Cellular Physiology and Biochemistry

Cell Physiol Biochem 2018;48:2103-2113

DOI: 10.1159/000492552

Published online: August 9, 2018

Accepted: July 31, 2018

Original Paper

Suckling Piglet Intestinal Enterocyte Nutrient Metabolism Changes

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Key Words

Differentiated enterocytes • Intestine • Nutrient metabolism • Piglet • Suckling period

Abstract

Background/Aims: Intestinal morphology and the types of enterocytes are changed in piglets during the suckling period, but it is unclear whether these changes are associated with metabolic changes in epithelium. The present study was conducted to test the hypothesis that glucose, fatty acids, and amino acid metabolism in differentiated piglet enterocytes changed during suckling. ***Methods:*** Twenty-four piglets (Duroc × [Landrace × Yorkshire]) from 8 litters (3 piglets/litter) were selected. A single piglet from each litter was randomly selected and euthanized at days 7, 14, and 21. Differentiated enterocytes (DE) were isolated from their mid-jejunum. Isobaric tags for relative and absolute quantification and subsequent liquid chromatography-tandem mass spectrometry were used to identify and measure protein synthesis. ***Results:*** The results showed that various activities, including: cellular processes; metabolic processes; biological regulation; pigmentation; and, localization, in DEs changed during suckling. Metabolic process analyses revealed that protein expression related to glycolysis and citrate cycle was decreased from day 7 to day 14. The number of differentiated enterocytes of 21 d piglets increased compared to 7 d piglets. Most of the proteins involved in fatty acid and amino acids metabolism had decreased DE expression between day 7 and day 14. Some, but not all, detected proteins down-regulated in DEs of 21 day piglets compared to 7 day piglets. ***Conclusion:*** These results indicate that glucose, fatty acids, and amino acids metabolism changed during suckling. This may provide useful information for designing feed formulas and regulating piglet intestinal growth and development.

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Introduction

Piglet gastrointestinal systems develop intensively during their first few weeks [1, 2]. The most intensive changes occur in the intestinal epithelium [2, 3]. Villi, and crypt, depth increase rapidly post-partum. Villi shape changes during the early postnatal period [1, 4, 5]. Intestinal enterocytes, which constitute more than 90% of all intestinal epithelial cells, play key roles in digesting and absorbing luminal nutrients. Vacuolated fetal-type enterocytes are replaced by adult-type enterocytes in piglets during suckling [6]. Studies to date that were conducted to investigate piglet intestinal development have focused on intestinal growth, morphology, and functional changes [3-6]. It remains unclear whether intestinal morphology or enterocyte type changes in suckling piglets associate with epithelial metabolic change.

The intestine has a high rate of nutrient utilization via the portal-drained viscera (PDV) which includes the stomach, intestines, pancreas, and spleen. It contributes about 5% of body weight and accounts for 20-35% of whole-body protein synthesis and energy expenditure. Most nutrients are utilized by the intestinal epithelium [7-9]. About one-third of all essential dietary amino acids are consumed by the intestine during first-pass metabolism. It has been reported that small intestine growth and development depends largely on luminal nutrient composition [3, 4]. Understanding nutrient metabolism in the intestinal epithelium is central to designing milk replacement formulas and regulating gastrointestinal system growth and development [3].

Using a label-free quantitative proteomics approach, Hansson et al. found that carbohydrate, amino acids, lipid, fatty acids, and steriod metabolisms changed in mice intestinal epithelial cells during suckling [10]. There are marked differences between early life rodent and piglet intestinal maturation stages [11]. The intestinal epithelium undergoes continual renewal along the crypt-villus axis. Nutrient metabolism differs between villus and crypt epithelial cells in piglets [2, 12, 13]. Differentiated enterocytes, located in the upper villi, are the primary intestinal epithelial cells involved in nutrient digestion and absorption [2, 14]. This experiment is premised on thehypothesis that glucose, fatty acids, and amino acids metabolism in DEs in piglets change during suckling. The current study purposed to investigate nutrient metabolism in sucking piglet upper villus enterocytes.

Materials and Methods

The experimental design and procedures in this study were reviewed and approved by the Animal Care and Use Committee of the Institute of Subtropical Agriculture, Chinese Academy of Science.

Reagents

DL-β-Hydroxybutyrate sodium salt was purchased from J&K Chemical (Ltd., USA). Trypsin was procured from Promega (Madison, WI, USA). iTRAQ-reagent was purchased from Applied Biosystems (Foster City, CA, USA). Bovine serum alumin (BSA, fraction V), phenylmethylsulfonyl fluoride (PMSF), dithiothreitol (DTT), and other chemical were obtained from Sigma-Aldrich (St. Louis, MO, USA) unless otherwise stated.

Animals and intestinal upper villus enterocytes isolation

Twenty-four piglets (Duroc × [Landrace × Yorkshire]) from 8 litters (3 piglets per litter) were selected based on their BW and gender. Sows had free access to feed and drinking water. Piglets had free access to nipples and water during the experiment. One piglet from each litter was maintained under general anesthesia and intravenously euthanized via a jugular injection of 4% sodium pentobarbital solution (40 mg/kg body weight) at 7, 14, and 21 days of age. The DEs were isolated from jejunum upper villi using distended intestinal sac method as previously described [15, 16]. Ice-cold physiological saline solution rinsed jejunum segments are incubated with oxygenated phosphate buffered saline at 37 °C for 30 min. Intestine segments were incubated in an oxygenated isolation buffer (5 mM Na2EDTA, 10 mM HEPES pH

7.4, 0.5 mM DTT, 0.25% BSA, 2.5 mM D-glucose, 2.5 mM L-glutamine, 0.5 mM dl-β-hydroxybutyrate sodium salt, oxygenated with an O_2/CO_2 mixture (19:1, v/v)) at 37 °C for 40 min to isolate upper villus enterocytes. The isolation buffers were collected and centrifuged at 400 g for 4 min at 4 °C. The collected enterocytes were twice washed with an oxygenated cell suspension buffer (10 mM HEPES, 1.5 mM $CaCl_2$, 2.0 mM $MgCl_2$, pH 7.4). The isolated upper villus enterocytes were confirmed by testing alkaline phosphatase activity and proliferating cell nuclear antigen (PCNA) expression.

Sample preparation and isobaric labeling

A lysis buffer (7 M urea, 2 M thiourea, 4% w/v 3-[(3-Cholamidopropyl) dimethylammonio] propanesulfonate, 20 mMTributyl phosphate, and 0.2% Bio-lyte (pH 3-10)) and a protease inhibitor cocktail (Roche Diagnostics Ltd, Mannheim, Germany) re-suspended and disrupted the isolated enterocytes. The lysate was then treated with DNAse I and RNAse A at final concentrations of 1 mg/mL and 0.25 mg/mL, respectively. The protein solution was centrifugally separated from the cell debris at 12, 000 × g, 4 °C for 5 min. A Ready Prep 2-D Cleanup Kit (Bio-Rad Laboratories, USA) was used to further purify the crude protein. Purified protein underwent a reductive alkylation reaction. The protein concentration was measured using a 2-D Quant Kit (GE Healthcare, USA). Trypsin digestion and iTRAQ labeling were performed according to manufacturer protocol (Applied Biosystems, Foster City, CA, USA). A sample of 100 μg total protein from each was reduced, alkylated, and digested overnight with trypsin (Promega, Madison, WI, USA) at 37 °C. It was then labeled with iTRAQ-reagents (Applied Biosystems, Foster City, CA, USA) as follows: 7d, iTRAQ reagent 115; 14d, iTRAQ reagent 116; 21d, iTRAQ reagent 117.

Peptide fractionation and LC-MS/MS acquisition

The isotopically-labeled samples were pooled. An Ultremex SCX column, containing 5-μm particles (Phenomenex, USA), fractionated the pooled samples into 12 fractions. A Strata X C18 column (Phenomenex, USA) desalted the eluted fractions. The desalted fractions were then vacuum-dried. Average peptide concentration in each fraction was adjusted to 0.25 μg/μL. Dried peptides were stored at -80 °C until used for MS analysis. The analytical separation was performed using a nanospray ion source (Waters, USA) system (coupled with Triple TOF). Symmetry C18- packed microfluidic traps (5 μm, 180 μm × 20 mm) and nanofluidic columns were employed in online trapping, desalting. BEH130 C18-packed (1.7 μm, 100 μm × 100 mm) nanofluidic columns were used for analytical separation. Water/acetonitrile/formic acid (A: 98/2/0.1%; B: 2/98/0.1%) composed solvents were used. A portion of the 2.25 μg (9 μL) sample was loaded, trapped, and desalted. Analytical separation was established by maintaining 5% B for 1 min at a 300 nL/min flow rate. The following then occured: a linear gradient to 35% B occurred at 40 min, this gradient increased to 80% B at 45 mi; and was maintained for 5 min after the peptide elution window. Initial chromatographic conditions were restored in 2 min.

A Triple TOF 5600 System (AB SCIEX, USA), fitted with a Nanospray III source (AB SCIEX, USA) and a pulled quartz tip as the emitter (New Objectives, USA), was used for data acquisition. Data was acquired under the condition of an ion spray voltage of 2.5 kV, curtain gas of 30 Psi, nebulizer gas of 15 Psi, and an interface heater temperature of 150 °C. The MS was operated with a RP ≥ 30, 000 FWHM for the TOF MS scans. Information dependant acquisition (IDA) survey scans were acquired in 250 ms and as many as 30 product ion scans were collected if they exceeded a 120 counts per second (counts/s) threshold with a 2+ to 5+ charge-state. Total cycle time was fixed to 3.3 s and the Q2 transmission window was 100 Da for 100%.

Four time bins were summed for each scan at a pulser frequency value of 11 kHz via monitoring of the 40 GHz multichannel TDC detector with four-anode/channel detection. A sweeping collision energy setting of 35 ± 5 eV coupled with iTRAQ adjusted rolling collision energy was applied to all precursor ions for collision-induced dissociation. Dynamic exclusion was set for 1/2 of the peak width (18 s). The precursor was then refreshed off the exclusion list.

Database analysis and quantification

Mascot software (version 2.3.02, Matrix Science) was used to identify and quantify proteins. Searches were made against the NCBI non-redundant database consisting of *Sus scrofa* proteins. Spectra from the 12 fractions of each sample were combined into one MGF (Mascot generic format) file after the raw data was loaded. The MGF file was searched using the following search parameters: i) trypsin was selected as the enzyme with one missed cleavage allowed; ii) the fixed modifications of carbamidomethylation were set as

Cys; iii) peptide tolerance was set as 0.05 Da; and, iv) MS/MS tolerance set at 0.1 Da. An automatic decoy database search strategy was used to estimate the false discovery rate (FDR). False positive matches divided by the total matches were employed to calculate FDR. The final FDR was less than 1.5%. Search results were passed through additional filters prior to data exportation. The filters used for protein identification were: 1) significance threshold $P<$ 0.05 (with 95% confidence); and, 2) ion score or expected cutoff $<$ 0.05 (with 95% confidence). The filters for protein quantitation were: 1) "median" was used for the protein ratio type; 2) the minimum precursor charge was set as 2+ and the minimum peptides were set as 2. Only 2 or more than 2 unique peptides were used to quantify proteins using IQuant (BGI-Shenzhen, China). The median intensities were set as normalization, and outliers were removed automatically. In the present study, a protein with more than 1.2-fold or less than 0.8-fold difference between day 7 and day 14, or day 21, and a *P*-value ≤ 0.05 (using t-test) was deemed differentially expressed (IQuant, China).

Bioinformatics analysis

The Blast2GO program was used for functional annotations of the differentially expressed proteins against non-redundant database consisting of *Sus scrofa* proteins [17]. The differentially-expressed protein biological process ontology was performed using WEGO program [18]. The metabolic pathway analysis was performed using the Kyoto Encyclopedia of Genes and Genomes (KEGG) Pathway database.

Results

Gene ontology category and KEGG pathway analysis

We identified 2417 unique proteins. Of these, 1269 were differentially synthesized between day 7 and day 14; or day 7 and day 21. Differentially-expressed protein functional annotations were classified in terms of biological process with a WEGO program for the overrepresentation of specific gene ontology (GO) terms. Differentially-expressed proteins were primarily involved in cellular process, metabolic process, biological regulation, pigmentation, localization, multicellular organismal process, cellular component organization, establishment of localization, response to stimulus, developmental process (Fig. 1). A KEGG pathway enrichment analysis showed that the differentially expressed proteins were primarily involved in ribosome, carbon metabolism, protein processing in endoplasmic reticulum,

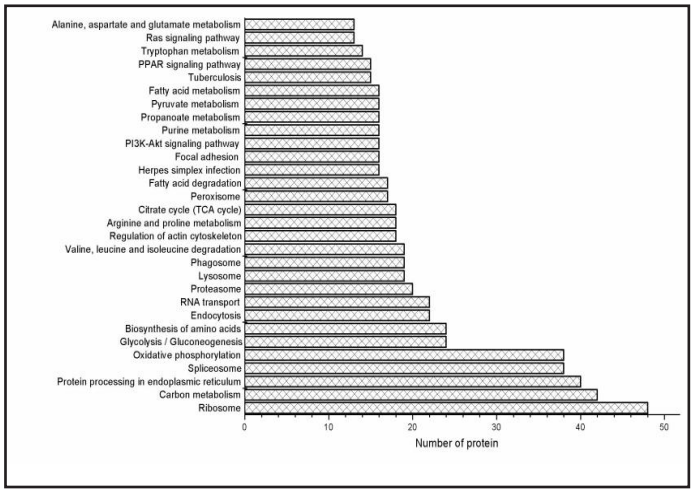

Fig. 1. Functional categories of differentially-expressed proteins in suckling pig jejunal upper villus enterocytes. Gene ontology (GO) biological processes for significantly differentially-expressed proteins performed using WEGO.

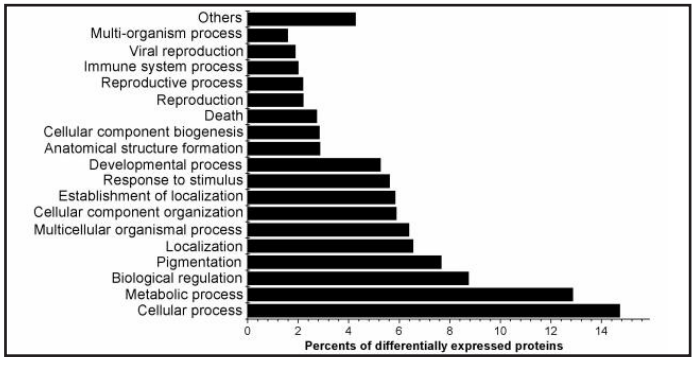

Fig. 2. Top Kyoto Encyclopedia of Genes and Genomes pathways enriched with differentially-expressed suckling piglet jejunal upper villus enterocyte proteins.

Table 1. Abundance changes of proteins involved in glycolysis injejunal upper villus enterocytes of suckling piglets

Proteins	KEGG Orthology	Fold change	
		D14 vs D7	D21 vs D7
Alcohol dehydrogenase (NADP+)	K00002	0.85	1.56
L-lactate dehydrogenase	K00016	1.25	1.43
S-(hydroxymethyl)glutathione dehydrogenase / Alcohol dehydrogenase	K00121	0.72	1.54
Aldehyde dehydrogenase (NAD+)	K00128	0.58	0.93
Glyceraldehyde 3-phosphate dehydrogenase	K00134	0.74	1.31
Aldehyde dehydrogenase family 9 member A1	K00149	0.94	1.28
Pyruvate dehydrogenase E1 component subunit alpha	K00161	1.68	2.25
Pyruvate dehydrogenase E1 component subunit beta	K00162	0.57	1.26
Hexokinase	K00844	1.75	1.65
6-phosphofructokinase 1	K00850	1.45	1.43
Pyruvate kinase	K00873	1.55	2.26
Phosphoenolpyruvate carboxykinase (GTP)	K01596	0.70	0.77
Fructose-bisphosphate aldolase, class I	K01623	1.62	1.67
Enolase	K01689	0.97	1.22
Aldose 1-epimerase	K01785	0.70	1.36
Triosephosphate isomerase (TIM)	K01803	0.93	1.33
Glucose-6-phosphate isomerase	K01810	1.19	1.26
2,3-bisphosphoglycerate-dependent phosphoglycerate mutase	K01834	0.88	1.41
Acetyl-CoA synthetase	K01895	1.25	1.25
Fructose-1,6-bisphosphatase I	K03841	0.84	0.79
Aldehyde dehydrogenase family 7 member A1	K14085	0.68	1.03
Phosphoglucomutase / phosphopentomutase	K15779	0.97	1.35

Table 2. Abundance changes of proteins involved in fatty acid metabolism injejunal upper villus enterocytes of suckling piglets

Proteins	KEGG Orthology	Fold change	
		D14 vs D7	D21 vs D7
3-hydroxyacyl-CoA dehydrogenase	K00022	0.83	2.32
Acyl-CoA oxidase	K00232	0.99	1.37
Acyl-CoA dehydrogenase	K00249	0.68	0.96
Long-chain-acyl-CoA dehydrogenase	K00255	0.75	0.72
Acetyl-CoA C-acetyltransferase	K00626	0.53	1.00
Fatty acid synthase, animal type	K00665	0.95	1.50
Long-chain acyl-CoA synthetase	K01897	0.77	0.75
Acetyl-CoA acyltransferase	K07509	0.74	1.18
Enoyl-CoA hydratase	K07511	0.58	0.95
Acetyl-CoA acyltransferase 1	K07513	0.92	1.30
Enoyl-CoA hydratase / 3-hydroxyacyl-CoA Dehydrogenase / 3,2-trans-enoyl-CoA isomerase	K07514	1.00	1.29
Enoyl-CoA hydratase / long-chain 3-hydroxyacyl-CoA dehydrogenase	K07515	1.45	1.02
Peroxisomal trans-2-enoyl-CoA reductase	K07753	1.25	1.08
Carnitine O-palmitoyltransferase 1	K08765	0.45	0.98
Very long chain acyl-CoA dehydrogenase	K09479	0.79	0.74

Table 3. Abundance changes of proteins involved in amino acids metabolism in jejunal upper villus enterocytes of suckling piglets

Proteins	KEGG Orthology	Fold change	
		D14 vs D7	D21 vs D7
Enoyl-CoA hydratase / 3-hydroxyacyl-CoA Dehydrogenase / 3,2-trans-enoyl-CoA isomerase	K07514	1.00	1.29
2-oxoglutarate dehydrogenase E1 component	K00164	0.77	1.29
Aldehyde dehydrogenase family 9 member A1	K00149	0.94	1.28
D-amino-acid oxidase	K00273	0.69	1.27
Carbamoyl-phosphate synthase (ammonia)	K01948	0.82	1.26
Methylmalonyl-CoA/ethylmalonyl-CoA epimerase	K05606	1.30	1.24
Alanine transaminase	K00814	1.88	1.18
Acetyl-CoA acyltransferase	K07509	0.74	1.18
3-oxoacid CoA-transferase	K01027	0.68	1.14
Glucosamine--fructose-6-phosphate aminotransferase (isomerizing)	K00820	1.12	1.13
Aldehyde dehydrogenase family 7 member A1	K14085	0.68	1.03
Enoyl-CoA hydratase / long-chain 3-hydroxyacyl-CoA dehydrogenase	K07515	1.45	1.02
Acetyl-CoA C-acetyltransferase	K00626	0.53	1.00
Argininosuccinate synthase	K01940	0.27	0.99
Acyl-CoA dehydrogenase	K00249	0.68	0.96
Enoyl-CoA hydratase	K07511	0.58	0.95
Aldehyde dehydrogenase (NAD+)	K00128	0.58	0.93
Glutamate dehydrogenase (NAD(P)+)	K00261	0.67	0.91
Cytosolic nonspecific dipeptidase	K08660	0.64	0.85
Ornithine--oxo-acid transaminase	K00819	0.56	0.84
Kynurenine---oxoglutarate transaminase / cysteine-S-conjugate beta-lyase / glutamine---phenylpyruvate transaminase	K00816	0.76	0.79
Catalase	K03781	0.85	0.77
Omega-amidase	K13566	0.74	0.76
Diamine oxidase	K11182	0.67	0.76
Propionyl-CoA carboxylase beta chain	K01966	0.87	0.76
Argininosuccinate lyase	K01755	0.53	0.73
Hydroxymethylglutaryl-CoA lyase	K01640	0.64	0.72
Isovaleryl-CoA dehydrogenase	K00253	0.83	0.59
Succinate-semialdehyde dehydrogenase	K00139	0.62	0.58
Aspartate aminotransferase, cytoplasmic	K14454	1.42	0.57
Cytosol aminopeptidase	K11142	0.39	0.44
3-hydroxyanthranilate 3,4-dioxygenase	K00452	0.55	0.44
Glycine amidinotransferase	K00613	0.47	0.40
Alanine-glyoxylate transaminase / (R)-3-amino-2-methylpropionate-pyruvate transaminase	K00827	0.32	0.36

spliceosome, oxidative phosphorylation, glycolysis/gluconeogenesis, biosynthesis of amino acids, endocytosis, and RNA transport (Fig. 2). These results suggest that the expression of proteins involved in various metabolic pathways in piglet jejunal differentiated enterocytes changes during suckling. This present study focused on proteins related to nutrient metabolism, including glycolysis, fatty acid metabolism, amino acid metabolism, and citrate cycle.

Glycolysis

A total of 22 differentially-expressed proteins involved in glycolysis were identified in piglet jejunal upper villus enterocytes during suckling (Table 1). The expression of L-lactate dehydrogenase, pyruvate dehydrogenase E1 component subunit alpha, hexokinase, 6-phosphofructokinase 1, pyruvate kinase, fructose-bisphosphate aldolase (class I), and acetyl-CoA synthetase was up-regulated in jejunal upper villus enterocytes of 14 d old piglets compared with that in 7 d old piglets (Table 1). However, the expression of S-(hydroxymethyl) glutathione dehydrogenase/Alcohol dehydrogenase, aldehyde dehydrogenase (NAD^+), glyceraldehyde 3-phosphate dehydrogenase, pyruvate dehydrogenase E1 component subunit beta, phosphoenolpyruvate carboxykinase (GTP), aldose 1-epimerase, and aldehyde dehydrogenase family 7 member A1 was down-regulated in jejunal upper villus enterocytes of 14 d old piglets compared with that in 7 d old piglets (Table 1). The expression of most of the proteins related to glycolysis was up-regulated in jejunal upper villus enterocytes of 21 d old piglets compared with that in 7 d old piglets (Table 1).

Fatty acid metabolism

Fifteen proteins involved in fatty acid metabolism were differentially expressed in piglet jejunal upper-villus enterocytes during suckling (Table 2). Most of the proteins involved in fatty acid metabolism were down-regulated in jejunal upper villus enterocytes of 14 d old piglets compared with that in 7 d old piglets (Table 2). The expression of 3-hydroxyacyl-CoA dehydrogenase, acyl-CoA oxidase, Fatty acid synthase (animal type), acetyl-CoA acyltransferase 1, and enoyl-CoA hydratase/3-hydroxyacyl-CoA dehydrogenase/3, 2-trans-enoyl-CoA isomerase was up-regulated in jejunal upper villus enterocytes of 21 d old piglets compared with that in 7 d old piglets, while the expression of long-chain-acyl-CoA dehydrogenase, long-chain acyl-CoA synthetase, and very long chain acyl-CoA dehydrogenase was down-regulated in jejunal upper villus enterocytes of 21 d old piglets compared with that in 7 d old piglets (Table 2).

Amino acid metabolism

The down-regulation of 34 differentially-expressed proteins involved in amino acid metabolism were identified in suckling piglet jejunal upper villus enterocytes (Table 3). Most of the proteins involved in amino acid metabolism (e.g. alanine, aspartate and glutamate metabolism, valine, leucine and isoleucine degradation, arginine and proline metabolism, and tryptophan metabolism) were down-regulated in jejunal upper villus enterocytes of 14 d old piglets compared with that in 7 d old piglets (Table 3; Table 4-7). More enzymes involved in alanine, aspartate and glutamate metabolism, valine, leucine and isoleucine degradation, arginine and proline metabolism, and tryptophan metabolism expressed up-regulation than down-regulation in 21-day piglet jejunal upper villus enterocytes than in 7-day piglets (Table 4-7).

Table 4. Abundance changes of proteins involved in alanine, aspartate and glutamate metabolism injejunal upper villus enterocytes of suckling piglets

Proteins	KEGG Orthology	Fold change	
		D14 vs D7	D21 vs D7
Succinate-semialdehyde dehydrogenase	K00139	0.62	0.58
Glutamate dehydrogenase (NAD(P)+)	K00261	0.67	0.91
Alanine transaminase	K00814	1.88	1.18
Alanine-glyoxylate transaminase / (R)-3-amino-2-methylpropionate-pyruvate transaminase	K00827	0.32	0.36
Argininosuccinate lyase	K01755	0.53	0.73
Adenylosuccinate synthase	K01939	0.93	1.36
Argininosuccinate synthase	K01940	0.27	0.99
Carbamoyl-phosphate synthase (ammonia)	K01948	0.82	1.26
Asparagine synthase (glutamine-hydrolysing)	K01953	0.69	1.67
4-aminobutyrate aminotransferase / (S)-3-amino-2-methylpropionate transaminase	K13524	0.94	1.46
Omega-amidase	K13566	0.74	0.76
Aspartate aminotransferase, cytoplasmic	K14454	1.42	0.57

Table 5. Abundance changes of proteins involved in valine, leucine and isoleucine degradation injejunal upper villus enterocytes of suckling piglets

Proteins	KEGG Orthology	Fold change	
		D14 vs D7	D21 vs D7
3-hydroxyisobutyrate dehydrogenase	K00020	0.71	0.49
3-hydroxyacyl-CoA dehydrogenase	K00022	0.83	2.32
Aldehyde dehydrogenase (NAD+)	K00128	0.58	0.93
Malonate-semialdehyde dehydrogenase (acetylating) / methylmalonate-semialdehyde dehydrogenase	K00140	1.18	2.96
Aldehyde dehydrogenase family 9 member A1	K00149	0.94	1.28
Acyl-CoA dehydrogenase	K00249	0.68	0.96
Isovaleryl-CoA dehydrogenase	K00253	0.83	0.59
Acetyl-CoA C-acetyltransferase	K00626	0.53	1.00
3-oxoacid CoA-transferase	K01027	0.68	1.14
Hydroxymethylglutaryl-CoA lyase	K01640	0.64	0.72
Propionyl-CoA carboxylase beta chain	K01966	0.87	0.76
Methylmalonyl-CoA/ethylmalonyl-CoA epimerase	K05606	1.30	1.24
Acetyl-CoA acyltransferase	K07509	0.74	1.18
Enoyl-CoA hydratase	K07511	0.58	0.95
Acetyl-CoA acyltransferase 1	K07513	0.92	1.30
Enoyl-CoA hydratase / 3-hydroxyacyl-CoA dehydrogenase / 3,2-trans-enoyl-CoA isomerase	K07514	1.00	1.29
Enoyl-CoA hydratase / long-chain 3-hydroxyacyl-CoA dehydrogenase	K07515	1.45	1.02
4-aminobutyrate aminotransferase / (S)-3-amino-2-methylpropionate transaminase	K13524	0.94	1.46
Aldehyde dehydrogenase family 7 member A1	K14085	0.68	1.03

Citrate cycle

Seventeen proteins involved in citrate cycle differentially expressed in piglet jejunal upper villus enterocytes during suckling (Table 8). The majority of these differentially-expressed proteins involved in citrate cycle down-regulated in 14-day jejunal upper villus piglet enterocytes than in 7-day piglets (Table 8). Most of the differentially-expressed proteins involved in citrate cycle up-regulated in 21-day jejunal upper villus piglet enterocytes than in 7-day piglets (Table 8).

Discussion

Visceral tissues, especially intestinal mucosa, have high nutrient metabolism rates [7]. Our results suggest that various biological processes in piglet DE changed during suckling. Differentially-expressed proteins were highly enriched in metabolic processes. This may be due to high intestinal enterocyte nutrient metabolism rates. Metabolic process changes in sucking pig intestinal differentiated enterocytes may be partly result from changes in the type of intestinal enterocytes resulting from vacuolated fetal-typeenterocytes replacement by non-vacuolatedadult-type in suckling piglet small intestines [6]. More research is needed to study the mechanism that affects metabolic processes of differentiated enterocytes in suckling piglets.

We further analyzed the specific metabolic pathways using KEGG pathway enrichment analysis. The results showed that the differentially-expressed proteins were primarily involved in energy metabolism (e.g., carbon metabolism, oxidative phosphorylation,glycolysis/ gluconeogenesis) and amino acids metabolism. Hansson et al. showed that biological processes related to carbohydrate metabolism, tricarboxylic acid pathway, fatty acid metabolism, amino acid metabolism, and electron transport changed in mice intestinal epithelial cells during suckling [10]. Although the intestinal epithelial cells (whole intestinal epithelial cells vs. DE) and experimental animals (mouse vs. pig) used in prior studies differ from the present experiment, the changed metabolic processes are similar. These results indicate that suckling animals may have similar metabolic changes in intestinal epithelial cells.

Intestinal mucosa nutrient metabolism is very complex. Nutrient sources for intestinal mucosa are provided by both arterial and luminal substrates [19]. The intestinal mucosa is the first tissue with the capability to use luminal nutrients. Nutrient metabolism in intestinal enterocytes can be altered by nutrients composition in the diet [20, 21]. Nutrients metabolism in villus enterocytes differs from that in crypt

Table 6. Abundance changes of proteins involved in arginine and proline metabolism injejunal upper villus enterocytes of suckling piglets

Proteins	KEGG Orthology	Fold change	
		D14 vs D7	D21 vs D7
Aldehyde dehydrogenase (NAD^+)	K00128	0.58	0.93
Aldehyde dehydrogenase family 9 member A1	K00149	0.94	1.28
Glutamate dehydrogenase ($NAD(P)^+$)	K00261	0.67	0.91
D-amino-acid oxidase	K00273	0.69	1.27
Monoamine oxidase	K00274	0.71	1.52
Glycine amidinotransferase	K00613	0.47	0.40
Spermine synthase	K00802	0.90	1.38
Ornithine--oxo-acid transaminase	K00819	0.56	0.84
Creatine kinase	K00933	0.98	1.67
Argininosuccinate lyase	K01755	0.53	0.73
Argininosuccinate synthase	K01940	0.27	0.99
Carbamoyl-phosphate synthase (ammonia)	K01948	0.82	1.26
Cytosolic nonspecific dipeptidase	K08660	0.64	0.85
Cytosol aminopeptidase	K11142	0.39	0.44
Diamine oxidase	K11182	0.67	0.76
Delta-1-pyrroline-5-carboxylate synthetase	K12657	0.85	1.31
Aldehyde dehydrogenase family 7 member A1	K14085	0.68	1.03
Aspartate aminotransferase, cytoplasmic	K14454	1.42	0.57
Argininosuccinate lyase	K01755	0.53	0.73
Argininosuccinate synthase	K01940	0.27	0.99
Carbamoyl-phosphate synthase (ammonia)	K01948	0.82	1.26

Table 7. Abundance changes of proteins involved in tryptophan metabolism injejunal upper villus enterocytes of suckling piglets

Proteins	KEGG Orthology	Fold change	
		D14 vs D7	D21 vs D7
3-hydroxyacyl-CoA dehydrogenase	K00022	0.83	2.32
Aldehyde dehydrogenase (NAD^+)	K00128	0.58	0.93
Aldehyde dehydrogenase family 9 member A1	K00149	0.94	1.28
2-oxoglutarate dehydrogenase E1 component	K00164	0.77	1.29
monoamine oxidase	K00274	0.71	1.52
3-hydroxyanthranilate 3,4-dioxygenase	K00452	0.55	0.44
Acetyl-CoA C-acetyltransferase	K00626	0.53	1.00
Kynurenine---oxoglutarate transaminase / cysteine-S-conjugate beta-lyase / glutamine---phenylpyruvate transaminase	K00816	0.76	0.79
Catalase	K03781	0.85	0.77
Enoyl-CoA hydratase	K07511	0.58	0.95
Enoyl-CoA hydratase / 3-hydroxyacyl-CoA dehydrogenase / 3,2-trans-enoyl-CoA isomerase	K07514	1.00	1.29
Enoyl-CoA hydratase / long-chain 3-hydroxyacyl-CoA dehydrogenase	K07515	1.45	1.02
Diamine oxidase	K11182	0.67	0.76
Aldehyde dehydrogenase family 7 member A1	K14085	0.68	1.03

Table 8. Abundance changes of proteins involved in citrate cycle injejunal upper villus enterocytes of suckling piglets

Proteins	KEGG Orthology	Fold change	
		D14 vs D7	D21 vs D7
Malate dehydrogenase	K00025	1.78	2.66
Malate dehydrogenase	K00026	0.84	1.31
Isocitrate dehydrogenase (NAD^+)	K00030	0.80	1.80
Isocitrate dehydrogenase	K00031	0.72	1.36
Pyruvate dehydrogenase E1 component subunit alpha	K00161	1.68	2.25
Pyruvate dehydrogenase E1 component subunit beta	K00162	0.57	1.26
2-oxoglutarate dehydrogenase E1 component	K00164	0.77	1.29
Succinate dehydrogenase (ubiquinone) flavoprotein subunit	K00234	1.22	0.95
Succinate dehydrogenase (ubiquinone) iron-sulfur subunit	K00235	1.09	1.55
2-oxoglutarate dehydrogenase E2 component (dihydrolipoamide succinyltransferase)	K00658	1.60	1.40
Phosphoenolpyruvate carboxykinase (GTP)	K01596	0.70	0.77
citrate synthase	K01647	0.35	0.53
Fumarate hydratase, class II	K01679	0.56	0.81
Aconitate hydratase	K01681	0.50	0.75
Succinyl-CoA synthetase alpha subunit	K01899	0.71	0.68
Succinyl-CoA synthetase beta subunit	K01900	0.57	1.00
Pyruvate carboxylase	K01958	1.54	1.42

enterocytes. Glucose and fatty acids metabolism increases in villus enterocytes compared to cryptenterocytes [22, 23]. In addition to glucose and fatty acids, amino acids, particularly glutamate and glutamine, are important energy sources for intestinal enterocytes [24, 25]. Stoll et al. showed that enteral glucose, arterial glucose, enteral glutamate, and arterial glutamine contributed 15%, 29%, 36%, and 19% of the total CO_2 production in piglet PDV, respectively [25]. The present experimental results show that the expression of most of the proteins involved in fatty acid metabolism and alanine, aspartate and glutamate metabolism decreased in 14-day piglet DE compared to 7-day piglets. Less than one-third of the proteins related to glycolysis decreased in 14-day piglet differentiated enterocytes compared to 7-day piglets. The majority of all proteins related to glycolysis up-regulated in intestinal differentiated enterocytes in of 21-day piglets compared to 7-day piglets. Only a few proteins involved in fatty acid metabolism and alanine, aspartate and glutamate metabolism up-regulated in intestinal differentiated enterocytes of 21-day piglets compared to 7-day piglets.

These results indicate that energy metabolism in intestinal differentiated piglet enterocytes changes during suckling. The changes in glucose, fatty acids, and alanine, aspartate and glutamate metabolism were different between early (7-14 d) and late (14-21 d) suckling piglets. Glucose, fatty acids and glutamate metabolism have different patterns in piglet intestinal DE during suckling. The nutrients for villus enterocytes, especially for upper villus enterocytes, are thought to be provided mainly by luminal substrates. Thus metabolism inupper villus enterocyte metabolism in suckling piglets can be affected by sow milk composition [26, 27]. Various experiments suggest that the contents of lactose, fatty acids, and glutamine and glutamate were changed in the sow milk [28-30]. Different sow milk may be a confounding factor in the protein expression differences found in this study. Gastrointestinal development is also regulated by genetic influences. Age-related changes in enzymes and nutrient transporters are genetically programmed and are little affected by diet [2]. This study cannot address whether these nutrient metabolism changes during suckling relate to maturity, sow milk, or genetics.

Besides the amino acids, including glutamate, glutamine, aspartate, and asparagine, that play key roles in generating energy, piglet intestines also consume many other amino acids [27]. Stoll et al. showed that 61%, 35%, 35%, and 32% of the dietary Thr, Lys, Phe, and Leu, respectively, were consumed by PVD tissues during the first-pass metabolism. Only 12%, 18%, 18%, and 12% of the total first-pass metabolism of Thr, Lys, Phe, and Leu were recovered in mucosal protein [31]. They concluded that about one-third of all dietary essential amino acids consumed by the intestine during the first-pass metabolism, and that more amino acids were catabolized by the mucosal cells compared to amino acids which were incorporated into mucosal protein [31].

The results of the present study showed that the metabolism of various of amino acids change in suckling piglet intestinal differentiated enterocytes. In addition, the metabolism of most of the amino acids decreased from day 7 to day 14, while only part of the detected proteins down-regulated in intestinal differentiated enterocytes of 21-day piglets compared to 7-day piglets. Consistent with energy metabolism, amino acids metabolism changes in suckling pig intestinal differentiated enterocytes may also depend on luminal nutrients as protein amounts in sow milk increased during suckling [28, 30].

The intestinal first-pass metabolism of dietary nutrients is independent of dietary nutrients intake [27]. It has been reported that dietary protein intake has little effect on the first-pass metabolism of essential dietary amino acids in the intestine, but may suppress arterial amino acid utilization [20, 27]. Therefore, understanding intestinal nutrients metabolism is very important for designing milk replacements for early-weaned piglets. For example, the gastrointestine may consume most of the dietary amino acids when piglets were given low-protein diets. This would restrict muscle and other organ grown and piglet overall growth [32]. The growth and development of the gastrointestinal system may be inhibited if piglets are provided with very low nutrient-content diets as luminal nutrients are essential to the gastrointestinal system. Dietary nutrients play key roles in affecting intestinal metabolism [19]. Maintaining intestinal function is critical because any decrease in enteral

nutrients results in reduced growth and intestinal mucosa dysfunction even when piglets are provided an equal amount of nutrients parenterally [20]. The present experiment suggests that nutrient metabolism changes in suckling piglet intestinal differentiated enterocytes of piglets provides useful information for designing piglet feed formula and regulating piglet intestinal growth and development. Intestinal adaptation, which occurs following the loss of a major portion of the small intestine, has important implications to patient potential [33]. The adaptive process may be different for intestinal crypt and villus tips because the crypt and villus epithelial cells have different functions and nutrient sources [26, 33]. There results of this study may also provide useful information for improving neonatal intestinal adaption.

In conclusion, the present study shows that various cellular processes in piglet intestinal differentiated enterocytes change during suckling. The metabolism of glucose, fatty acids, and alanine, aspartate and glutamate change with variously. The changes in glucose, fatty acids, and alanine, aspartate and glutamate metabolism also differ between early (7-14 day) and late (14-21 day) sucklings. These results reveal metabolic energy changes in intestinal differentiated enterocytes and may provide useful information for designing piglet feed formulas and regulating piglet intestinal growth and development.

Abbreviations

DE (differentiated enterocytes); BSA (bovine serum alumin); PMSF (phenylmethylsulfonyl fluoride); DTT (dithiothreitol); PCNA (proliferating cell nuclear antigen); IDA (information dependant acquisition); MGF (mascot generic format); FDR (false discovery rate); KEGG (Kyoto encyclopedia of genes and genomes); GO (gene ontology); PDV (portal-drained viscera).

Acknowledgements

The authors would like to thank Dr. Chengbo Yang for superb technical assistance. The present study was jointly supported by National Natural Science Foundation of China (31330075, 31301988, 31402089), Key Programs of Frontier Scientific Research of the Chinese Academy of Sciences (QYZDY-SSW-SMC008), Natural Science Foundation of Hunan Province (2017JJ1020), Young Elite Scientists Sponsorship Program by CAST (YESS20160086).

Disclosure Statement

The authors have declared that no conflict of interests exists.

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