Leucine supplementation during late gestation globally altered placental metabolism and nutrient transport via modulation of the PI3K/AKT/mTOR signalling pathway

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Research

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Abstract

Background

Our previous study found that sow dietary leucine supplementation significantly improved fetal intrauterine growth and newborn piglet birth weight. But we still have limited knowledge how leucine regulated placental functions to promote the nutrient supply to fetus to support its intrauterine development.

Methods

150 sows at day 90 of gestation were divided into three groups and fed with either control diet (CON), CON + 0.4% Leu or CON + 0.8% Leu separately until parturition. Placental metabolomics, full spectrum amino acids and nutrient transporters were systematically analyzed.

Results

Leu supplementation lead to an altered placental metabolism with higher amount of metabolites related to glycolysis and fatty acids oxidation, and more amino acids accumulation in placenta. Besides, nutrient transporters including amino acids transporters, glucose transporters and fatty acids transporters in placenta were globally enhanced and several enzymes related to energy metabolism including hexokinase, succinate dehydrogenase, lactate dehydrogenase, glycogen phosphorylase and hydroxyaryl-CoA-dehydrogenase were significantly increased with no change in antioxidative status in the groups with Leu supplementation. Furthermore, Leu supplementation significantly increased phosphorylation of PI3K, Akt, and mTOR in placenta compared with control group.

Conclusions

Leu supplementation during late gestation globally altered placental metabolism, nutrient transport (amino acids, glucose, and fatty acids) via modulation of the PI3K/Akt/mTOR signalling pathway.

Background

Placenta, an extremely critical organ that connects mother and fetus during pregnancy, performs a variety of functions including nutrients supplying (e.g., amino acids, oxygen and water) from mother to fetus, as well as metabolites (e.g., ammonia and CO₂) removing from fetus to mother throughout the pregnant process to keep the fetus alive and development [1–3]. Besides, placenta also plays crucial roles in biosynthesis and metabolism of steroids to modulate both maternal and fetal physiology and metabolism [4, 5].

In mammals, foetal intrauterine development and maturity primarily depends on the performance of placenta [6]. Previous investigation clearly proved that insufficiency of placenta function could result in intrauterine growth restriction in varieties of animals. In sows, inadequate placental capacity limits optimal fetal development since day 25 of gestation and continue to the farrowing [7]. This problem is more severe in modern industry due to that much higher prolific breeds with more than 15 fetuses per sow were widely used in these days than 30 years ago [8, 9].

Maternal diet has been shown to strongly influence the placental development, nutrient transport capacity and metabolism, with subsequent effects on fetal growth and health during pregnancy [10]. Leucine (Leu), an essential amino acid for protein synthesis, has been reported to regulate several cellular processes such as protein synthesis, tissue regeneration, and metabolism [11]. Our previous study found that Leu supplementation in sow diet during late

pregnancy (day 70–110) significantly increased fetal birth weight and decreased the occurrence of intrauterine growth restriction by improving fetal protein synthesis, but we still have limited knowledge how Leu exert its effect through placenta to regulate fetal development.

Considering that fetal growth is primarily determined by nutrient availability, which is intimately related to placental nutrient transport [12, 13], we hypothesize the changes in placenta caused by dietary Leu supplementation might be the main contribution to the improved nutrient supplying from maternal to fetus and then effect on fetal development. Nutrients in maternal circulation were accumulated in placenta via specific transporters and then some of them are metabolized within placenta to supply energy for organ development and maintenance or converted to other active substance which could regulate placental function within placenta or further transported to fetal circulation. Another part of nutrients is directly transported through placenta into fetal circulation to participate fetal development during pregnancy. Thus, in present study we determined the placental metabolics, gene expression of global nutrient transporters and energy metabolism related enzymes to systematically investigate the impact of maternal Leu supplementation on placenta function with the purpose to reveal the underlying mechanism of improved fetal growth induced by maternal dietary Leu supplementation during late pregnancy.

Materials And Methods

Animals, experimental diets, and sample collection

All the animal procedures used during this experiment were approved by the South China Agricultural University Animal Care and Use Committee. 150 healthy sows (Landrace × Large White, average weight =260 kg) were assigned to one of three treatments with 50 replications in each group according to the principle of parity (4-5), similar weight, historical reproductive performance and body condition. The basal experimental diets were formulated to meet the nutrient requirements of swine (NRC 2012). The composition and nutrient content of treatment diets are presented in Table 1, 2. Three treatments are listed as follows: (1) corn and soybean basal diets (CON); (2) CON + 0.40% Leu; (3) CON + 0.80% Leu. Leu (purity ≥ 98.5%) was obtained from Jizhou Huayang Chemical Co. Ltd. (Jizhou, China). During experiment, the sows were fed twice a day (7:00 and 14:00) and could drink water freely. During sow delivery, the placenta excreted was collected in time after 75% alcohol was used for surface disinfection. About 100 g placenta samples were obtained from areas rich in blood vessels each sow. The samples were cut into pieces, sub-packaged and stored in liquid nitrogen until analysis. Blood samples were collected aseptically in tubes from the jugular vein and kept at 37°C for 1 h and then centrifuged at 3,000 g at 4 °C for 15 min. The serum was separated and stored at –80°C until analysis.

Placental metabolomics analysis

After pretreatment of placental samples, the samples were subjected to HPLC- MS/MS (Ultimate 3000 - API, 3200 Q TRAP) for detection in positive ion modes. 100 µL serum was added into 400 µL methanol and acetonitrile solution (1:1), and the mixture was centrifuged at 13,200 g at 4 °C for 12 min after vortexing for 30 s. The supernatant was filtered using an Oasis PRiME HLB SPE (30 mg, 1 mL; Waters, Millford, MA, USA) and transferred into a fresh tube. The extract was dried in a vacuum concentrator, the dried metabolite for resolution was added 100 µL acetonitrile and water (1:1). Vortex for 30 s, ice water bath ultrasound for 10 min, the samples were centrifuged at 4 °C for 13,200 g for 10 min. 50 µL supernatant was taken out into injection flask for LC-MS detection. The serum was analyzed using an MSLab 45+AA-C18 C18 (15µm, 50× 4.6mm) column at 35 °C, the flow rate at 0.35 mL min⁻¹ and the injection volume at 2.0 µL. The adduct was set to M+Na, M+H, M+K, 2M+Na, 2M+H, 2M+K, M+H-H₂O, and the molecular weight tolerance was set to 20 ppm. The mobile phase was consisted of water (A) and 0.10% formic acid in acetonitrile (B). The MS parameters were as follows: onspray voltage = 3500 V, Ion Source Gas1 = 40 psi, Ion Source Gas2 = 80 psi and source temperature = 650 °C. The Human Metabolome Database and the Metlin database were used to identify the qualitative differential metabolites.

Serum full spectrum amino acids analysis

50 µL serum sample was placed in a 1.5 mL EP tube and added 50 µL mixed amino acid standard substance and 50 µL protein precipitator (containing NVL). The mixture was mixed for 30 s and centrifuged 13,200 g at 4°C for 4 minutes. 10 µL supernatant was obtained and added 50 µL marked buffering solution (containing 20 µmol /L orthoalvic acid) and 20 µL derivatization reagent, then incubated at 55°C for 15min. Concentrations of serum free amino acids were determined using HPLC- MS/MS (Ultimate3000 – API, 3200 Q TRAP). MSLab 45+AA-C18 (15µm, 50× 4.6mm) was used for separation and 5 µl of the sample was injected onto the column. The column temperature was set at 50 °C. Mobile phase A was 0.1% formic acid in water and mobile phase B was 0.1% formic acid in acetonitrile at a flow rate of 1 mL/min. The LC gradient condition was 0-2 min of mobile phase B at 10%, 3 min at 70%, 4-5 min at 100%, 6-7 min at 10%. After HPLC analysis, the mass spectrometer was used to analyze. The mainly operating parameters were as follows: ion source = +ESI, scan mode = MRM, curtain gas = 20 psi, collision gas = medium, ion spray = +5500 V, ion source temperature = 500 °C, nebulizing gas = 55 psi, drying gas = 60 psi and entrance potential = 10 V[14].

Real-time PCR

The total RNA was isolated from placenta tissues using the RNA extraction kit (LS040, Promega, Shanghai, China) followed the manufacturer's instruction. The RNA purity and concentration were checked by electrophoresis on 1.0% agarose gel (130 V, 18 min) . The purity of RNA (A260/A280) for all RNA samples were greater than 1.8 by using a spectrophotometer. The cDNA synthesis was performed from the RNA reverse transcription reaction using the PrimeScript First Strand cDNA Synthesis Kit (RR047A, Takara, Dalian, China) according to the manufacturer's protocol. Real time quantitative PCR was carried out on an ABI Prism 7500 sequence detection system (Applied Biosystems, Carlsbad, CA, USA) consisting of 20 µL reaction volume. The PCR reaction protocol was consisted of Initial denaturation one cycle at 94 °C for 1 min, amplification forty cycles at 94 °C for 30 s , 60 °C for 30 s and 72 °C for 20 s and melting one cycle at 95 °C for 60 s, 60 °C for 15 s and 72 °C for 30 s. The primers used as shown in Table 3.

Western blot

Protein was extracted from placente tissue frozen in liquid nitrogen and cleaved by adding a mixture of RIPA lysis buffer (Beyotime, Shanghai, China) and the protease inhibitor PMSF (Beyotime, Shanghai, China). The protein concentration from the supernatant was determined by a BCA Protein Assay Kit (P0010, Beyotime, Jiangsu, China) after the supernatant was separated using the centrifuge. The equal amount of protein (30 µg) was separated on 8-12% SDS-PAGE gels, and then electrically transferred to polyvinylidene difluoride (PVDF) membranes. The PVDF membranes were washed 5 times for 5 min with TBST after blocking 5% skimmed milk powder at room temperature for 1h. Then the PVDF membranes were incubated with the different primary antibody [mTOR (2983S, Cell Signaling Technology, USA), p-mTOR (5536S, Cell Signaling Technology, USA), PI3K (4249S, Cell Signaling Technology, USA), p-PI3K (17366S, Cell Signaling Technology, USA), Akt (4685S, Cell Signaling Technology, USA), P-Akt (9271S, Cell Signaling Technology, USA) and β-actin (bs-0061R, Bioss, China)] at 4 degrees for 12h. Repeat the steps of washing with TBST, the PVDF membranes were incubated with corresponding secondary antibodies (511203, ZenBio, China) for 1h at room temperature, followed by testing the target Protein bands using an enhanced chemiluminescence kit (P1020, Applygen, Beijing, China) with the ImageQuant LAS 4000 mini system.

Antioxidant status

The antioxidant status of serum, including glutathione peroxidase (GSH-PX), glutathione transferase (GSH-Tr), superoxide dismutase (SOD), catalase (CAT) and malonaldehyde (MDA) were tested by commercially available kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, China).

Energy metabolism related enzyme activities

About 30 mg placenta was taken and homogenized in phosphate buffer (1 : 40; EDTA-Na 2 mmol/L, glucose 5 mmol/L, mercaptoethano 5l mmol/L in potassium phosphate buffer 100mmol/L; pH 7.2). All operations were done in an ice bath. The homogenate was centrifuged at 2,000 g in a refrigerated centrifuge for 10 min. The supernatants were collected for determination of the enzyme activities of hexokinase, succinate dehydrogenase, lactate dehydrogenase, glycogen phosphorylase and hydroxyaryl-CoA-dehydrogenase using commercial kits according to manuals. All enzyme activities were expressed as units per gram of protein (U/g Protein).

Data Analysis

SIMCAP software (Version 14.0, Umetrics, Umeå, Sweden) was used for data modeling and analysis of placental metabolomics and full spectrum of amino acids. Data were mean-centered using Pareto scaling and models were built on partial least-square discriminant analysis (PLS-DA). PLS-DA allowed the determination of discriminating metabolites using the variable importance on projection (VIP). The VIP score indicates the contribution of a variable to the discrimination between all the classes of samples and the VIP values over 1 are considered as significant. The *P* value was calculated by one-way analysis of variance (ANOVA) using SPSS 22.0 software (IBM Inc., United States). Metabolites with VIP values more than 1.0 and *P* value less than 0.05 were statistically significant. The data of PCR, western blot, antioxidant status and energy metabolism related enzyme activities were analyzed with one way ANOVA followed the procedure using SPSS 22.0 software. *P* less than 0.10 was considered as an upward or downward trend and *P* less than 0.05 was considered as the judgment criterion for the significance of difference.

Results

Metabolite profiling

To assess the effects of Leu in metabolic pathways of the placenta, we performed an untargeted metabolomic analysis using a panel of LC-MS protocols. Totally 40 differential metabolites were identified between control group and the groups with 0.4% Leu, 46 differential metabolites between control group and groups with 0.8% and 35 differential metabolites between groups with 0.4% and 0.8% Leu. Totally 25 common differential metabolites were found among these three groups. PLS-DA of placental metabolites showed a clear separation among the sows from control group, control + 0.4% Leu, and control + 0.8% Leu (Figure 1A, 1B, 1C and 1D). Figure 1E illustrates the identified common differential metabolites with more than 2 folds change and further Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis indicated 9 pathways including fatty acid metabolism; alanine(Ala), aspartate, and glutamate metabolism; glycolysis/gluconeogenesis; valine (Val), Leu, and isoleucine (Ile) degradation; ABC transporters; cell cycle; regulation of lipolysis in adipocytes; apoptosis; and one carbon pool by folate, were involved in the regulation of Leu in placenta. Figure 1F summarized the 25 common differential metabolites among three groups with VIP scores higher than 1. The groups added with Leu were significantly decreased in metabolites of palmitic acid, 13S- hydroxy- octadecadienoic acid and 9S- hydroxy- octadecadienoic acid compared with control group. On contrast, prostaglandins (PGs), polyamines, putrescine, spermidine and spermine were increased from the groups fed with 0.4% and 0.8% Leu.

Full spectrum of amino acids in placenta

Amino acids accumulated in placenta is the main supply of amino acid for fetal intrauterine growth during pregnancy. LC-MS were used to evaluate the effect of Leu on full spectrum of amino acids in placenta and PLS-DA analysis showed a clear separation among the sows from control group, control + 0.4% Leu, and control + 0.8% Leu (Figure 2A, 2B, 2C and 2D). Figure 2E illustrates the identified common differential amino acids between three groups. Sow dietary Leu supplementation improved 24 amino acids accumulation in placenta: Leu, Val, Ile, glutamine (Gln), glutamic acid (Glu), aspartic acid (Asp), Ala, arginine (Arg), glycine (Gly), taurine (Tau), lysine (Lys), serine (Ser), histidine (His), proline (Pro), cysteine (Cys), phenylalanine (Phe), methionine (Met), aminoadipic acid (Aad), phosphoserine (PSer), N-histidine (NHis),

citrulline (Cit), hydroxyproline (HPro), tryptophan (Trp) and homoarginine (Harg) (VIP scores > 1). The VIP scores of Leu, Val, Ile, Glu and Gln were highest among all the amino acid.

Gene expression of amino acids, fatty acids and glucose transporters in placenta

Placenta plays an important role in nutrient transport from maternal to fetus during pregnancy, which process are mainly regulated by specific amino acids transporters, fatty acids transporters and glucose transporters located on placenta. Figure 3A, 3B and 3C shows the gene expression of main nutrient transporters in pig placenta and we found that most of amino acid transporters (Figure 3A) were upregulated with the supplementation of Leu including SLC7A1, SLC7A2, SLC7A8, SLC7A11, SLC15A1, SNAT1, LAT1, 4F2hc and rBAT. The gene expression of glucose transporters (Figure 3B) including SLC2A1, SLC2A2, SLC2A3 and SLC2A6 were also increased in the placenta from the sows fed with 0.4% and 0.8% Leu, but SLC2A5 and SLC2A8 were changed unapparently. We interestingly found that fatty acid transporters (Figure 3C) were differentially regulated by Leu in present study. The expression of the genes involved in fatty acid cross-membrane transport were increased with Leu supplementation, including FATP1, FATP2, FATP3, FATP4, CD36, FABP3, FABP5 and FABP7, but the expression of those genes related to intracellular transport showed no significant change.

Enzymes related to energy metabolism in placenta

The expression of enzymes activities are shown in Figure 4. Compared with the control group, hexokinase, glycogen phosphorylase, lactated hydrogenase, hydroxyacyl-CoA-dehydrogenase and succinate dehydrogenase was expressed at a significantly higher level in 0.4 % and 0.8 % Leu group.

Protein expression of mTOR signal pathway

Western Blot were used to detect the expression of proteins related to mTOR signal pathway and the results indicates that the ratio of T-PI3K/P-PI3K, T-Akt/P-Akt and T-mTOR/P-mTOR were up-regulated with the supplementation of Leu. The phosphorylation of PI3K and Akt were significantly higher in the 0.8 % Leu groups than in the 0.4 % Leu groups (Figure 5).

Oxidative indices

No significant differences in blood oxidative status including GSH-PX, GSH-Tr, SOD, CAT and MDA were observed in different sows fed with either control diet or supplemented with 0.4% and 0.8% Leu in present study ($P \geq 0.05$, Figure 6).

Discussion

Placenta, a crucial organ linking maternal and fetal for nutrients and waste exchange, undergoes extensive metabolism for its development and function during pregnancy. The interaction between nutrients transport and metabolism within placenta is directly related to the nutrient supply from maternal to fetal to regulate fetal development. To our knowledge, this is the first study providing preliminary data on the influence of maternal nutrition to a broad range of metabolites and global nutrients transporter in placenta in mammals. Important differences in metabolism and transport of lipids, glucose and amino acids were observed in placentas from those sows fed with Leu during late pregnancy.

Amino acids transport and metabolism

The quality and relative composition of amino acids delivered to fetus through placenta is directly related to fetal growth during pregnancy due to the importance of amino acids as basic substance for protein synthesis[15]. In present study, amino acids full spectrum analyses indicates that sow dietary Leu supplementation widely improved amino acids accumulation within placenta, which implying more amino acids supply to fetuses. In porcine placenta, branch chain amino acids (BCAAs), including Leu, Val, Ile, could be degraded to donate their amino groups to synthesize Gln, which is important precursor for protein, purine and pyrimidines synthesis to supply to fetuses[16-18]. In addition, Gln also could be

used in the fetus to produce Glu, Asp, and Ala [19, 20]. Thus, Gln accumulated in the placenta either from maternal circulation or placental synthesis, serves as a major nitrogen supply for fetus. Further study found that the amount of Gln in placenta were increased with higher dietary intake of BCAAs during pregnancy [21]. In our present study, we also found that the amount of Gln was significantly increased more than 2 folds in placentas from the sows fed with 0.4% Leu compared with control group. In porcine placenta, those amino acids including Glu, Gln, Ile, Val and Leu showed the highest amount among all amino acids and their accumulation play important role for both placental and fetal development [22]. In the present study, we interestingly found that Leu, Val, Ile, and Glu were enhanced with the addition of Leu and showed the highest VIP scores among all differential amino acids, which might be growth stimulator for improved fetal development during pregnancy [23].

Amino acids from maternal circulation are accumulated within the placenta by active transport systems for further transportation or metabolism [24, 25]. Amino acids transport across placenta are also important determinants of the amino acid composition in the fetal circulation [26] and impaired amino acid transport could lead to placental metabolic disorder and restricted fetal growth [27, 28, 29]. In present study, we determined the gene expression of global amino acids transporters in placenta and found that most of transporters were significantly upregulated with the supplementation of Leu. Tau, an essential nutrient in fetal development, need to be mainly supplied by maternal via placenta due to inadequate synthesis from placenta and fetus [30]. Reduced activity of a placental Tau transporter were related with fetal intrauterine growth restriction and impaired placental function [31, 32]. In the present study, the increased level of Tau in placenta from the sows fed with Leu might attribute to elevated transporters expression in those placentas. In addition, Arg could not be metabolized in porcine placenta because of the lack of arginase [33, 34]. The increased Arg within placenta with Leu supplementation might also attribute to the elevated transporters and would be further transported across placenta to promote Arg supply from maternal to fetus.

Metabolic analysis indicated that amino acid metabolism related to polyamines in placenta were significantly altered by supplementation of Leu in sow diets. Polyamines, which plays essential roles for DNA and protein synthesis in animal cells, are key regulator of placental growth and mammalian embryo early development [29]. Porcine placenta could convert Pro to polyamines by providing the bulk of the carbon skeleton and nitrogen in putrescine, spermidine, and spermine [33]. In the present study, several metabolites related to polyamines including putrescine, spermidine and spermine were up-regulated in the placenta from those sows fed with Leu. In addition, we found the placental glutathione were greatly increased in treatment sow. Glutathione, synthesized in placenta from glutamate, Gly and Cys, are transported to fetus to provide antioxidative protect during development [35]. The enhanced glutathione in placenta has a potential to improve fetal growth during pregnancy.

Fatty acids metabolism and transport

Essential fatty acids and their long-chain polyunsaturated fatty acids play crucial roles for fetal intrauterine development due to their contribution in formation and dynamic properties of biological membranes during pregnancy [36-39]. In placenta, most of fatty acids require facilitated transport by several plasma membrane-located transport/binding proteins such as fatty acid translocase (FAT/CD36), plasma membrane fatty acid binding protein (FABPpm), fatty acid transport protein (FATP 1-6) and intracellular fatty acid binding protein 1-9 (FABP 1-9) [40]. The family of FATPS and FAT/CD36 have been demonstrated to mediate cellular uptake of long-chain and very long chain fatty acids to facilitate fatty acid influx across biological membranes [41]. Previous studies reported that increased expression of CD36 and FATP4 were certainly associated with higher fatty acids uptake in placenta [42, 43]. In present study, the mRNA expression of CD36, FATP1 and FATP2 were significantly enhanced in the placenta from the sows fed with Leu, implying more fatty acid influx from maternal circulation to fetus. Polyunsaturated fatty acids (PUFAs) are essential for fetal development because they are the precursors of eicosanoids and also are essential constituents of the membrane lipids that maintain cellular and organelle integrity [37, 44]. During pregnancy, intrauterine transfer from maternal across placenta is the only supply of PUFAs to the fetus [45]. We found that Leu supplementation resulted in placental higher levels several essential fatty acids

(EFA) and their derivatives including arachidonoc, decosahexaenoic acid, and cholesterol in present study, which might be the results of increased gene expression of CD36 and FATP family.

After inside the placenta, fatty acids could be directly transported into the fetal circulation or bound to FABPs to participate in cellular metabolism within placenta [46]. Selective cellular metabolism of certain fatty acids in placenta could contribute to new synthesized lipid supplying from mother to fetus, conversion of certain proportion of arachidonic acid to PGs, incorporation of some fatty acids into phospholipids and triacylglycerols, and the oxidation of fatty acids[47]. Metabolics analysis showed that sow dietary Leu supplementation leads to altered placental lipid metabolism. Placenta has been shown to oxidize fatty acids as an important metabolic fuel to supply energy during the whole gestational process [48, 49] and FABP 3, 4, 5 were upregulated in placenta during fatty acids oxidation [50, 51, 52]. On the other hand, long-chain fatty acid metabolites may exert toxic effects on cellular functions and cause cell injury [53]. In present study, gene expression of FABP 3, FABP 5 and FABP 7 were significantly enhanced in placenta from those sows fed with Leu supplementation. But metabolic analysis showed decreased level of several metabolites related to lipid oxidation including 9S-hydroxy-octadecadienoic acid (HODE) and 13S-HODE, in treatment placenta compared with control. These results indicated that Leu supplementation upregulated fatty acid oxidation to produce more energy to support placental function without boosting cellular transport.

In addition, we interestingly found PGs which is a crucial regulator for pregnancy and has been linked to induction of fetal organ maturation [54, 55], were upregulated with the leu supplementation. During pregnancy, placenta is the major source of PGs within intrauterine tissues. This result might suggest a new regulation effect of leu to PGs synthesis.

Glucose transport and metabolism

Glucose is the main metabolic fuel for fetal intrauterine growth and is one of the major nutrients transported from maternal circulation [56]. In pig placenta, SLC2A family of glucose transporters including 14 members (SLC2A1-SLC2A14), are responsible for transporting glucose in addition to multiple other sugars from maternal to fetus and SLC2A1 and SLC2A3 play the prominent role among these transporters [57, 58]. In present study, we globally determined all transporters belong to SLC2A family and not surprisingly found that SLC2A1 and SLC2A3 were dramatically increased with leu supplementation, suggesting more glucose delivery between maternal and fetus.

Interestingly that although glucose is the major metabolic fuel, but fructose shows the most abundant hexose sugar in porcine endometria and conceptuses to support conceptus development as a substrate in many metabolic pathway [59]. In porcine placenta, glucose could be actively converted to fructose and then enter the hexosamine biosynthesis pathway and stimulate trophoblast proliferation or transported into fetus to supply energy through glycolytic pathway [60]. Because that fructose is undetected in blood of pregnant gilts and sows, placental synthesis from glucose is the main source of fructose for intrauterine fetus during pregnancy [61]. We interestingly found that placenta glucose and fructose amount was both greatly higher in the sows fed with 0.4% Leu compared with control group, but the expression of fructose transporters SLC2A5 and SLC2A8 showed no difference induced by addition of Leu [62]. This result suggested that the promoted fructose synthesis from glucose induced by Leu in placenta just only was metabolized within placenta to provide energy, but no contribution to fetal supply.

Energy metabolism and mTOR signal pathway

Constant and abundant source of energy supply is necessary in placenta for its specialized roles, both nutrient transportation and the synthesis of placental protein hormones, as well as for its nonspecific functions [63]. Previous studies indicated that intensity of energy metabolism in placenta was greatly decreased in intrauterine growth restriction and resulted in disturbances of the placental morphological structure and functional insufficiency. [64]. In present study, the activities of enzymes in placenta involved in energy metabolism including carbohydrate metabolism (hexokinase, glycogen phosphorylase and lactate dehydrogenase), tricarboxylic acid cycle enzyme (succinate dehydrogenase) and fatty

acid oxidation (hydroxyacyl-CoA-dehydrogenase) were all significantly enhanced with Lue supplementation in sow diet, implying more intensive energy metabolism to provide ATP for placenta development and function.

The mechanisms by which the placenta adjusts signals to meet appropriate intrauterine fetal growth are complex [5, 65]. The mammalian target of **rapamycin** (mTOR) protein is a Ser/threonine-specific protein kinase that belongs to the PI3K-related kinase (PIKKs) family, which functions in the regulation of cell growth and protein transcription by serving as a nutrient sensor during gestation, and a downstream target of the PI3K and PKA pathways [66, 67]. Previous studies have shown that the inhibition of mTOR activation results in impaired placental function and elevated expression of mTOR as compensation of Intrauterine growth retardation (IUGR) [68]. In current study, we found that mTOR pathway is higher activated in placenta from the sows fed with Leu, demonstrated by elevated levels of p-PI3K, p-Akt, and p-mTOR, implying that PI3K/Akt/mTOR signal pathway was involved in the regulation of leu to placenta nutrient transport and metabolism.

Conclusion

The present study suggests that dietary Lue supplementation during late gestation improved amino acids, fatty acids, and glucose transport cross placenta, and globally altered placental metabolism to enhance glycolysis and fatty acids oxidization to supply energy to support more nutrient transport. In addition, PI3K/AKT/mTOR signal pathway was involved in the regulation of leu to placenta nutrient transport and metabolism. It would be of great interest in future studies to clarify the mechanism of regulation of PI3K/Akt/mTOR by Leu in an in vitro model.

Abbreviations

Aad: Amino adipic acid; Akt: Protein kinase B; Ala: Alanine; ANOVA: One-way analysis of variance; Arg: Arginine; Asp: Aspartic acid; CAT: Catalase; Cit: Citrulline; Cys: Cysteine; EFA: Essential fatty acids; Gln: Glutamine; Glu: Glutamic acid; Gly: Glycine; GSH-Px: Glutathione peroxidase; GSH-Tr: Glutathione transferase; Harg: Homoarginine; His: Histidine; HODE: Hydroxy-octadecadienoic acid; HPLC-MS/MS: High performance liquid chromatography–tandem mass spectrometry; Hpro: Hydroxyproline; IUGR: Intrauterine growth retardation; Ile: Isoleucine; KEGG: Kyoto Encyclopedia of Genes and Genomes; Leu: Leucine; Lys: Lysine; MDA: Malonaldehyde; Met: Methionine; mTOR: Mammalian target of rapamycin; NHis: N-histidine; PGs: Prostaglandins; Phe: Phenylalanine; PI3K: Phosphatidylinositol 3-kinase; PLS-DA: Partial least-square discriminant analysis; Pro: Proline; PSer: Phosphoserine; PUFAs: Polyunsaturated fatty acids; PVDF: Polyvinylidene difluoride; Ser: Serine; SOD: Superoxide dismutase; Tau: Taurine; Trp: Tryptophan; Val: Valine; VIP: Variable importance on projection

Declarations

Ethics approval and consent to participate

All the animal procedures used during this experiment were approved by the South China Agricultural University Animal Care and Use Committee.

Consent for publication

Not applicable.

Conflicts of interest

The authors state there is no conflict of interest.

Availability of data and materials

The data analyzed during the current study are available from the corresponding author on reasonable request.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

CC, WG, SZ and FC conceived and designed the experiments; CC, CW and PZ performed the experiments; CC, JW, ZM and XZ used software to analyze and examine the data. CC and FC wrote and revised the article. All authors read and approved the final version of the manuscript.

Acknowledgment

Not applicable.

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Tables

Table 1. Composition of the diet (as-fed basis)

Ingredient (%)	Added leucine level (%)		
	CON	0.40	0.80
Corn	66.00	66.00	66.00
Soybean meal	18.5	18.5	18.5
Soybean oil	1.00	1.00	1.00
Wheat bran	9.50	9.50	9.50
Limestone	1.20	1.20	1.20
Dicalcium phpsphate	0.50	0.50	0.50
Salt	0.30	0.30	0.30
L-lysine HCL(78%)	0.12	0.12	0.12
DL-methionine(100%)	0.02	0.02	0.02
L-threonine(98.5%)	0.06	0.06	0.06
L-tryptophan(98.5%)	0.02	0.02	0.02
L-valine(98.5%)	0.07	0.07	0.07
L-leucine(98.5%)	0.00	0.40	0.80
Vitamin-mineral premix ^a	0.20	0.20	0.20
Choline chloride(50.0%)	0.30	0.30	0.30
Phytase	0.02	0.02	0.02
Mold removal agent	0.10	0.10	0.10
Mold inhibitors	0.05	0.05	0.05
antioxidant	0.04	0.04	0.04
carrier	2.00	1.60	1.20
Total	100.00	100.00	100.00

Note: ^aVitamin and mineral premix (by per kilogram of diet): vitamin A, 13 000 IU; vitamin D3, 4000 IU; vitamin E, 60 mg; vitamin K3, 4 mg; vitamin B1, 4 mg; vitamin B2, 10 mg; vitamin B6, 4.8 mg; vitamin B12, 0.034 mg; niacin, 40 mg; pantothenic acid, 20 mg; folic acid, 2 mg; biotin, 0.16 mg; 80 mg of Fe (as FeSO₄·H₂O); 5 mg of Cu (as CuSO₄· 5H₂O); 51 mg of Zn (as ZnSO₄·H₂O); 20.5 mg of Mn (as MnSO₄· H₂O); 0.14 mg of I (as Ca(IO₃)₂); and 0.15 mg of Se (as sodium selenite).

Table 2. Nutritional value of the diet (as-fed basis)

Nutritional level (%) ^a	Added leucine level (%)		
	CON	0.40	0.80
Digestible energy,MJ/kg	13.35	13.31	13.27
Crude protein	14.99	15.19	15.40
Calcium	0.86	0.86	0.86
Total phosphorus	0.64	0.64	0.64
nonphytic acid phosphorus	0.39	0.39	0.39
Salt	0.36	0.36	0.36
Arginine	0.96	0.95	0.95
lysine	0.79	0.79	0.79
methionine	0.24	0.24	0.24
Methionine+cysteine	0.46	0.46	0.46
threonine	0.06	0.06	0.06
Tryptophan	0.18	0.18	0.18
Valine	0.74	0.74	0.74
Isoleucine	0.56	0.56	0.56
Leucine	1.30	1.70	2.10
Phenylalanine	0.71	0.71	0.71
Phenylalanine+tyrosine	1.21	1.21	1.21

^aCalculated values according to the tables of feed composition and nutritive values in China.

Table 3. Primer sequences used in Real-time PCR

Genes	Accession	Sequencens5'-3'	
SLC1A4	XM_003125088	F:AGACCTCTCTTTGATCCTGGC	R:TGTTTCCTCCTCTGATTTGCA
SLC7A1	NW_003611328.1	F:GCCTGAGAGCAAGACCAAAC	R:GCCGTAGCCGAAGTAGATGA
SLC7A2	EU155140	F:GCCCCAGAATCAGCAAAAAGTA	R:GATGCTGAAGGCTGGCAAAA
SLC7A5	NM_003486	F:CTCTTCCTGATCGCCGTCTC	R:CTTCTGACACAGGACGGTCGT
SLC7A6	NM_003983	F:CTGCCGCCTGCATGTGT	R:TGTGCCCCACTTGACATAGG
SLC7A7	EU047705	F:TTTGTTTCCCAAGTTGCA	R:GCAGCTTCCTGGCATTGC
SLC7A8	AF135830	F:TCGCTGTGACTTTTGGAGAGA	R:CGGGAGGAGGTGAAGAGG
SLC7A10	XM_003127014	F:TGGACCCTCGAAAGAACCTAC	R:TGATCCCTCCGAAAGTGGA
SLC7A11	AB271957	F:AATGTGGCCTACTTCACGACC	R:CCTCCCGAGATGCAACATAGA
SLC15A1	NM_214347.1	F:TTCGTTTATCCCTTGAGCATCTTCTTC	R:GTGGTAGATGGCAGTGGACAGATTG
SNAT1	XM_003355629	F:AAGAACCTGGGCTATCTCGG	R:TGTTGCGTTAGGACTCGTTG
SNAT2	NM_018976	F:GTTACCTTTGGTGATCCAGGC	R:ACCAATGACACCAGCAGAACC
LAT1	NM_003486	F:GCCCATTGTCACCATCATC	R:GAGCCCACAAAGAAAAGC
4F2hc	XM_003353809	F:CTCGAACCCACCAAGGAC	R:GAGGTGAGACGGCACAGAG
rBAT	EU587017	F:TTTCCGCAATCCTGATGTTC	R:GGGTCTTATTCACTTGGGTC
SGLT1	NM_001164021	F:CATCATCGTCCTGGTCGTC	R:CATCATCGTCCTGGTCGTC
SGLT3	NM_214182	F:CCTTGGGATTGGACCTTTATC	R:CGTTTGCGGAGGGTGTCTGTGT
SGLT5	NM_001012297	F:GCGTCCAGATCGGCAGAACCT	R:GCGTCCAGATCGAGAACCT
CD36	DQ192230.1	F:GGA CTATTGCTGGTGCTGT	R:GTCTGTAACTTCCGTGCCTGT
FATP1	NM_001083931.1	F:GGCAACAGACGTGATCTATGAC	R:AGCGGCTGGCTGAAAAC
FATP2	JX092264.1	F:TCTAACACGGATGGGGTCG	R:AGGGCAGGAGTGGAAGT
FATP3	XM_001929591.2	F:AGGTCTCAGCCGAAGTGGAT	R:TGGGAGGGCGAGGTAGAT
FATP4	XM_003353676.1	F:AGCCGCATCCTGTCCTTT	R:GACATCCTTGGCGATCTTTT
FABP3	AY569332.1	F:CTGGGAGTGGAGTTTGATGAGAC	R:CCATGGGTGAGTGTGAGGAT
FABP4	NM_001002817.1	F:TGAAAGGTGTCACGGCTAC	R:TCGGGACAATACATCCAACAGAG
FABP5	AY841270.1	F:CTGGGACAGAAGTTTGAAGAGAC	R:GACCCGAGTGCAGGTGACATT
FABP7	NM_001025229.1	F:GACTCAAAGCACATTCAAGAAC	R:GCAACCACATCACCAAAAGTAA
SLC2A1	XM_021096908.1	F:GCTCCTGGTCCTGTTCTTCATCTTC	R:CTCGGGTGTCTTGTGCTTTGG
SLC2A2	NM_001097417.1	F:CCATTGTCACAGGCATTCTTGTTAGTC	R:CACAGCAGATAGACCAAGCAGGATG
SLC2A3	XM_021092391.1	F:TCTCCATCATGCTCCAGCTCTCC	R:AATAGTGGCATAGATTGGCTCCTGAAC
SLC2A5	XM_021095282.1	F:CTCTTCATCACCGTTGGCATCCTC	R:GGGAAGAAGGGCAGGCAAAGAAG
SLC2A6	XM_003353701.4	F:GGCTCCTGTCTGTGCTGATTGC	R:ATGGTGGCTCCTGAGACGAAGAG
SLC2A8	XM_003480608.4	F:CTTCGTGTCGCGCTCTCACTG	R:CCAGGCTGCTCTCCTTGAACCTG

Figures

Figure 1

A) 2D scores plot showing PLS-DA discrimination between placentas from sows fed with control diet, control + 0.4% Leu and control + 0.8% Leu; B) 2D scores plot showing PLS-DA discrimination between placentas from sows fed with control diet vs control + 0.4% Leu; C) 2D scores plot show in PLS-DA discrimination between placentas from sows fed with control diet vs control + 0.8% Leu; D) 2D scores plot showing PLS-DA discrimination between placentas from sows fed with control + 0.4% Leu vs control + 0.8% Leu; E) Differential metabolites with more than 2 folds change of KEGG pathway analysis; F) The colored boxes on the right indicate the abundance of different metabolites in control diet, control + 0.4% Leu, and control + 0.8% Leu. VIP scores are based on the PLS-DA model.

Figure 2

Biological signaling pathway were identified by using KEGG pathway analysis. A) Biological pathway between control group, control + 0.4% Leu and control + 0.8% Leu; B) Biological pathway between control group and control + 0.4% Leu; C) Biological pathway between control group and control + 0.8% Leu; D) Biological pathway between control + 0.4% Leu and control + 0.8% Leu; E) Graphical presentation of abundance of different amino acid with VIP scores.

Figure 3

A) Effects of supplementation with 0.4% and 0.8% Leu of sows on mRNA expression of amino acid transporters in placente; B) Effects of supplementation with 0.4% and 0.8% Leu of sows on mRNA expression of glucose transporters in placente; C) Effects of supplementation with 0.4% and 0.8% Leu of sows on mRNA expression of fatty acids transporters in placente.

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Figure 4

Effects of supplementation 0.4% and 0.8% Leu in late pregnancy on the expression of enzymes activities, including hexokinase, glycogen phosphorylase, lactated hydrogenase, hydroxyaccyl- CoA- dehydrogenas and succinatede hydrogenase. Data are showed as means $\pm$ SEM (n = 6), Different letters (a, b, c) indicate significant differences ($P < 0.05$).

Figure 5

Effects of supplementation 0.4% and 0.8% Leu in late pregnancy on expressions of mTOR signal pathway proteins, including P-PI3K/T-PI3K, P-Akt/T-Akt and P-mTOR/T-mTOR. Data are shown as means $\pm$ SEM (n = 6), Different letters (a, b, c) indicate significant differences ($P < 0.05$).

Figure 6

Effects of supplementation 0.4% and 0.8% Leu in late pregnancy on blood oxidative status, including GSH-Px, GSH-Tr, CAT, MDA. Data are showed as means $\pm$ SEM (n = 6).