUT2* gene was higher ($P=0·03$) in HP formula-fed piglets than in AP formula-fed piglets at the end of the suckling period. Moreover, this expression was greater ($P=0·05$) in LBW piglets than in NBW piglets. The expression of the insulin receptor gene did not differ between the dietary groups at 28 d of age, nor did that of *PEPCK* and *G6Pase*. At 1 month after weaning, the expression of *GLUT2*, *G6Pase* and *PEPCK* genes were not different between the groups.

In the skeletal muscle of 28-d-old piglets, the expression of insulin receptor genes did not differ between the groups (0·67 (SE 0·08) *v.* 0·69 (SE 0·08) for HP *v.* AP, $P>0·05$). At 1 month later, the expression of the insulin receptor gene was higher ($P<0·05$) in LBW pigs than in NBW pigs, whereas expression of the *GLUT4* gene was not different between the groups (Fig. 3).

In subcutaneous and pAT, the expressions of genes encoding GLUT4 and insulin receptor did not differ between the dietary groups in 28-d-old piglets[27], whereas *GLUT4* gene expression was higher 1 month later ($P<0·05$) in HP pigs than in AP pigs in scAT but not in pAT, with no effect of birth weight (Fig. 3). Levels of insulin receptor mRNA were higher ($P<0·05$) or tended to be higher ($P<0·1$) in LBW pigs than in NBW pigs in scAT and pAT, respectively (Fig. 3).

Discussion

The present study was designed to evaluate whether a high protein intake during the suckling period increases postprandial insulin secretion and overstimulates the pancreas in a period of still intense maturation of the organ, which may result in permanent morphological changes of the endocrine pancreas and in metabolic perturbations later in life. We demonstrated that a high protein intake did not modify postprandial insulin secretion and pancreas anatomy and had no consequences on glucose tolerance in the post-weaning period.

During the formula-feeding period, however, piglets receiving the HP formula displayed higher basal insulin concentration and concomitantly higher HOMA-IR, though within the physiological range (HOMA-IR < 2·4), than piglets receiving the AP formula. They also displayed higher relative and absolute growth rates. This result is in accordance with a recent study in rat pups, in which a HP diet from day 7 to day 15 of the suckling period induced higher growth rate and basal insulin:glucose ratio compared with a low-protein diet[42]. In human subjects, term infants fed a HP

Table 7. Metabolic outcomes of the euglycaemic–hyperinsulinaemic clamp in 50-d-old piglets fed the adequate-protein (AP) or high-protein (HP) formula between 7 and 28 d of age and a standard diet thereafter

(Means values with their standard errors)

	NBW				LBW						
	AP (*n* 13)		HP (*n* 13)		AP (*n* 13)		HP (*n* 13)		Effects		
	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Diet	BW	I
M (mg/min per kg)	16·6	1·3	15·8	1·7	16·7	1·2	16·8	1·4	NS	NS	NS
Insulin (μIU/ml)	67·4	8·7	66·4	6·9	57·1	6·6	59·5	6·5	NS	NS	NS
Disposition index*	0·295	0·044	0·279	0·051	0·363	0·055	0·305	0·031	NS	NS	NS

NBW, normal birth weight; LBW, low birth weight; BW, birth weight; I, interaction between diet and BW; *M*, glucose metabolic clearance (average of glucose infusion rates over the period of 90–120 min from the start of the clamp); NS, $P>0·1$.

* Ratio of *M* to the average plasma insulin concentration during the same period of time.

Table 8. Endocrine pancreas features of 28 and 70-d-old pigs fed the adequate-protein (AP) or high-protein (HP) formula between 7 and 28 d of age and a standard diet thereafter

(Mean values with their standard errors)

	NBW				LBW						
	AP (*n* 5)		HP (*n* 5)		AP (*n* 5)		HP (*n* 5)		Effects		
	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Diet	BW	I
Endocrine pancreas of 28-d-old piglets											
Endocrine tissue (%)	1·62	0·38	1·03	0·59	1·02	0·20	1·24	0·25	NS	NS	NS
Number of islets (per 0·47 cm^2 tissue)	405	100	169	84	242	19	279	41	NS	NS	NS
Mean islet diameter (μm)	46·6	1·6	49·7	4·0	46·5	2·1	47·4	1·1	NS	NS	NS
Endocrine pancreas of 70-d-old pigs											
Endocrine tissue (%)	0·82	0·18	0·56	0·15	0·77	0·11	0·80	0·16	NS	NS	NS
Number of islets (per 0·47 cm^2 tissue)	84·7	11·1	60·2	10·2	84·5	13·7	89·0	8·7	NS	NS	NS
Mean islet diameter (μm)	75·2	2·9	74·0	4·9	77·0	3·5	73·0	5·3	NS	NS	NS

NBW, normal birth weight; LBW. low birth weight; BW, birth weight; I, interaction (diet × BW); NS, $P > 0·1$.

formula between 4 and 6 months of age displayed higher growth rate and urinary C-peptide concentration at 6 months of age than infants fed an isoenergetic AP formula[43]. The authors postulated that the higher urinary C-peptide concentration was due to higher insulinotropic amino acid supply in the HP diet. However, in 8-year-old boys, 7 d of the HP diet increased fasting C-peptide, insulin and HOMA-IR when proteins where provided by milk but not by meat, with a similar increase in fasting plasma insulinotropic amino acid with both milk and meat[22]. Therefore, stimulation of basal insulin secretion by insulinotropic amino acid has not been supported. In the present study, we did not evidence higher fasting C-peptide concentration in piglets receiving the HP formula compared with piglets receiving the AP formula, arguing for a lesser basal insulin clearance in HP-fed piglets than in AP-fed piglets. In rats, a HP diet provided from weaning to adulthood[44] or for 2 weeks in adulthood[45] increased both fasting insulin and glucose, indicating a lesser insulin sensitivity. Adipose tissue *in vitro* was actually less sensitive to the action of insulin on glucose uptake[44], and the authors have also hypothesised that basal hyperglycaemia was due to increased gluconeogenesis in the presence of higher supply of amino acids[45]. In the present study, the higher basal insulin concentration in HP-fed piglets was not due to a modification in the liver sensitivity to insulin since the expressions of insulin receptor and gluconeogenic hormones PEPCK and G6Pase were not different in HP- and AP-fed piglets. The only modification observed in the liver was an increase in the glucose transporter GLUT2 in HP-fed piglets, suggesting a higher glucose transport in hepatic cells. We have recently demonstrated that 28-d-old piglets fed a HP diet during suckling showed a decreased expression of fatty acid synthase gene in the pAT compared with piglets fed an AP diet, without modification of the expression of GLUT4[27]. Since fatty acid synthase expression is up-regulated by insulin[46], this could indicate that the pAT would be less sensitive to insulin in HP-fed piglets compared with AP-fed piglets and would contribute to the lesser overall insulin sensitivity in HP-fed piglets during the suckling period. However, this hypothesis has to be taken with caution since the decreased expression of fatty acid synthase gene was more pronounced in LBW piglets than in NBW piglets and could also be attributed to a delay in gene expression in LBW piglets fed the HP diet rather than to hyperinsulinaemia[47]. However, at 1 month after weaning, the only difference between the dietary groups was an increased mRNA expression of GLUT4 in the scAT of HP piglets, with no consequences on glucose tolerance and overall insulin sensitivity as assessed by the IVGTT and EHC, suggesting that the early modification in insulin clearance has no longer consequences.

In the present study, enhancing by 48 % the protein supply (whey and casein) of the meal did not modify the postprandial C-peptide concentration taken as an indicator of insulin secretion. Postprandial concentrations of the intestinal insulinotropic hormones CCK, GIP and GLP-1 were not different between the dietary groups either. This could account for a surprising result since proteins are known to stimulate insulin secretion, with milk proteins being among the most efficient[12,14]. This is due to their strong propensity to induce the early release of insulinotropic amino acid[12] and the insulinotropic hormones CCK, GIP and GLP-1[12,16]. Furthermore, the HP diet has been described to sensitise endocrine β-cells to glucose[20] and arginine[45]. However, these results are highly dependent on the nature of the HP diet: proteins alone do not elicit insulin secretion *in vivo* and there seems to be a threshold below which additional proteins to a glucose meal do not stimulate insulin secretion[48]. One hypothesis to explain the present results may therefore be that the amount of proteins added in the HP formula was not sufficient to elicit complementary insulin secretion. Furthermore, we took the option to formulate the AP and HP diets as isoenergetic diets and the protein supply in the HP diet was made in partial substitution from fat and carbohydrates, keeping the carbohydrate:fat ratio constant in both diets, resulting in 10 % less lactose in the HP formula than in the AP formula. The higher supply of lactose in the AP formula may have masked the effect of the higher protein supply in the HP diet, the lactose insulinotropic properties equalling the effects of the added proteins on insulin secretion in the HP diet. The postprandial plasma concentration of α-amino-nitrogen did not differ between the AP and HP meals (data not shown). This may indicate that the additional amino acids of the HP diet had

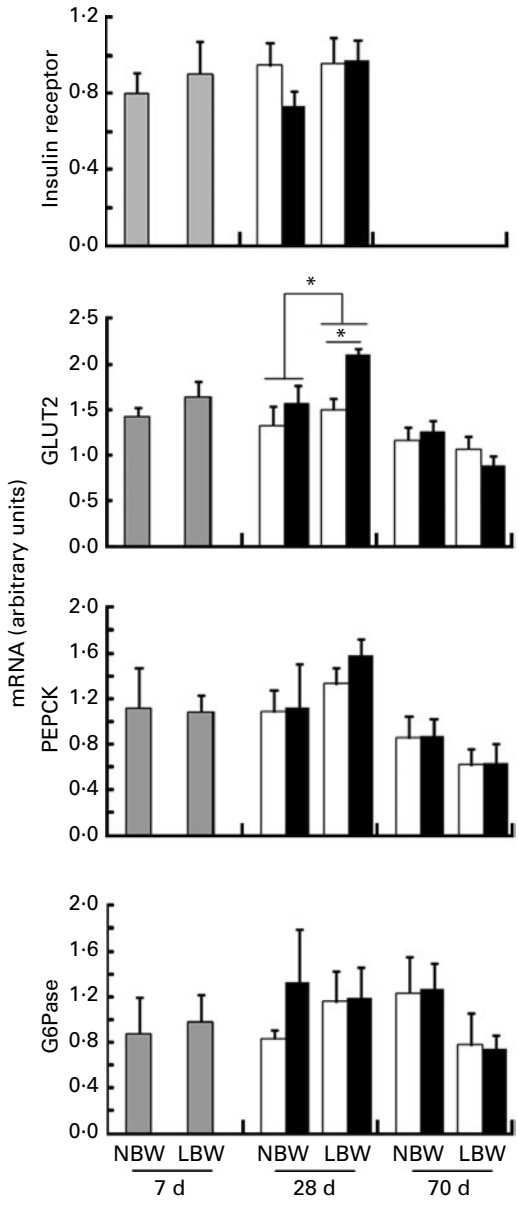

Fig. 2. Relative levels of insulin receptor, GLUT2, phosphoenol pyruvate carboxykinase (PEPCK) and glucose-6-phosphatase (G6Pase) mRNA in the liver of 7-d-old piglets, 28-d-old piglets and 70-d-old pigs fed the adequate-protein (□) or high-protein (■) formula between 7 and 28 d of age and fed a standard diet thereafter. Values are means, with their standard errors represented by vertical bars (n 6). * Mean values were significantly different ($P<0{\cdot}05$). NBW, normal birth weight; LBW, low birth weight.

been cleared quicker from the bloodstream or that they not had been absorbed properly. This latter hypothesis is, however, unlikely since piglets fed the HP formula displayed as expected a higher growth during the suckling period compared with piglets fed the AP formula.

In the present study, no difference in the endocrine pancreas morphology was observed under the HP diet either during the suckling period or 1 month after weaning. This was not surprising since the HP diet did not induce higher insulin demand on the pancreas.

The weight at birth had little impact on the studied parameters. We demonstrated a greater *GLUT2* gene expression in the liver of LBW piglets than that of NBW piglets at 28 d, which might reflect a higher hepatic glucose transport capacity in LBW piglets than in NBW piglets. At 70 d, LBW pigs had greater expression of insulin receptor compared with NBW pigs in muscle and scAT, with no consequence on overall insulin sensitivity. Notable effects of LBW have been demonstrated on glucose tolerance in 12 months old pigs, but not in 3 months old piglets[25], so 70 d of age in the present study may be too early to screen the long-term effect of LBW on glucose tolerance. Therefore, it is not excluded that a high protein intake early in life would worsen the LBW adverse metabolic outcomes later in life. Some recent results in intra-uterine growth retardation, rat pups did demonstrate that a HP diet had more adverse consequences on metabolism in the long term than an AP diet (Delamaire *et al.*, unpublished results).

In summary, the present study demonstrates in a piglet model that increasing by 48 % the protein content of the diet during the suckling period has no consequences on postprandial insulin secretion and pancreas maturation. We demonstrated that the short- and longer-term consequences of the HP diet on glucose metabolism are also very weak. Therefore, the hypothesis stipulating that higher growth observed during the suckling period under the HP diet may be due to higher insulin secretion is not validated in the present study, which accurately screened postprandial insulin secretion in an

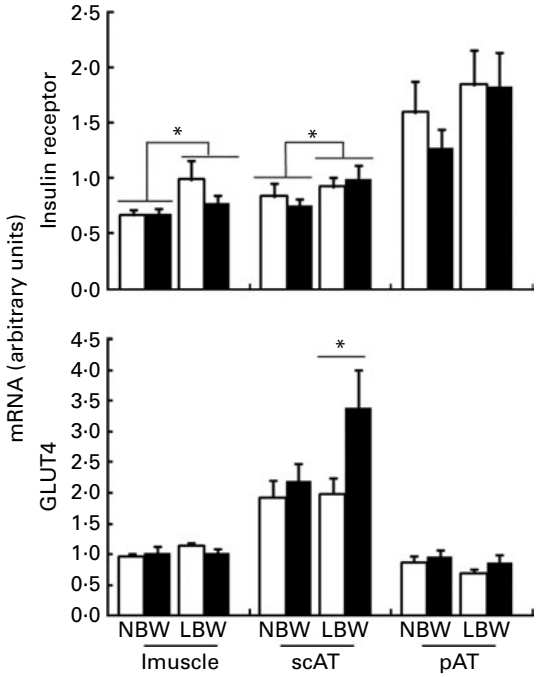

Fig. 3. Relative levels of insulin receptor and GLUT4 in longissimus muscle (lmuscle) and in subcutaneous (sc) and perirenal (p) adipose tissues (AT) in 70-d-old pigs fed the adequate-protein (□) or high-protein (■) formula between 7 and 28 d of age and fed a standard diet thereafter. Values are means, with their standard errors represented by vertical bars (n 6). * Mean values were significantly different ($P<0{\cdot}05$). NBW, normal birth weight; LBW, low birth weight.

animal model. Further studies are needed to elucidate the mechanisms behind the higher growth under HP diets and their actual longer-term consequences on metabolism.

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