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RESEARCH ARTICLES

# Aspartate alleviates liver injury and regulates mRNA expressions of TLR4 and NOD signaling-related genes in weaned pigs after lipopolysaccharide challenge☆

Weibo Leng[a], Yulan Liu[a,*], Haifeng Shi[a], Shuang Li[a], Huiling Zhu[a], Dingan Pi[a], Yongqing Hou[a], Joshua Gong[b]

[a]Hubei Key Laboratory of Animal Nutrition and Feed Science, Wuhan Polytechnic University, Wuhan 430023, China
[b]Guelph Food Research Centre, Agriculture and Agri-Food Canada, Guelph, Ontario, Canada N1G 5C9

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**Abstract**

Pro-inflammatory cytokines play a critical role in many models of liver injury. In addition, aspartate (Asp) plays an important role in many biological and physiological processes including liver physiology. We hypothesized that Asp could alleviate lipopolysaccharide (LPS)-induced liver injury. Forty-eight weanling pigs were assigned to four treatments including: (1) non-challenged control; (2) LPS challenged control; (3) LPS+0.5% Asp; (4) LPS+1.0% Asp. After 20-d feeding with control (0% Asp), 0.5% or 1.0% Asp supplemented diets, pigs were injected with saline or LPS. At 4 (early phase) and 24 h (late phase) post-injection, blood and liver samples were obtained. Asp attenuated liver injury indicated by reduced serum aspartate aminotransferase activity and increased ratio of serum alanine aminotransferase and aspartate aminotransferase at 24 h, and less severe histological liver damage induced by LPS challenge at 4 or 24 h. In addition, Asp supplementation to LPS challenged pigs decreased mRNA expressions of tumor necrosis factor (TNF)-α and cyclooxygenase-2 linearly and quadratically at 4 h, and increased mRNA expressions of these pro-inflammatory mediators linearly and quadratically at 24 h. Finally, Asp decreased mRNA expression of toll-like receptor 4 (TLR4) signaling related genes (TLR4, myeloid differentiation factor 88, IL-1 receptor-associated kinase 1, TNF-α receptor-associated factor (6), nucleotide-binding oligomerization domain protein (NOD) signaling related genes (NOD1, NOD2 and receptor-interacting serine/threonine-protein kinase 2) and nuclear factor-κB p65 linearly or quadratically at 4 h. However, Asp increased mRNA expressions of these signaling molecules linearly or quadratically at 24 h. These results indicate that, at early and late phases of LPS challenge, Asp exerts opposite regulatory effects on mRNA expression of hepatic pro-inflammatory cytokines and TLR4 and NOD signalling related genes, and improves liver integrity.


*Keywords:* Aspartate; Weaned pigs; Lipopolysaccharide; Pro-inflammatory cytokines; Toll-like receptor 4; Nucleotide-binding oligomerization domain protein; Liver injury

## 1. Introduction

The liver is a metabolically active organ and the primary detoxifying site in the body [1]. However, multiple factors such as pathogenic bacteria and virus, lead to activation of macrophages (Kupffer cells) with an excessive production of pro-inflammatory cytokines such as interleukin (IL)-1β, IL-6 and tumor necrosis factor-α (TNF-α), consequently lead to liver impairment and liver dysfunction [2,3]. Among these cytokines, TNF-α is the key mediator of hepatotoxicity in many models of liver injury, including those involving the lipopolysaccharide (LPS) [4]. Therefore, modulating the release of liver pro-inflammatory cytokines may play a beneficial role in alleviating these liver disorders.

Aspartate (Asp) is an acidic amino acid. Traditionally, Asp is considered as a nutritionally non-indispensable amino acid in mammals [5,6]. However, growing literatures have shown that Asp plays an important role in many biological and physiological processes including liver physiology [7,8]. Of particular interest, Asp is required for the recycling of the citrulline produced by nitric oxide synthase into arginine (Arg) in macrophage [5,9]. This citrulline-Arg cycle helps maintain an adequate intracellular concentration of Arg for sustaining a high rate of nitric oxide (NO) production in macrophages in response to immunological challenges [9]. Accumulating evidence suggests that Arg could attenuate liver injury [10–12]. Yanni et al. [13] reported that oral supplementation with Asp inhibited atherogenesis and fatty liver disease in cholesterol-fed rabbit. However, the mechanism(s) underlying the beneficial effect of Asp on liver remain to be elucidated.

Transmembrane toll-like receptors (TLRs) and cytoplasmic nucleotide-binding oligomerization domain proteins (NODs) are important

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* Corresponding author. Tel./fax: +8627 83956175.
*E-mail address:* yulanflower@126.com (Y. Liu).

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pattern recognition receptors that recognize pathogen associated molecular patterns (PAMPs) and play critical roles in modulating innate and adaptive immune responses [14,15]. As the best-characterized member of the TLR family, TLR4 is activated by a small amount of LPS [14]. Among NOD family, NOD1 and NOD2 are specialized NODs, which can connect with the LPS and peptidoglycan, and trigger signal transduction pathway [16]. Interaction of PAMPs with TLRs or NODs can initiate downstream signaling events that leads to the activation of nuclear factor-κB (NF-κB), which then stimulates the expression of inflammatory genes including IL-1β, IL-6 and TNF-α [14,15]. Consequently, the overproduction of pro-inflammatory cytokines induce host tissue injury.

Accordingly, we hypothesized that Asp would affect liver integrity by modulating the production of pro-inflammatory cytokines via regulation of TLR and NOD signaling pathways. LPS has been suggested to play a role in various types of liver diseases, including alcoholic or non-alcoholic fatty liver [17,18], alcoholic or non-alcoholic steatohepatitis [17,18], ischemic liver injury [19] and liver cirrhosis [20,21]. When binding with TLR4/MD-2/CD14 receptor complex, LPS stimulates Kupffer cells (macrophages in liver) to produce a variety of pro-inflammatory cytokines, which mediates inflammatory response and results in liver damage [2]. So, in our current experiment, *Escherichia coli* LPS was injected to establish the model of liver injury according to Chen et al. [2] and Li et al. [10]. In addition, we made use of a piglet model, a well-characterized animal model for studying human nutrition and physiology [22,23]. Our objective was to investigate whether dietary supplementation of Asp could alleviate LPS-induced liver injury, and to elucidate its molecular mechanism(s). Our findings will not only aid in understanding the mode of Asp's actions in the liver of piglets, but also have important implications for the development of new interventions to ameliorate liver injury in inflammatory condition in human.

## 2. Materials and methods

### 2.1. Animal care and experimental design

This research protocol was approved by the Animal Care and Use Committee of Hubei Province, China. Forty-eight crossbred pigs (Duroc×Large White×Landrace) weighing 8.07 ±0.75 kg were balanced for ancestry and initial body weight across four treatment groups. There were twelve replicate pens for each treatment groups. Pigs were individually caged in 1.80×1.10 m pen in a temperature-controlled nursery barn (25 to 27°C) and allowed ad libitum access to feed and water. The basal diet (Table 1) was formulated in accordance with National Research Council (NRC) requirements for all nutrients [24].

The experiment included four treatments ($n$=12): (1) non-challenged control (CONTR, pigs receiving a control diet and injected with 0.9% sterile saline); (2) LPS-challenged control (LPS, pigs receiving the same control diet and challenged with *E. coli* LPS); (3) LPS+0.5% Asp treatment (pigs receiving a 0.5% Asp diet and challenged with LPS); (4) LPS+1.0% Asp treatment (pigs receiving a 1.0% Asp diet and challenged with LPS). The Asp doses (L-aspartic acid, purity >99%; Amino Acid Bio-Chemical, Wuhan, China) were chosen according to our preliminary study, which showed that 0.5% and 1.0% Asp attenuated LPS-induced decrease of weight gain in weanling piglets [25]. We added 0.67%, 0.34% and 0% alanine (purity >99 %; Amino Acid Bio-Chemical) to the control, 0.5% Asp and 1.0% Asp diets respectively, to obtain isonitrogenous diets. Alanine was commonly chosen for the isonitrogenous control in many studies because alanine is not toxic and is not a substrate for Asp synthesis, but is extensively catabolized by pigs [26,27]. After 20-day feeding with control, 0.5% and 1.0% Asp supplemented diets, the challenged group was injected intraperitoneally with *E. coli* LPS (*E. coli* serotype 055: B5; Sigma Chemical, St. Louis, MO, USA) at 100 μg/kg body weight (BW), and the unchallenged group was injected with the same amount of 0.9% NaCl solution. The LPS dose (100 μg/kg BW) was used in accordance with our previous studies [2,10,25]. During 4 or 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 blood and liver variables. The pigs were allowed ad libitum access to water.

### 2.2. Blood and liver sample collections

After 4 or 24 h saline or LPS injection, blood and liver samples were harvested from half of the pigs ($n$=6) in four treatment groups. The procedures of blood and liver sample collections were the same as Chen et al. [2]. Blood samples were centrifuged to separate serum. Serum was stored at −80°C for further analyses of amino acid concentrations and biochemical parameters. One fragment of liver samples was stored in fresh 4% paraformaldehyde/phosphate-buffered saline at least for 24 hours, and then embedded in paraffin. The remaining portions were frozen immediately in liquid nitrogen, and then stored at -80°C for subsequent analysis.

Table 1
Ingredient composition of diets (as fed basis)[a]

| Items | Content |
|---|---|
| Ingredients | g/kg |
| Corn | 574 |
| Soybean meal (44% CP) | 224 |
| Wheat middling | 50 |
| Fish meal | 36 |
| Soy protein concentrate | 14 |
| Fat powder[b] | 20 |
| Milk-replacer powder | 30 |
| Limestone | 9.4 |
| Dicalcium phosphate | 12.2 |
| Salt | 3.4 |
| Alanine[c] | 6.7 |
| Cornstarch[c] | 3.3 |
| Acidifier[d] | 2.0 |
| L-Lysine. HCl (78.8% Lysine) | 2.7 |
| DL-Methionine (99% threonine) | 1.0 |
| L-Threonine (98% threonine) | 0.8 |
| Butylated hydroquinone | 0.5 |
| Vitamin and mineral premix[e] | 10 |
| Nutrient composition | g/kg |
| Digestible energy[f,g] (MJ/kg) | 13.6 |
| Crude protein[h] | 203 |
| Calcium[h] | 8.0 |
| Total phosphorus[h] | 7.0 |
| Total lysine[g] | 13 |
| Total methionine + cysteine[g] | 6.5 |

[a] The basal diet was formulated in accordance with NRC requirements for all nutrients [24].
[b] A rumen-stable fat powder, purchased from Berg + Schmidt, German.
[c] In the 0.5% Asp diet, 0.67% alanine and 0.33% cornstarch were replaced by 0.5% Asp, 0.34% alanine and 0.16% cornstarch. In the 1.0% Asp diet, 0.67% alanine and 0.33% cornstarch was replaced by 1.0% Asp. All diets were isonitrogenous.
[d] A compound acidifier including lactic acid and phosphoric acid, provided by Wuhan Fanhua Biotechnology, Wuhan, China.
[e] The vitamin and mineral premix (defatted rice bran as carrier) provided the following amounts per kilogram of complete diet: retinol acetate, 2700 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 ($Na_2SeO_3 \cdot 5H_2O$).
[f] Based on diets containing cornstarch.
[g] Calculated.
[h] Analyzed.

### 2.3. Serum amino acid concentrations

Serum concentrations of Asp and the related amino acids were measured by high-performance liquid chromatography methods involving precolumn derivatization with *o*-phthaldialdehyde as previously described [28].

### 2.4. Liver morphology

After a 24 h fixation, the liver segments were dehydrated, embedded, and stained with hematoxylin and eosin. The method for measurements of hepatic injury was according to Li et al. [10].

### 2.5. Serum biochemical parameters

The activities of serum alanine aminotransferase (ALAT), aspartate aminotransferase (ASAT), glutamyl transpeptidase (GGT) and alkaline phosphatase (AKP) were analyzed as previously described [2].

### 2.6. Western blotting measurement

The methods for protein immunoblot analysis were the same as Chen et al. [2]. Briefly, the liver samples (150–200 mg) ($n$=6) were homogenized and centrifuged to collect the supernatants for Western blot and protein assay. An equal amount of hepatic proteins were separated on a polyacrylamide gel and transferred onto