Open Access
Asian-Australas J Anim Sci
Vol. 31, No. 4:548-555 April 2018
https://doi.org/10.5713/ajas.17.0426
pISSN 1011-2367 eISSN 1976-5517

AJAS
Asian-Australasian Journal of Animal Sciences

The effect of dietary asparagine supplementation on energy metabolism in liver of weaning pigs when challenged with lipopolysaccharide

Ping Kang[1], Yulan Liu[1,*], Huiling Zhu[1], Jing Zhang[1], Haifeng Shi[1], Shuang Li[1], Dinan Pi[1], Weibo Leng[1], Xiuying Wang[1], Huanting Wu[1], and Yongqing Hou[1]

* **Corresponding Author:** Yulan Liu
Tel: +86-27-83956175, **Fax:** +86-27-83956175,
E-mail: yulanflower@126.com

[1] Hubei Collaborative Innovation Center for Animal Nutrition and Feed Safety, Hubei Key Laboratory of Animal Nutrition and Feed Science, Wuhan Polytechnic University, Wuhan 430023, China

ORCID
Ping Kang
https://orcid.org/0000-0003-1665-8862
Yulan Liu
https://orcid.org/0000-0003-3320-2381

Submitted Jun 3, 2017; Revised Sept 8, 2017; Accepted Oct 22, 2017

Objective: This experiment was conducted to investigate whether asparagine (Asn) could improve liver energy status in weaning pigs when challenged with lipopolysaccharide.
Methods: Forty-eight weaned pigs (Duroc×Large White×Landrace, 8.12±0.56 kg) were assigned to four treatments: i) CTRL, piglets received a control diet and injected with sterile 0.9% NaCl solution; ii) lipopolysaccharide challenged control (LPSCC), piglets received the same control diet and injected with *Escherichia coli* LPS; iii) lipopolysaccharide (LPS)+0.5% Asn, piglets received a 0.5% Asn diet and injected with LPS; and iv) LPS+1.0% Asn, piglets received a 1.0% Asn diet and injected with LPS. All piglets were fed the experimental diets for 19 d. On d 20, the pigs were injected intraperitoneally with *Escherichia coli* LPS at 100 μg/kg body weights or the same volume of 0.9% NaCl solution based on the assigned treatments. Then the pigs were slaughtered at 4 h and 24 h after LPS or saline injection, and the liver samples were collected.
Results: At 24 h after LPS challenge, dietary supplementation with 0.5% Asn increased ATP concentration (quadratic, $p<0.05$), and had a tendency to increase adenylate energy charges and reduce AMP/ATP ratio (quadratic, $p<0.1$) in liver. In addition, Asn increased the liver mRNA expression of pyruvate kinase, pyruvate dehydrogenase, citrate synthase, and isocitrate dehydrogenase β (linear, $p<0.05$; quadratic, $p<0.05$), and had a tendency to increase the mRNA expression of hexokinase 2 (linear, $p<0.1$). Moreover, Asn increased liver phosphorylated AMP-activated protein kinase (pAMPK)/total AMP-activated protein kinase (tAMPK) ratio (linear, $p<0.05$; quadratic, $p<0.05$). However, at 4 h after LPS challenge, Asn supplementation had no effect on these parameters.
Conclusion: The present study indicated that Asn could improve the energy metabolism in injured liver at the late stage of LPS challenge.

Keywords: Asparagine; Energy Metabolism; Lipopolysaccharide; Piglets

INTRODUCTION

L-asparagine (Asn) is non-essential amino acid. However, previous studies have demonstrated that weaning rats required Asn over a short period [1], and Asn could stimulate growth and protein synthesis of weaning rats. In addition, Asn can stimulate enterocyte proliferation [2,3] and improve jejunal enterocyte ornithine decarboxylase (ODC) activity [3,4]. Asparagine can be synthesized from aspartate and glutamine (or ammonia) by asparagine synthetase in mammals [5,6]. However, in liver, the rate of Asn synthesis is much less because of the lower Asn synthetase activity [7].

Asn has similar structure to aspartate, and it can be deaminated to form aspartate in an ATP-dependent amidation reaction [8]. Liver plays a pivotal role in the metabolism of the

whole-body energy [9], such as stimulating fatty acid oxidation and glucose generation. LPS challenge could induce liver injury [10], which could cause insufficient energy supply in the liver [11]. Xu et al [12] reported that energy was necessary for a successful stress response. Our previous study has shown that dietary supplementation of aspartate improved energy status in lipopolysaccharide (LPS)-injured liver of weaning pigs [13]. Accordingly, we hypothesized that Asn could influence the energy metabolism in the liver in LPS-challenge weaning pigs. In this study, we measured the hepatic adenylate purine concentrations and the mRNA expression of key enzymes involved in glycolysis, tricarboxylic acid (TCA) cycle and fatty acid oxidation as well as AMP-activated protein kinase (AMPK) signaling pathway to examine the influence of Asn on energy metabolism in the liver in LPS-challenged weaning pigs.

MATERIALS AND METHODS

Animals

The Animal Care and Use Committee of Wuhan Polytechnic University approved the animal use protocol for this research. This experiment was conducted in the Hubei Key Laboratory of Animal Nutrition and Feed Science (Wuhan Polytechnic University, Wuhan, China). In this study, a total of 48 weaned castrated male pigs (Duroc×Large White×Landrace, 8.12±0.56 kg, weaned at 21±2 d of age) were purchased from the Hubei Zhengda Swine Co., Ltd (Wuhan, Hubei, China), and individually housed in the stainless steel cages (length 1.08 m, width 0.98 m, height 0.84 m). All pigs were housed in an environmentally controlled room (temperature 28°C and natural light), and allowed *ad libitum* access to water and feed during a 19-d experimental period.

Experimental design

Based on the BW, forty-eight piglets were randomly assigned to four treatments (two sampling times), i) non-challenged control (CTRL; piglets received a control diet and injected with 0.9% NaCl solution); ii) lipopolysaccharide (LPS)-challenged control (LPSCC; piglets received the same control diet and injected with *Escherichia coli* LPS); iii) LPS+0.5% Asn treatment (piglets received a 0.5% Asn diet and injected with LPS); and iv) LPS+1.0% Asn treatment (piglets received a 1.0% Asn diet and injected with LPS). There were six replicates (n = 6) for each treatment in each sampling time, and each replicate had one pig. In this study, the dose of the Asn (L-Asn, purity >99%; Amino Acid Bio-Chemical Co, Wuhan, China) and LPS (*Escherichia coli* serotype 055: B5, Sigma Chemical Inc., St. Louis, MO, USA) were determined on the basis of our previous studies [14]. The control diet was prepared to meet or exceed NRC [15] nutrient requirements (Table 1), and the diets were isonitrogenous by supplementation of L-alanine (purity >99%; Amino Acid Bio-Chemical Co, China). During the entire 19 d feeding trial (pre-challenge), there were no differences in average daily gain, average daily feed intake and feed:gain ratio among the four treatment groups (data not shown). On d 19, the LPS-challenged pigs were injected intraperitoneally with 500 μg/mL *Escherichia coli* LPS at 100 μg/kg BW (0.2 mL/kg BW), and the pigs in control group were injected with the same volume of 0.9% NaCl solution. LPS injection caused fever, somnolence, anorexia, vomiting, and shivering within 1 h in all pigs. At 4 h or 24 h (n = 6) after LPS or saline injection, pigs were slaughtered under anesthesia with an intravenous injection of sodium pentobarbital (50 mg/kg BW), and a portion of the liver (approximately 3 g) was re-

Table 1. Ingredients and nutritional composition of diet (as fed basis)

Items	Content
Ingredients (%)	
Corn	55.50
Soybean meal (44% crude protein)	22.00
Wheat bran	3.00
Fish meal	5.50
Corn oil or fish oil	5.00
Soy protein concentrate	2.50
Milk-replacer powder	3.00
Limestone	0.70
Dicalcium phosphate	1.00
Salt	0.20
L-Lysine·HCl (78.8% Lysine)	0.27
Acidifier[1)]	0.20
Butylated hydroquinone	0.05
Preservative[2)]	0.05
Sweetener[3)]	0.03
Vitamin and mineral premix[4)]	1.00
Nutrient composition (g/kg)	
Digestible energy[5)] (MJ/kg)	14.0
Crude protein[6)]	202
Calcium[6)]	9.0
Phosphorus[6)]	7.0
Lysine[6)]	13.5
Methionine+cysteine[6)]	7.2
Aspartate+asparagine[6)]	16.9

[1)] A compound acidifier including lactic acid and phosphoric acid, provided by Wuhan Fanhua Biotechnology Company, Wuhan, China.
[2)] A compound mould inhibitor including calcium propionate, fumaric acid, fumaric acid monoethyl ester and sodium diacetate, provided by Sichuan Minsheng Pharmaceutical Co., Ltd, Chengdu, China.
[3)] A compound sweetener including saccharin sodium and disodium 5'-guanylate, provided by Wuhan Fanhua Biotechnology Company, Wuhan, China.
[4)] The premix provided the following amounts per kilogram of complete diet: retinol acetate, 2,700 μg; cholecalciferol, 62.5 μg; dl-α-tocopheryl acetate, 20 mg; menadione, 3 mg; vitamin B_{12}, 18 μg; riboflavin, 4 mg; niacin, 40 mg; pantothenic acid, 15 mg; choline chloride, 400 mg; folic acid, 700 μg; thiamin, 1.5 mg; pyridoxine, 3 mg; biotin, 100 μg; Zn, 80 mg ($ZnSO_4 \cdot 7H_2O$); Mn, 20 mg ($MnSO_4 \cdot 5H_2O$); Fe, 83 mg ($FeSO_4 \cdot H_2O$); Cu, 25 mg ($CuSO_4 \cdot 5H_2O$); I, 0.48 mg (KI); Se, 0.36 mg ($Na_2SeO_3 \cdot 5H_2O$).
[5)] Calculated. [6)] Analyzed.

moved and frozen in liquid nitrogen immediately. One part of the liver sample (approximately 1 g) was used for measuring ATP, ADP, and AMP concentrations, and another part (approximately 2 g) was then stored at –80°C for mRNA and protein expression analysis.

ATP, ADP, and AMP concentrations in the liver

The ATP, ADP, and AMP concentrations in the liver were measured with high performance liquid chromatography (HPLC) according to a published method [16]. Briefly, the supernatant was prepared from the liver. As for the HPLC, the detection wavelength was 260 nm, and the column temperature and the pump flow was 35°C and 1.0 mL/min, respectively. Total adenine nucleotide (TAN) and adenylate energy charges (AEC) were calculated using the following equations: TAN = ATP+ADP+AMP; AEC = (ATP+0.5ADP)/(ATP+ADP+AMP) [16].

mRNA abundance analysis by real-time PCR

Total RNA was isolated from pig liver by Trizol (#9108, TaKaRa Biotechnology [Dalian] Co., Ltd., Dalian, China). cDNA was synthesized with Prime Script RT reagent kit with gDNA eraser according to the producer instructions (#RR047A, TaKaRa Biotechnology [Dalian] Co., Ltd., China). The expression of the target genes were analyzed by the real-time polymerase chain reaction (PCR) with an Applied Biosystems 7500 Real-Time PCR System (Applied Biosystems, Life Technologies, Foster City, CA, USA) using a SYBR Premix Ex TaqTM (Tli RNaseH Plus) qPCR kit (#RR420A, TaKaRa Biotechnology [Dalian] Co., Ltd., China). The PCR program was as follows: 95°C for 30 s, followed by 40 cycles of 95°C for 5 s and 60°C for 34 s. The target genes were amplified by using the forward and reverse primers, which were designed with Primer Premier 6.0 and synthesized by TaKaRa Biotechnology (China, Table 2), and their mRNA expression relative to housekeeping gene (glyceraldehyde-3-phosphate dehydrogenase) were calculated according to the $2^{-\Delta\Delta CT}$ method [17].

Protein abundance analysis by Western blot

The method for quantification of liver protein expression was carried out with Western blot as previously described [13]. Briefly, equal amounts of proteins in liver supernatant fluids were separated on 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and then transferred to blotting membrane for immunoblotting. The specific primary antibodies included rabbit phosphorylated AMPKα (pAMPKα; Thr172; 1:1,000, #2532) and rabbit anti-total AMPKα (tAMPKα; 1:1,000, #2535) (Cell Signaling Technology Inc., Danvers, MA, USA).

Statistical analysis

The experimental data were analyzed using SPSS 17.0 software (SPSS Inc., Chicago, IL, USA). T test was used to determine whether there was a significant difference between the data obtained in the LPS group (LPSCC, 0% Asn) and the data obtained in the control group, in order to determine the effect of LPS challenge on the weaned piglets. Linear and quadratic polynomial contrasts were used to determine the response to Asn supplementation among LPS-challenged pigs. All data were expressed as means±SE. The statistical significance level for all analyses was set at $p \leq 0.05$ and $0.05 < p < 0.10$ were considered as trends.

RESULTS

Concentrations of ATP, ADP, and AMP in the liver

As shown in Table 3, LPS challenge had no effect on the ATP, ADP, and AMP concentrations ($p > 0.1$) at 4 or 24 h post-in-

Table 2. Specific primer sequences for pigs used for real-time polymerase chain reaction

Gene	Forward (5′-3′)	Reverse (5′-3′)	Gene Bank No.	Sequences bp
Hexok 2	CTCATCACAACCGTTACCA	TGTCATTAGTGTCCTCATCC	NM_001122987.1	119
L-PFK	CTGCACCGCATCATGGA	CCCCATCACCTCCAGAACA	XM_003358990.1	84
PK	TCACTCCACAGACCTCAT	TACCTAGCCACCTGATGT	XM_003356683.1	123
PDH	GCAGACTTACCGTTACCAT	GATAGCCGAGTTCTTCCAA	XM_003360244.2	248
ACO	CTCGCAGACCCAGATGAAAT	TCCAAGCCTCGAAGATGAGT	AF185048	218
L-CPT-1	GGACCGCCACCTGTTCTGCCTCTA	GCCCCCTCCGCTCGACACATAC	AF288789	175
CS	TCTCAGCTCAGTGCAGCCATTACA	CTGCAACACAAGGTAGCTTTGCGA	NM_214276	145
ICDH β	TGTGGTTCCTGGTGAGAG	CGAGATTGAGATGCCGTAG	XM_003361597.1	149
ICDH γ	GGTGGAGAGCCTCAAGAT	TGGTGGTGTTGTCTACGA	XM_003360495.1	218
AMPKα1	AAATCGGCCACTACATCCTG	GGATGCCTGAAAAGCTTGAG	NM_001167633.1	187
AMPKα2	AACATGGACGGGTTGAAGAG	CGCAGAAACTCACCATCTGA	NM_214266.1	193
Sirt1	CTGGAACAGGTTGCAGGAAT	CCTAGGACATCGAGGAACCA	EU030283	144
PGC1α	GATGTGTCGCCTTCTTGTTC	CATCCTTTGGGGTCTTTGAG	NM_213963	93
GAPDH	CGTCCCTGAGACACGATGGT	GCCTTGACTGTGCCGTGGAAT	AF017079	194

Hexok 2, hexokinase 2; *L-PFK*, 6-phosphofructokinase (liver type-like); *PK*, pyruvate kinase; *PDH*, pyruvate dehydrogenase; *ACO*, acyl-coenzyme A oxidase; *L-CPT-1*, liver carnitine palmitoyltransferase I; *CS*, Citrate synthase; *ICDH β*, isocitrate dehydrogenase β; *ICDH γ*, isocitrate dehydrogenase γ; *AMPK*, AMP-activated protein kinase; *Sirt1*, silent information regulator 1; *PGC1α*, peroxisome proliferator activated receptor gamma coactivator-1α; *GAPDH*, glyceraldehyde-3-phosphate dehydrogenase.

Table 3. The effect of Asn supplementation on the ATP production in liver at 24 h after *Escherichia coli* lipopolysaccharide challenge in weaned pigs[1)]

Item	CTRL[2)]	LPSCC[2)]	LPS+0.5% Asn[2)]	LPS+1.0% Asn[2)]	SEM	p value		
						Control vs LPSCC	Linear	Quadratic
4 h post-challenge								
ATP (μg/g wet wt)	280	143	206	172	67	0.129	0.558	0.340
ADP (μg/g wet wt)	143	122	142	133	32	0.308	0.789	0.865
AMP (μg/g wet wt)	443	419	291	391	51	0.611	0.759	0.101
TAN[3)] (μg/g wet wt)	866	684	639	695	85	0.086	0.836	0.684
AEC[4)]	0.38	0.31	0.42	0.35	0.06	0.220	0.629	0.182
AMP/ATP	2.18	3.17	1.54	3.17	0.91	0.230	0.918	0.171
24 h post-challenge								
ATP (μg/g wet wt)	256	218	375	223	54	0.514	0.399	0.008
ADP (μg/g wet wt)	176	138	211	86	41	0.426	0.725	0.025
AMP (μg/g wet wt)	365	447	431	392	60	0.147	0.467	0.713
TAN[3)] (μg/g wet wt)	796	802	1017	701	114	0.962	0.93	0.038
AEC[4)]	0.42	0.36	0.47	0.39	0.05	0.235	0.254	0.063
AMP/ATP	1.67	2.25	1.28	1.77	0.41	0.271	0.133	0.098

Asn, asparagine; LPSCC, lipopolysaccharide challenged control; LPS, lipopolysaccharide; SEM, standard error of the mean; TAN, total adenine nucleotide; AEC, adenylate energy charges.
[1)] Values are means ± SE, n = 6 (1 pig/pen).
[2)] CTRL (non-challenged control), piglets receiving a control diet and injected with 0.9% NaCl solution; LPSCC (LPS challenged control), piglets receiving the same control diet and injected with *Escherichia coli* LPS; LPS+0.5% Asn, piglets receiving a 0.5% Asn diet and injected with LPS; LPS+1.0% Asn, piglets receiving a 1.0% Asn diet and injected with LPS.
[3)] TAN = ATP+ADP+AMP.
[4)] AEC = (ATP+0.5ADP)/(ATP+ADP+AMP).

jection. Dietary supplementation with Asn had no effect on the concentrations of ATP, ADP, and AMP at 4 h after LPS challenge ($p>0.1$), however, dietary Asn supplementation increased ATP and ADP concentrations (quadratic, $p<0.05$), and had a tendency to increase AEC (quadratic, $p<0.1$) and reduce AMP/ATP ratio (quadratic, $p<0.1$) at 24 h after LPS challenge.

mRNA expression of genes related to the energy metabolism

As shown in Table 4, at 4 h post-injection, LPS challenge up-regulated the mRNA expression of liver hexokinase (Hexok) 2, and down-regulated the mRNA expression of 6-phosphofructokinase (liver type-like) (L-PFK), pyruvate dehydrogenase (PDH), acyl-coenzyme A oxidase, and liver carnitine palmitoyltransferase (L-CPT)-1 ($p<0.05$). Asn supplementation had no effect on mRNA expression of the key enzymes involved in glycolysis, TCA cycle and fatty acid oxidation after 4 h LPS challenge ($p>0.1$). However, at 24 h post-challenge, LPS challenge decreased mRNA expressions of PDH and citrate synthase (CS) ($p<0.05$). Asn supplementation increased pyruvate kinase (PK), PDH, CS, and isocitrate dehydrogenase (ICDH) β mRNA expression (linear, $p<0.05$; quadratic, $p<0.05$), and had a tendency to increase Hexok 2 mRNA expression (linear, $0.05<p<0.1$) at 24 h after LPS challenge.

mRNA expression of AMPKα1, AMPKα2, Sirt1, and PGC1α

As shown in Table 5, LPS challenge decreased the mRNA expression of AMP-activated protein kinase (AMPK) α1, AMPKα2, and silent information regulator (Sirt)1 at 24 h post-LPS challenge. However, Asn supplementation had no effect on the mRNA expression of AMPKα1, AMPKα2, Sirt1, and PGC1α at 4 h or 24 h after LPS challenge.

Protein phosphorylation and abundance of AMPKα

As shown in Table 6, LPS challenge had no effect on the pAMPKα/tAMPKα ratio ($p>0.05$) at 4 and 24 h post-injection. However, Asn supplementation increased this ratio at 24 h after LPS challenge (linear, $p<0.05$; quadratic, $p<0.05$).

DISCUSSION

Many amino acids can be used as an energy source by the body, such as glutamine [18], proline [19,20], arginine [21], and aspartate [13,22]. Yi et al [23] found dietary N-acetylcysteine supplementation also could alleviate liver injury in lipopolysaccharide-challenged piglets by improving energy metabolism.

ATP is the main energy source in a variety of cellular functions. Ramesh et al [24] reported that Asn increased the ODC activity, which primarily controls the intracellular concentrations of polyamines. Fausto [25] also found that Asn could increase ODC activity in regenerating liver. Polyamines play an essential role in regulating energy metabolism [26]. In our study, Asn supplementation increased ATP concentration at

Table 4. The effect of Asn supplementation on the liver carbohydrate metabolism and tricarboxylic acid cycle at 24 h after *Escherichia coli* LPS challenge in weaned pigs[1)]

Item	CTRL[2)]	LPSCC[2)]	LPS+0.5% Asn[2)]	LPS+1.0% Asn[2)]	SEM	p value		
						Control vs LPSCC	Linear	Quadratic
4 h post-challenge								
Carbohydrate metabolism								
Hexok 2	1.00	15.9	21.3	25.57	5.13	0.001	0.120	0.293
L-PFK	1.00	0.44	0.69	0.52	0.14	0.002	0.291	0.163
PK	1.00	1.67	1.46	1.54	0.27	0.399	0.573	0.787
PDH	1.00	0.90	0.80	0.81	0.11	0.032	0.305	0.571
Fatty acid oxidation								
ACO	1.00	0.45	0.60	0.37	0.12	0.001	0.888	0.112
L-CPT-1	1.00	0.35	0.42	0.22	0.14	0.003	0.483	0.158
Tricarboxylic acid cycle								
CS	1.00	0.98	0.89	0.84	0.10	0.806	0.189	0.428
ICDH β	1.00	0.90	0.94	0.77	0.12	0.397	0.460	0.348
ICDH γ	1.00	0.83	0.79	0.77	0.10	0.194	0.511	0.811
24h post-challenge								
Carbohydrate metabolism								
Hexok 2	1.00	0.59	1.30	1.15	0.33	0.095	0.069	0.134
L-PFK	1.00	0.64	0.59	0.60	0.17	0.169	0.519	0.771
PK	1.00	0.90	1.25	1.14	0.11	0.486	0.010	0.007
PDH	1.00	0.67	0.81	0.95	0.10	0.027	<0.001	0.001
Fatty acid oxidation								
ACO	1.00	0.69	0.65	0.70	0.18	0.258	0.868	0.741
L-CPT-1	1.00	0.32	0.16	0.24	0.30	0.163	0.211	0.194
Tricarboxylic acid cycle								
CS	1.00	0.68	0.99	0.95	0.11	0.039	0.014	0.033
ICDH β	1.00	0.66	0.72	0.91	0.15	0.145	0.008	0.004
ICDH γ	1.00	0.72	0.71	0.67	0.11	0.078	0.570	0.760

Asn, asparagine; LPS, lipopolysaccharide; LPSCC, lipopolysaccharide challenged control; SEM, standard error of the mean; Hexok 2, hexokinase 2; L-PFK, 6-phosphofructokinase (liver type-like); PK, pyruvate kinase; PDH, pyruvate dehydrogenase; ACO, acyl-coenzyme A oxidase; L-CPT-1, liver carnitine palmitoyltransferase I; CS, Citrate synthase; ICDH β, isocitrate dehydrogenase β ; ICDH γ, isocitrate dehydrogenase γ.

[1)] Values are means, n = 6 (1 pig/pen).

[2)] CTRL (non-challenged control), piglets receiving a control diet and injected with 0.9% NaCl solution; LPSCC (LPS challenged control), piglets receiving the same control diet and injected with *Escherichia coli* LPS; LPS+0.5% Asn, piglets receiving a 0.5% Asn diet and injected with LPS; LPS+1.0% Asn, piglets receiving a 1.0% Asn diet and injected with LPS.

Table 5. The effect of Asn supplementation on the liver mRNA expression of AMPKα1, AMPKα2, Sirt1, and PGC1α at 4 h or 24 h after LPS challenge in weaned piglets[1)]

Item	CTRL[2)]	LPSCC[2)]	LPS+0.5% Asn[2)]	LPS+1.0% Asn[2)]	SEM	p value		
						Control vs LPSCC	Linear	Quadratic
4 h post-challenge								
AMPKα1	1.00	0.94	1.02	0.82	0.14	0.627	0.623	0.355
AMPKα2	1.00	0.99	1.01	0.89	0.19	0.945	0.715	0.798
Sirt1	1.00	1.17	1.06	0.91	0.24	0.495	0.326	0.580
PGC1α	1.00	0.36	0.39	0.14	0.22	0.073	0.189	0.094
24 h post-challenge								
AMPKα1	1.00	0.54	0.64	0.64	0.16	0.053	0.281	0.542
AMPKα2	1.00	0.47	0.44	0.53	0.17	0.052	0.723	0.659
Sirt1	1.00	0.61	0.46	0.60	0.13	0.039	0.608	0.277
PGC1α	1.00	0.98	0.72	0.91	0.26	0.930	0.612	0.634

Asn, asparagine; AMPK, AMP-activated protein kinase; Sirt1, silent information regulator 1; PGC1α, peroxisome proliferator activated receptor gamma coactivator-1α; LPS, lipopolysaccharide; LPSCC, lipopolysaccharide challenged control; SEM, standard error of the mean.

[1)] Values are means, n = 6 (1 pig/pen).

[2)] CTRL (non-challenged control), piglets receiving a control diet and injected with 0.9% NaCl solution; LPSCC (LPS challenged control), piglets receiving the same control diet and injected with *Escherichia coli* LPS; LPS+0.5% Asn, piglets receiving a 0.5% Asn diet and injected with LPS; LPS+1.0% Asn, piglets receiving a 1.0% Asn diet and injected with LPS.

Table 6. The effect of Asn supplementation on the liver pAMPKα/tAMPKα ratio (AU) at 24 h after *Escherichia coli* LPS challenge in weaned piglets[1)]

Item	CTRL[2)]	LPSCC[2)]	LPS+0.5% Asn[2)]	LPS+1.0% Asn[2)]	SEM	p value		
						Control vs LPSCC	Linear	Quadratic
4 h post-challenge	16.79	16.11	19.02	20.90	5.22	0.897	0.326	0.586
24 h post-challenge	23.27	25.91	25.13	44.49	7.92	0.692	0.044	0.050

Asn, asparagine; AMPK, AMP-activated protein kinase; LPS, lipopolysaccharide; LPSCC, lipopolysaccharide challenged control; SEM, standard error of the mean.
[1)] Values are means, n = 6 (1 pig/pen).
[2)] CTRL (non-challenged control), piglets receiving a control diet and injected with 0.9% NaCl solution; LPSCC (LPS challenged control), piglets receiving the same control diet and injected with *Escherichia coli* LPS; LPS+0.5% Asn, piglets receiving a 0.5% Asn diet and injected with LPS; LPS+1.0% Asn, piglets receiving a 1.0% Asn diet and injected with LPS.

24 h after LPS challenge, which might have resulted from the increased polyamines concentration. Moreover, the rise of ATP level indicated that Asn could play an important role on recovery of injured liver.

The ATP generation in the liver mainly depends on the respiratory chain and glycolysis [27,28], which could meet the energy requirement during the early response to a stress. The Hexok catalyzes glucose phosphorylation, which is the first step of glycolysis. In the present study, we found that Asn supplementation had a tendency to increase Hexok 2 mRNA expression after 24 h LPS challenge, which could stimulate the subsequent glycolysis steps to produce more ATP to improve the liver's response to stress after LPS challenge. Six-phosphofructokinase (PFK) is one of the key enzymes of glycolysis. ATP and AMP can down-regulate and up-regulate the activity of PFK, respectively [29]. However, Reinhart and Lardy [30] reported that this enzyme in liver could be inactive because of the lower physiological concentrations of ATP and AMP. This result is consistent with our findings that Asn supplementation could increase ATP level after LPS challenge, however, the increased ATP level could not be enough to stimulate liver PFK expression.

The PK is also a key regulatory enzyme in glycolysis. In erythrocytes, PK insufficiency can decrease ATP level, which might shorten their life span [31]. Roy et al [32] reported that PK deficiency increased susceptibility to *Salmonella typhimurium* infection in mice. In this study, Asn supplementation increased the PK mRNA expression at 24 h after LPS challenge, which might in turn augment the ATP production and have a positive function to alleviate the liver stress after LPS challenge. The PDH is not only the key enzyme in the TCA cycle, but also the first component enzyme of pyruvate dehydrogenase complex. PDH catalyzes pyruvate to produce acetyl coenzyme A (acetyl-CoA). This reaction is a main link between glycolysis and TCA cycle. In this study, Asn increased the PDH mRNA expression at 24 h after LPS challenge. Theoretically, this increased mRNA expression might increase the production of acetyl-CoA, which can produce the energy through the TCA cycle. These results are also in accordance with the result that Asn supplementation increased the ATP production.

The CS is the first enzyme in TCA cycle. Zhang et al [33] reported that the increased Asn could stimulate the CS activity. In this study, Asn supplementation increased the CS mRNA expression at 24 h after LPS challenge, which might result from the high Asn circulation concentration. Accordingly, the increased CS mRNA expression could stimulate TCA cycle to produce more ATP to improve the injured liver. The ICDH are also the key enzymes in TCA cycle. There are three ICDH in liver, including one cytosolic and two mitochondrial ones. The latter, including ICDHβ and ICDHγ, are the key enzymes against oxidative damage in mitochondria. Schanbacher et al [34] reported that ICDH level increased in cattle's serum of hepatic injury model. However, in the present study, Asn supplementation had no effect on their mRNA expression.

Mammalian AMPK is a main regulator of energy control [35], which can be activated by the high concentration of the AMP and the reversible phosphorylation [36]. AMPK is a heterotrimer consisting seven subunits [37]. The major phosphorylated site is the Thr172 phosphorylation within the AMPK α-subunit. In response, the activated AMPK can stimulate catabolic pathways to produce more ATP in the face of energy stress. Our previous studies have shown that Asn supplementation could improve the injured intestine induced by LPS via the AMPK signaling pathway [38,39]. In this study, we found that 1.0% Asn supplementation had no effect on the AMPK mRNA expression at 4 and 24 h after LPS challenge, however, it increased liver pAMPKα/tAMPKα ratio at 24 h, which was in response to the decreased AMP-to-ATP ratio, indicating that Asn could increase AMPK phosphorylation to produce more ATP to improve energy status in the injured liver induced by LPS challenge.

Asn can be deaminated to form aspartate (Asp) in an ATP-dependent amidation reaction [8]. Our previous study had shown that Asp could decrease the expressions of hepatic pro-inflammatory mediators at 4h after LPS challenge, which could not stimulate the positive effects of Asp on the injured liver induced by LPS. In contrast, Asp could attenuate this injury resulting from the increased expressions of hepatic pro-inflammatory mediators at 24 h after LPS challenge. These responses might be part of the reason that Asn improved the energy status to attenuate the injured liver just at 24 h after LPS challenge

due to the increased expressions of hepatic pro-inflammatory mediators. Moreover, because our previous study found only one time-point (4 h) might not the most appropriate time point to measure all the intestinal pro-inflammatory mediators following the LPS challenge, in this study, we chose two time points for liver sample collection to analyze energy metabolism in liver based on our found in Asp [13].

CONCLUSION

In the present study, we showed that dietary supplementation with Asn had no effect on the energy status at 4 h after LPS injection. However, Asn could improve the energy status in injured liver at 24 h after LPS challenge, as evidenced by increased liver content of ATP, which might result from increasing the activity of TCA cycle and glycolysis, and Asn supplementation could stimulate AMPK phosphorylation. The results indicated that Asn could play an important role in increasing energy production just at the late stage of LPS challenge. Furthermore, in our study, it remained unclear whether the positive effects on energy metabolism were mediated directly by Asn itself or by its metabolites.

CONFLICT OF INTEREST

We certify that there is no conflict of interest with any financial organization regarding the material discussed in the manuscript.

ACKNOWLEDGMENTS

This study was financially supported by the National Natural Science Foundation of China (31422053 and 31372318).

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