Effects of Asparagine on Growth Performance and Hypothalamic-Pituitary-Adrenal Axis Toll-Like Receptor 4 and Nucleotide-Binding Oligomerization Domain Signaling Pathways in Lipopolysaccharide-Challenged Weaned Piglets: Postprint

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Abstract

This study aimed to investigate the effects of asparagine (Asn) on growth performance and mRNA expression of key genes in the Toll-like receptor 4 (TLR4) and nucleotide-binding oligomerization domain (NOD) signaling pathways in the hypothalamic-pituitary-adrenal (HPA) axis of lipopolysaccharide (LPS)-challenged weaned piglets. Twenty-four healthy (21±2)-day-old "Duroc × Landrace × Large White" weaned piglets [(8.12±0.56) kg] were selected and randomly allocated to 4 groups based on similar body weight: 1) control group (basal diet); 2) LPS group (basal diet + LPS); 3) LPS+0.5% Asn group (basal diet + 0.5% Asn + LPS); 4) LPS+1.0% Asn group (basal diet + 1.0% Asn + LPS). Each group had 6 replicates with 1 pig per replicate. On day 20 of the experiment, pigs in the LPS, LPS+0.5% Asn, and LPS+1.0% Asn groups were injected with 100 g/kg BW of LPS, while pigs in the control group were injected with an equal volume of physiological saline. The experimental period lasted 20 days. The results showed that compared with the control group, LPS challenge and dietary Asn supplementation had no significant effect on the growth performance of LPS-challenged weaned piglets ($P>0.05$). LPS challenge significantly increased the mRNA expression of TLR4, myeloid differentiation factor 88 (MyD88), NOD2, receptor-interacting serine/threonine-protein kinase 2 (RIP2), and nuclear factor- B (NF- B) in the HPA axis ($P<0.05$). Dietary Asn supplementation significantly decreased the mRNA expression of NF- B in the hypothalamus (linear, $P<0.05$) and tended to decrease the mRNA expression of TLR4 and NOD2 in the adrenal gland (linear, $P<0.10$). Furthermore, dietary Asn supplementation significantly decreased the mRNA expression of tumor necrosis factor receptor-associated factor 6 (TRAF6) in the pituitary

(quadratic, P<0.05) and significantly increased the mRNA expression of RIP2 in the adrenal gland (linear, P<0.05; quadratic, P<0.05). These results indicate that although Asn had no effect on the growth performance of LPS-challenged piglets, it may exert certain inhibitory effects on the hypothalamic TLR4 signaling pathway by decreasing the mRNA expression of NF- B in the hypothalamus.

Full Text

Abstract

This study investigated the effects of asparagine (Asn) on growth performance and key gene mRNA expression in the Toll-like receptor 4 (TLR4) and nucleotide-binding oligomerization domain (NOD) signaling pathways of the hypothalamus-pituitary-adrenal (HPA) axis in weaned piglets challenged with lipopolysaccharide (LPS). Twenty-four healthy (21±2)-day-old "Duroc×Landrace×Yorkshire" weaned piglets [(8.12±0.56) kg] were selected and randomly allocated to 4 groups: 1) control group (basal diet), 2) LPS group (basal diet + LPS), 3) LPS+0.5% Asn group (basal diet + 0.5% Asn + LPS), and 4) LPS+1.0% Asn group (basal diet + 1.0% Asn + LPS). Each group had 6 replicates with 1 pig per replicate. On day 20, pigs in the LPS, LPS+0.5% Asn, and LPS+1.0% Asn groups were injected with 100 g/kg BW of LPS, while control pigs received an equivalent volume of saline. The experiment lasted 20 days. Results showed that compared with the control group, LPS challenge and dietary Asn supplementation had no significant effects on growth performance of LPS-challenged weaned piglets (P>0.05). LPS challenge significantly increased mRNA expression of TLR4, myeloid differentiation factor 88 (MyD88), NOD2, receptor-interacting protein serine/threonine kinase 2 (RIP2), and nuclear factor- B (NF- B) in the HPA axis (P<0.05). Dietary Asn supplementation significantly decreased NF- B mRNA expression in the hypothalamus (linear, P<0.05) and tended to decrease TLR4 and NOD2 mRNA expression in the adrenal gland (linear, P<0.10). Additionally, Asn supplementation significantly decreased tumor necrosis factor receptor-associated factor 6 (TRAF6) mRNA expression in the pituitary (quadratic, P<0.05) and significantly increased RIP2 mRNA expression in the adrenal gland (linear, P<0.05; quadratic, P<0.05). These results suggest that although Asn did not affect growth performance in LPS-challenged piglets, it may exert inhibitory effects on the hypothalamic TLR4 signaling pathway by decreasing NF- B mRNA expression.

Keywords: asparagine; lipopolysaccharide; weaned piglets; hypothalamus-pituitary-adrenal axis; TLR4; NOD

Introduction

In modern swine production, early-weaned piglets are vulnerable to various adverse environmental factors such as bacterial and viral challenges, which can trigger immune stress. During immune stress, the immune system produces large

quantities of inflammatory cytokines including tumor necrosis factor- (TNF-), interleukin (IL)-6, and IL-1, leading to tissue damage (e.g., in intestine and liver) [1] and ultimately causing reduced growth performance. Therefore, nutritional interventions (such as supplementation with fatty acids and amino acids) that reduce excessive release of inflammatory mediators are important for alleviating immune stress in piglets.

Traditionally considered a non-essential amino acid, asparagine (Asn) has been shown to play important roles in regulating immune function [2]. Wu et al. [3] classified Asn as a member of the arginine (Arg) family of amino acids, as it can be converted to Arg and glutamine (Gln) in vivo through deamination and transamination pathways. Both Arg and Gln are known to regulate tissue function and alleviate inflammation. Previous studies from our laboratory demonstrated that Asn could relieve immune stress and tissue damage induced by LPS challenge in piglets [4] and improve impaired hepatic energy metabolism [5]. The hypothalamus-pituitary-adrenal (HPA) axis regulates numerous physiological processes including immunity, reproduction, and stress responses [6], playing a crucial role in stress and infection [7]. LPS stimulates peripheral immune cells to produce abundant inflammatory cytokines such as TNF- , IL-1, and IL-8]. Elevated levels of these cytokines activate the HPA axis, triggering immune stress responses [9-10]. Nutritional strategies that reduce inflammatory cytokines may help alleviate LPS-induced immune stress [11]. Based on these findings, we hypothesized that dietary Asn supplementation might mitigate LPS-induced inflammatory responses in the HPA axis, thereby relieving systemic stress.

Toll-like receptor 4 (TLR4) is a critical component of innate immunity. Its interaction with myeloid differentiation factor 88 (MyD88) initiates nuclear factor- B (NF- B) activation, which stimulates expression of inflammatory genes and promotes pro-inflammatory cytokine production. Similarly, nucleotide-binding oligomerization domain receptors (NOD) 1 and NOD2 can also activate inflammatory cytokine release. While these cytokines protect against pathogen invasion, they can also cause host tissue damage and neuroendocrine disruption. The HPA axis plays an important role in stress and infection, and LPS stimulation can activate TLR4 and NOD inflammatory signaling pathways in the HPA axis, leading to increased secretion of inflammatory factors such as TNF- [12]. Previous research showed that dietary fish oil could alleviate LPS-induced immune stress in weaned piglets by inhibiting TLR4 and NOD inflammatory signaling pathways in the HPA axis and reducing inflammatory factor secretion [12]. However, the effects of Asn on TLR4 and NOD signaling pathways in the HPA axis have not been reported. Therefore, this study investigated the effects of Asn on growth performance and TLR4 and NOD signaling pathways in the HPA axis of LPS-challenged weaned piglets to provide a theoretical basis for alleviating immune stress in piglets.

1. Materials and Methods

1.1 Experimental Material

Asparagine: Active ingredient >99.4%, purchased from Wuhan Amino Technology Co., Ltd.

1.2 Experimental Animals and Design

Twenty-four healthy (21±2)-day-old "Duroc×Landrace×Yorkshire" weaned piglets with an average body weight of (8.12±0.56) kg were selected and randomly divided into 4 groups according to similar body weight: 1) control group (basal diet), 2) LPS group (basal diet + LPS), 3) LPS+0.5% Asn group (basal diet + 0.5% Asn + LPS), and 4) LPS+1.0% Asn group (basal diet + 1.0% Asn + LPS). Each group had 6 replicates with 1 pig per replicate, and the experimental period lasted 20 days. The basal diet was isonitrogenously balanced with alanine. On day 20, pigs in the LPS, LPS+0.5% Asn, and LPS+1.0% Asn groups were injected intraperitoneally with 100 g/kg BW of LPS (E. coli serotype 055:B5, Sigma, USA), while the control group received an equivalent volume of saline.

1.3 Experimental Diets

Diet formulation followed NRC (1998) nutrient requirements for piglets. The composition and nutrient levels of the basal diet are shown in Table 1 .

1.4 Feeding Management

The animal trial was conducted at the Hubei Key Laboratory of Animal Nutrition and Feed Science. Before the experiment, pens were cleaned and washed, disinfected with 2% NaOH solution, sprayed with 1:200 diluted disinfectant, and fumigated with formaldehyde and potassium permanganate for several hours, followed by a 1-week purification period. Experimental piglets were housed in stainless steel cages (1.80 m × 1.10 m). During the trial, room temperature was maintained at 25-27°C with 12 h daily lighting, free ventilation, and ad libitum access to feed and water.

1.5 Sample Collection and Processing

Daily feed intake was recorded throughout the trial. Pigs were weighed after 12 h fasting on days 1 and 19 to calculate average daily gain (ADG), average daily feed intake (ADFI), and feed/gain ratio (F/G). On day 19, after 12 h fasting before LPS or saline injection, all pigs were slaughtered 4 h post-injection. The hypothalamus, pituitary, and adrenal glands were collected, rinsed with ice-cold saline, placed in cryovials, snap-frozen in liquid nitrogen, and stored at -80°C for subsequent analysis.

1.6 Detection Indicators and Methods

Tissue samples were homogenized in 1 mL RNAiso Plus reagent (Takara, Dalian, China) on ice, and total RNA was extracted according to the manufacturer' s instructions. cDNA synthesis was performed using the PrimeScript® RT reagent kit with gDNA eraser (Takara, Dalian, China) in a 20 L reaction system containing 4 L 5×PrimeScript® Buffer, 1 L PrimeScript® RT enzyme mix, 1.0 L RT primer mix, 10 L DNase-treated RNA, and RNase-free dH2O to 20 L. Reverse transcription parameters were: 37°C for 15 min, 85°C for 5 s, and 4°C hold.

Real-time quantitative PCR was performed using SYBR® Premix Ex TaqTM (Tli RNaseH Plus) (Takara, Dalian, China) in a 20 L reaction system containing 10.0 L SYBR® Premix Ex TaqTM (2×), 0.4 L ROX reference dye II (10×), 2.0 L cDNA, 6.8 L RNase-free dH2O, 0.4 L forward primer (10 mol/L), and 0.4 L reverse primer (10 mol/L). Amplification was performed on an ABI 7500 Real-time PCR system (Applied Biosystems) with the following conditions: 95°C for 30 s, followed by 40 cycles of 95°C for 5 s and 60°C for 34 s. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as the internal reference gene, and relative gene expression was analyzed using the 2-ΔΔCT method [13].

Specific primers were designed using Primer Premier 5.0 software and synthesized by Takara (Dalian, China). Primer sequences are listed in Table 2 .

1.7 Statistical Analysis

Data were analyzed using SPSS 22.0 software. Independent samples t-tests were used to compare the LPS group vs. control group (to determine LPS effects) and LPS group vs. LPS+0.5% Asn and LPS+1.0% Asn groups (to determine Asn effects). Linear and quadratic regression analyses were used to evaluate the effects of different Asn levels (0, 0.5%, 1.0%) in LPS-challenged piglets. Data are presented as means. P 0.05 was considered statistically significant, and 0.05<P 0.10 indicated a significant trend.

2. Results

2.1 Effects of Asn on Growth Performance of LPS-Challenged Weaned Piglets

As shown in Table 3 , LPS challenge and dietary Asn supplementation had no significant effects on growth performance of LPS-challenged weaned piglets compared with the control group (P>0.05).

2.2 Effects of Asn on Key Gene mRNA Expression in TLR4 and NOD Signaling Pathways in the Hypothalamus of LPS-Challenged Weaned Piglets

As shown in Table 4 , LPS significantly increased mRNA expression of TLR4, MyD88, NOD2, receptor-interacting protein serine/threonine kinase 2 (RIP2), and NF- B in the hypothalamus (P<0.05). Dietary Asn supplementation significantly decreased NF- B mRNA expression in the hypothalamus (linear, P<0.05) and tended to decrease interleukin-1 receptor-associated kinase 1 (IRAK1) mRNA expression (linear, P<0.10).

2.3 Effects of Asn on Key Gene mRNA Expression in TLR4 and NOD Signaling Pathways in the Pituitary of LPS-Challenged Weaned Piglets

As shown in Table 5 , LPS significantly increased mRNA expression of TLR4, MyD88, NOD1, NOD2, RIP2, NF- B, and TNF- in the pituitary (P<0.05). Dietary Asn supplementation significantly decreased tumor necrosis factor receptor-associated factor 6 (TRAF6) mRNA expression in the pituitary (quadratic, P<0.05) and increased NOD1 mRNA expression (linear, P<0.10; quadratic, P<0.05).

2.4 Effects of Asn on Key Gene mRNA Expression in TLR4 and NOD Signaling Pathways in the Adrenal Gland of LPS-Challenged Weaned Piglets

As shown in Table 6 , LPS significantly increased mRNA expression of TLR4, MyD88, IRAK1, NOD2, RIP2, NF- B, and TNF- in the adrenal gland (P<0.05). Dietary Asn supplementation tended to decrease TLR4 and NOD2 mRNA expression in the adrenal gland (linear, P<0.10) and significantly increased RIP2 mRNA expression (linear, P<0.05; quadratic, P<0.05).

3. Discussion

Immune stress is common in swine production and represents a major factor causing growth inhibition, resulting in significant economic losses. It is triggered by pathogens or non-pathogens (such as bacteria, viruses, and endotoxins) in the environment or by vaccination [14]. Highly activated immune stress leads to excessive secretion of inflammatory cytokines, causing tissue damage. Traditionally, inflammatory cytokines were thought to be produced primarily by the immune system, but recent studies have revealed that the neuroendocrine system, gastrointestinal tract, liver, muscle, and adipose tissue can also secrete inflammatory cytokines [15-17]. These cytokines cause behavioral and metabolic changes in animals through direct effects on target tissues or by acting on the neuroendocrine system, ultimately leading to growth retardation and reduced carcass quality [14]. Therefore, measures to moderately regulate excessive immune responses, particularly the overproduction of inflammatory cytokines, are

important for alleviating immune stress.

Nutritional regulation to mitigate immune stress has become a key research area in animal nutrition. Studies have shown that nutrients such as n-3 polyunsaturated fatty acids (PUFA), Arg, and conjugated linoleic acid can alleviate immune stress in pigs [15-19]. However, the regulatory mechanisms of immune stress remain unclear, limiting the specificity of nutritional interventions.

Toll-like receptors (TLRs) are pattern recognition receptors (PRRs) that recognize pathogen-associated molecular patterns (PAMPs) and play crucial roles in antibacterial defense and activation of innate immune responses [20-21]. TLR4 recognizes LPS and mediates inflammatory responses through signaling cascades involving MyD88, IRAK1, and TRAF6, ultimately triggering NF- B activation, which stimulates expression of inflammatory genes and promotes pro-inflammatory cytokine production. Many disease pathogenesis mechanisms are closely related to excessive activation of TLR signaling pathways [22], and TLR4 pathway activation is an important cause of immune stress in pigs [15-18]. Therefore, effective inhibition of the TLR4 signaling pathway is of practical significance for alleviating immune stress in piglets. In this study, LPS increased mRNA expression of TLR4, MyD88, NF- B in the hypothalamus, pituitary, and adrenal gland, and IRAK1 in the adrenal gland, indicating that LPS challenge activated the TLR4 signaling pathway in the HPA axis. Dietary Asn supplementation decreased IRAK1 mRNA expression in the hypothalamus, TLR4 mRNA expression in the adrenal gland, and TRAF6 mRNA expression in the pituitary, suggesting that Asn may inhibit the upstream TLR4 signaling pathway in the adrenal gland while inhibiting downstream pathways in the hypothalamus and pituitary.

Previous studies demonstrated that Arg can alleviate LPS-induced increases in hepatic TLR4 mRNA expression in weaned piglets [23]. As an Arg family amino acid that can be converted to Arg in vivo [2], Asn may inhibit TLR4 and downstream gene expression in the HPA axis through conversion to Arg, thereby alleviating LPS-induced inflammatory responses. Additionally, Asn supplementation showed a quadratic effect on TRAF6 mRNA expression in the pituitary, with 0.5% Asn being more effective than 1.0% Asn in inhibiting downstream TLR4 receptor signaling gene expression. Previous research also showed that Asn can alleviate LPS-induced liver injury [24] and improve damaged intestinal integrity [25] by decreasing mRNA expression of TLR4, IRAK1, TRAF6, NOD1, and NOD2. Therefore, Asn may alleviate LPS-induced inflammatory responses in the HPA axis by reducing mRNA expression of IRAK1, TLR4, and TRAF6.

In addition to the TLR4 pathway, nucleotide-binding oligomerization domain proteins (NODs) are another PRR family that plays key roles in recognizing PAMPs and regulating host innate immune responses [20-21]. Research has focused primarily on NOD1 and NOD2, which, similar to TLR4, can activate NF- B through the adaptor molecule RIP2, leading to upregulated transcription of pro-inflammatory cytokine genes [21]. To prevent excessive NOD pathway

activation and maintain homeostasis, negative regulators of NOD1 and NOD2 exist, such as Erbb2-interacting protein (ERBB2IP) and ACAP1 [26-27]. In this study, LPS challenge increased mRNA expression of NOD2, RIP2, NF- B in the hypothalamus; NOD1, NOD2, RIP2, NF- B in the pituitary; and NOD2, RIP2, NF- B in the adrenal gland, indicating activation of NOD signaling pathways. Dietary Asn supplementation decreased NOD2 mRNA expression in the adrenal gland and NF- B mRNA expression in the hypothalamus. However, Asn also increased RIP2 mRNA expression in the adrenal gland and tended to increase NF- B mRNA expression. Pi et al. [28] found that LPS challenge decreased hepatic ACAP1 mRNA expression, suggesting that excessive inflammatory responses may reduce expression of negative regulators. Similarly, high levels of dietary Asn may suppress expression of negative regulators in the NOD pathway, leading to increased NF- B mRNA expression. RIP2 is an upstream factor in the NOD signaling pathway that links NOD1/NOD2 to downstream NF- B. Although Asn increased RIP2 mRNA expression in the adrenal gland, it had minimal effect on downstream NF- B mRNA expression.

4. Conclusion

Although Asn did not affect growth performance in LPS-challenged piglets, it may exert inhibitory effects on the hypothalamic TLR4 signaling pathway by decreasing NF- B mRNA expression.

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Note: Figure translations are in progress. See original paper for figures.