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Dietary supplementation of aspartate enhances intestinal integrity and energy status in weanling piglets after lipopolysaccharide challenge☆

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Abstract

The intestine has a high requirement for ATP to support its integrity, function and health, and thus, energy deficits in the intestinal mucosa may play a critical role in intestinal injury. Aspartate (Asp) is one of the major sources of ATP in mammalian enterocytes via mitochondrial oxidation. We hypothesized that dietary supplementation of Asp could attenuate lipopolysaccharide (LPS)-induced intestinal damage via modulation of intestinal energy status. Twenty-four weanling piglets were allotted to one of four treatments: (1) nonchallenged control, (2) LPS-challenged control, (3) LPS+0.5% Asp treatment, and (4) LPS+1.0% Asp treatment. On day 19, pigs were injected with saline or LPS. At 24 h postinjection, pigs were killed and intestinal samples were obtained. Asp attenuated LPS-induced intestinal damage indicated by greater villus height and villus height/crypt depth ratio as well as higher RNA/DNA and protein/DNA ratios. Asp improved intestinal function indicated by increased intestinal mucosal disaccharidase activities. Asp also improved intestinal energy status indicated by increased ATP, ADP and total adenine nucleotide contents, adenylate energy charge and decreased AMP/ATP ratio. In addition, Asp increased the activities of tricarboxylic acid cycle key enzymes including citrate synthase, isocitrate dehydrogenase and alpha-oxoglutarate dehydrogenase complex. Moreover, Asp down-regulated mRNA expression of intestinal AMP-activated protein kinase α1 (*AMPKα1*), *AMPKα2*, silent information regulator 1 (*SIRT1*) and peroxisome proliferator–activated receptor gamma coactivator-1α (*PGC1α*) and decreased intestinal AMPKα phosphorylation. These results indicate that Asp may alleviate LPS-induced intestinal damage and improve intestinal energy status.

Keywords: Aspartate; Intestine; Energy status; Weanling piglets; Lipopolysaccharide

1. Introduction

The intestine is important in defense against harmful bacteria-derived endogenous and exogenous agents [1]. However, many factors such as infection and inflammation can result in intestinal injury and dysfunction [1–3]. Accumulating evidence suggests that the intestine has a high requirement for ATP to support its integrity, function and health [4,5], and thus, energy deficits in the intestinal mucosa may play a critical role in intestinal injury [6]. Amino acids rather than glucose have been shown to be the central fuel for intestinal mucosa [7]. Therefore, nutritional interventions (especially dietary supplementation of amino acids) targeting intestinal energy metabolism may exert protective effects in alleviating intestinal injury.

Aspartate (Asp) is an acidic amino acid and a member of the arginine family [8]. The amino acids of the arginine family, including glutamine, glutamate, proline, Asp, asparagine, ornithine, citrulline and arginine, are interconvertible via complex interorgan metabolism in most mammals [7–9]. Traditionally, Asp is classified as a nonessential amino acid in mammals [10]. However, emerging evidence shows that it plays a major role in energy metabolism, especially in coordination of mitochondrial and cytosolic biochemical processes [11]. Asp transaminase catalyzes the interconversion of Asp to oxaloacetate, which is a key intermediate in the tricarboxylic acid (TCA) cycle [12]. In addition, Asp is one of the major sources of ATP in mammalian enterocytes via mitochondrial oxidation [10]. Moreover, Sivakumar et al. [13] reported that Asp could sustain mitochondrial energy metabolism and protect cardiac mitochondria by increasing ATP production for myocardial activity in myocardial infarction-induced rats. However, until now, little research has been conducted to explore the protective effect of Asp in the intestine.

AMP-activated protein kinase (AMPK) is a serine/threonine protein kinase, which is composed of one catalytic (α) and two

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noncatalytic subunits (β and γ), and widely exists in eukaryotic cells [14,15]. When activated by phosphorylation of the α-subunit at threonine 172 (Thr^{172}), AMPK can directly mediate metabolic adaptations to the change of energy status [16]. In addition, AMPK can also translates this information into silent information regulator 1 (SIRT1)-dependent, which is shown to de-acetylate and affect the activity of the peroxisome proliferator activated receptor gamma coactivator-1α (PGC1α), culminating in the transcriptional regulation of mitochondrial and lipid metabolism genes [16–18]. These processes can turn on ATP-generating pathways including glycolysis and fatty acid oxidation, while synchronously turning off ATP-consuming pathways including gluconeogenesis and fatty acid synthesis [19].

Accordingly, we hypothesized that dietary supplementation of Asp would improve intestinal health by modulating intestinal energy status via regulation of AMPK signaling pathway. In our present study, *Escherichia coli* lipopolysaccharide (LPS) was injected as an inflammatory agent to establish the model of acute intestinal damage [2,3]. In addition, we made use of a piglet model, a good model for human nutritional and physiological studies [20,21]. Our aims were to investigate whether Asp could alleviate intestinal damage caused by LPS challenge and to elucidate its molecular mechanism(s).

2. Material and methods

2.1. Animal care and experimental design

The research protocol was approved by the Animal Care and Use Committee of Hubei Province, China. Twenty-four weanling castrated barrows [Duroc×Large White×Landrace, weaned at 21±1 days of age, 8.9±0.2 kg initial body weight (BW)] were randomly assigned to four treatments. The pigs with the same gender were used for keeping animal uniformity. Pigs were individual caged in 1.80×1.10-m pens and maintained in an environmentally controlled room. There were six replicate pens for each treatment. Each pen was equipped with a feeder and a nipple waterer to allow pigs to access both feed and fresh water. The basal diet (Supplemental Table 1) was formulated in accordance with NRC [22] recommendations for all nutrients.

The experiment included four treatments: (1) nonchallenged control (CONTR; piglets receiving a control diet and injected with 0.9% NaCl solution), (2) LPS-challenged control (LPS; piglets receiving the same control diet and injected with *E. coli* LPS), (3) LPS+0.5% Asp treatment (piglets receiving a 0.5% Asp diet and injected with LPS), and (4) LPS+1.0% Asp treatment (piglets receiving a 1.0% Asp diet and injected with LPS). The Asp doses (L-aspartic acid, purity >99 %; Amino Acid Bio-Chemical Co, Wuhan, China) were chosen according to our preliminary study, which showed that 0.5% and 1.0% Asp attenuated LPS-induced weight loss in weanling pigs [23]. We added 0.67%, 0.34% and 0% alanine (purity >99 %; Amino Acid Bio-Chemical Co) to the control, 0.5% Asp and 1.0% Asp diets, respectively, to obtain isonitrogenous diets. BW and feed consumption were recorded on days 1 and 19 before LPS or saline injection. After 19-day feeding with control, 0.5% and 1.0% Asp diets, the challenged groups were injected intraperitoneally with *E. coli* LPS (*E. coli* serotype 055: B5; Sigma Chemical Inc., St. Louis, MO, USA) at 100 μg/kg BW, and the unchallenged group was injected with the same volume of 0.9% NaCl solution. The LPS dose was used according to our previous experiments [2,3], which demonstrated that this dose of LPS caused acute intestinal injury in weaned pigs. During 24 h following LPS or saline injection, all pigs were pair-fed until slaughter in order to avoid the potential effects of LPS-induced feed intake reduction on intestinal variables. The pigs were allowed *ad libitum* access to water.

2.2. Intestinal sample collections

Twenty-four hours following LPS or 0.9% NaCl solution injection, all pigs were humanely killed, and the 3- and 10-cm segments were cut from the mid-jejunum and mid-ileum in accordance with our previous study [2]. Our previous research showed that, at 24 h postinjection, LPS caused intestinal injury and intestinal energy metabolic disruption indicated by decreased intestinal ATP content in weanling pigs [6]. Therefore, the time point of 24 h following injection with LPS or saline was chosen for intestinal sample collection.

The 3-cm intestinal segments were flushed and then fixed in 10% neutral-buffered formalin for analysis of intestinal morphology [2]. The 10-cm intestinal segments were opened and flushed, and then mucosal samples were collected [2]. The mucosal samples were rapidly frozen in liquid nitrogen and then stored at −80°C until analysis. The frozen intestinal mucosal samples were weighed, homogenized in ice-cold phosphate-buffered salien–EDTA (2.0 M NaCl, 0.05 M Na_3PO_4, 2×10^{-3} M EDTA, pH 7.4) with a ratio of 1:10 (wt/vol), and then centrifuged at 3500×*g* for 10 min at 4°C to collect the supernatant [2]. The supernatant was used for analysis of protein, DNA and RNA contents, disaccharidase activities and TCA cycle key enzyme activities.

2.3. Intestinal morphology analysis

After fixation for 24 h, the intestinal samples were dehydrated using a graded series of ethanol (70%–100%) and cleared with xylene, and then the samples were embedded in paraffin. Cross sections of the segments were cut at a thickness of approximately 5 μm with a microtome (American Optical Co., Scientific Instrument Division, Buffalo, NY, USA) and stained with hematoxylin and eosin [24]. The method for determination of villus height and crypt depth was described previously [2].

2.4. Analysis of intestinal mucosal protein, DNA and RNA contents

Protein content of intestinal mucosal supernatant was analyzed by the method of Lowry et al. [25]. Mucosal DNA content was measured by a fluorometric assay [26]. RNA was determined by spectrophotometry by a modified Schmidt-Tannhauser method [27].

2.5. Analysis of intestinal mucosal disaccharidase activities

The activities of lactase, maltase and sucrase were determined in accordance with Liu et al. [28] using a glucose kit (Nanjing Jiancheng Bioengineering Institute, Nanjing, China) to determine the amount of liberated glucose.

2.6. Analysis of intestinal mucosal ATP, ADP and AMP contents

Approximate 150 mg of frozen intestinal mucosal samples was homogenized in 2 ml of 1.5 mol/L perchloric acid. The homogenates were centrifuged at 3000×*g* for 5 min at 4°C to collect the supernatants. One milliliter of the supernatant was neutralized with 0.4 ml of 2 mol/L potassium carbonate and then centrifuged at 3000×*g* for 5 min at 4°C. The ATP, ADP and AMP contents were analyzed in accordance with the method of Hou et al. [6] using high-performance liquid chromatography. Total adenine nucleotide (TAN) and adenylate energy charges (AEC) were calculated in accordance with following equations [6].

$$TAN = ATP + ADP + AMP.\ \ AEC = (ATP + 0.5ADP)/(ATP + ADP + AMP).$$

2.7. Analysis of TCA cycle key enzyme activities of intestinal mucosa

The activities of citrate synthase, isocitrate dehydrogenase and alpha-oxoglutarate dehydrogenase complex were analyzed by commercially available porcine ELISA kits (Shanghai Yuanye Bio Technology Co., Shanghai, China). All variables were analyzed according to the manufacturer's guidelines. Briefly, 50 μl of standard solutions or the diluted intestinal mucosal supernatants was added to a separately identified well of the plates. Then 100 μl of horseradish peroxidase-conjugate reagent was added and incubated for 60 min at 37°C. Following incubation, the plates were washed five times with wash solutions. After washing, 50 μl chromogen solution A and 50 μl chromogen solution B were added. The plates were mixed and incubated for 15 min at 37°C. Then, 50 μl of stop solutions was added, and the plates were read at an absorbance of 450 nm using an ELISA plate reader (Model 550; Bio-Rad, Hercules, CA, USA).

2.8. mRNA abundance analysis

Total RNA was isolated from intestinal samples using the TRIzol reagent (TaKaRa Biotechnology (Dalian) Co., Ltd., Dalian, China) according to manufacturer's instructions. RNA concentration and purity were spectrophotometrically determined at OD260 and OD280. RNA integrity was verified by agarose gel electrophoresis. cDNA synthesis was performed using PrimeScript RT reagent kit with gDNA eraser (TaKaRa Biotechnology (Dalian) Co., Ltd.) according to the manufacturer's instructions. Quantitative analysis of polymerase chain reaction (PCR) was carried out on a ABI 7500 Real-Time PCR System (Applied Biosystems, Life Technologies) using a SYBR Premix Ex TaqTM (Tli RNaseH Plus) qPCR kit (TaKaRa Biotechnology (Dalian) Co., Ltd.) according to the manufacturer's guidelines. Cycling conditions were 95°C×30 s, followed by 40 cycles of 95°C×5 s and 60°C×34 s. The primer pairs used are shown in Supplemental Table 2. The expression of the target genes relative to housekeeping gene (GAPDH) was calculated by the formula $2^{-\Delta\Delta CT}$ according to the method of Livak and Schmittgen [29]. Our results demonstrated that there was no difference in GAPDH expression among tissues and treatments. Relative mRNA expression level of each target gene was normalized to the control group.

2.9. Protein abundance analysis

The method for quantification of intestinal mucosal protein expression was carried out as previously described [3]. Briefly, 150 mg of the intestinal mucosal samples was homogenized in 1 ml of lysis buffer and centrifuged at 12,000×*g* for 15 min at 4°C to collect the supernatants. Then the protein contents of the supernatants were measured using the BCA reagent [3,30]. Equal amounts of intestinal mucosal proteins were subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis and transferred to blotting membrane for immunoblotting [3,30]. Immunoblots were blocked with 3% bovine serum albumin in Tris-buffered saline/Tween-20 buffer for 60 min at room temperature and incubated overnight at 4°C with primary antibodies. Specific