Effects of Flaxseed Oil on Expression of Key Genes in TLR4 and NOD Signaling Pathways in the Liver of Lipopolysaccharide-Challenged Piglets: Postprint

Authors: Chen Shaokui, Liu Yulan, Wang Haibo, Wang Xiuying, Zhu Huiling, Zhang Jing, Wang Shuhui, Tu Zhixiao

Date: 2017-10-10T00:00:00+00:00

Abstract

This study was conducted to investigate the effects of flaxseed oil on the expression of key genes in the Toll-like receptor 4 (TLR4) and nucleotide-binding oligomerization domain (NOD) signaling pathways in the liver of lipopolysaccharide (LPS)-challenged piglets. Twenty-four weaned piglets were randomly allocated into four groups based on similar body weight: control group, LPS group, 2.5% flaxseed oil group (2.5% flaxseed oil + LPS), and 5.0% flaxseed oil group (5.0% flaxseed oil + LPS), with six replicates per group and one pig per replicate for a 21-day experimental period. The treatment groups were injected with LPS at 100 g/kg body weight, while the control group received an equal volume of physiological saline. Four hours post-injection, the piglets were euthanized, liver tissues were harvested, and the mRNA expression levels of key genes in the TLR4 and NOD signaling pathways and related inflammatory mediators were measured. The results showed that: 1) LPS challenge significantly increased the relative mRNA expression of hepatic tumor necrosis factor-α (TNF-α), cyclooxygenase-2 (COX-2), and heat shock protein 70 (HSP70) ($P<0.05$), while 2.5% flaxseed oil significantly decreased the relative mRNA expression of COX-2 and TNF-α ($P<0.05$), and 5.0% flaxseed oil significantly decreased the relative mRNA expression of TNF-α ($P<0.05$). 2) LPS challenge significantly increased the relative mRNA expression of hepatic TLR4, myeloid differentiation factor 88 (MyD88), interleukin-1 receptor-associated kinase 1 (IRAK1), NOD1, NOD2, receptor-interacting protein kinase 2 (RIPK2), and nuclear factor- B (NF- B) ($P<0.05$); 2.5% flaxseed oil significantly decreased the relative mRNA expression of NOD1 and NOD2 ($P<0.05$), and tended to decrease the relative mRNA expression of RIPK2 ($P<0.10$); 5.0% flaxseed oil significantly decreased the relative mRNA expression of NOD2 ($P<0.05$). These findings indicate that

LPS challenge induced an inflammatory response in piglets, and flaxseed oil may alleviate hepatic inflammatory response by suppressing the NOD signaling pathway.

Full Text

Effects of Flaxseed Oil on the mRNA Expression of Key Genes in TLR4 and NOD Signaling Pathways in Liver of Piglets Challenged with LPS

CHEN Shaokui, LIU Yulan*, WANG Haibo, WANG Xiuying, ZHU Huiling, ZHANG Jing, WANG Shuhui, TU Zhixiao

Hubei Key Laboratory of Animal Nutrition and Feed Science, Wuhan Polytechnic University, Wuhan 430023, China

Abstract

This experiment was conducted to investigate the effects of flaxseed oil on the mRNA expression of key genes in the toll-like receptor 4 (TLR4) and nucleotide-binding oligomerization domain (NOD) signaling pathways in the liver of piglets challenged with lipopolysaccharide (LPS). Twenty-four weaned piglets were randomly allocated to four groups based on similar body weight: control group, LPS group, 2.5% flaxseed oil group (2.5% flaxseed oil + LPS), and 5.0% flaxseed oil group (5.0% flaxseed oil + LPS), with six replicates per group and one piglet per replicate. The experimental period lasted 21 days. Piglets in the treatment groups were injected intraperitoneally with 100 g/kg body weight of LPS, while those in the control group received an equivalent volume of sterile saline. Four hours post-injection, all piglets were slaughtered and liver samples were collected to determine the mRNA expression levels of key genes in the TLR4 and NOD signaling pathways and related inflammatory mediators. The results showed that: (1) LPS challenge significantly increased the mRNA relative expression levels of tumor necrosis factor-α (TNF-α), cyclooxygenase-2 (COX2), and heat shock protein 70 (HSP70) in the liver ($P<0.05$). Dietary supplementation with 2.5% flaxseed oil significantly decreased the mRNA relative expression levels of COX2 and TNF-α ($P<0.05$), while 5.0% flaxseed oil significantly reduced the mRNA relative expression level of TNF-α ($P<0.05$). (2) LPS challenge significantly elevated the mRNA relative expression levels of TLR4, myeloid differentiation factor 88 (MyD88), interleukin-1 receptor-associated kinase 1 (IRAK1), NOD1, NOD2, receptor-interacting protein kinase 2 (RIPK2), and nuclear factor- B (NF- B) in the liver ($P<0.05$). Supplementation with 2.5% flaxseed oil significantly decreased the mRNA relative expression levels of NOD1 and NOD2 ($P<0.05$) and showed a tendency to reduce RIPK2 mRNA expression ($P<0.10$), while 5.0% flaxseed oil significantly lowered NOD2 mRNA relative expression ($P<0.05$). These findings indicate that LPS challenge induces an inflammatory response in piglets, and flaxseed oil may alleviate hepatic inflammation by inhibiting the NOD signaling pathway.

Keywords: piglets; flaxseed oil; LPS; liver; TLR4; NOD

The liver serves as the body' s metabolic center and a primary organ for detoxification and immune defense. In piglets, pathogenic and non-pathogenic antigens in the rearing environment can stimulate hepatic macrophages to produce and release large quantities of inflammatory mediators, leading to structural and functional liver damage that ultimately results in growth inhibition [1]. Therefore, regulating the excessive production of inflammatory mediators through nutritional interventions is crucial for alleviating liver injury and represents an effective strategy for mitigating inflammation.

Flaxseed oil is rich in α-linolenic acid (C18:3n-3), a precursor for eicosapentaenoic acid (EPA) (C20:5n-3) and docosahexaenoic acid (DHA) (C22:6n-3). Numerous studies have demonstrated that n-3 polyunsaturated fatty acids (PUFAs), such as EPA and DHA, exert important protective effects in various liver injury models, including acute chemical liver injury [1], cholestatic liver injury [2], diabetic liver injury [3], and fatty liver disease [4]. The protective effects of n-3 PUFAs may be attributed to their ability to suppress excessive production of inflammatory mediators [1-4]. However, the molecular mechanisms underlying the hepatoprotective effects of flaxseed oil remain unclear.

Toll-like receptor 4 (TLR4) and nucleotide-binding oligomerization domain (NOD) proteins are important families that regulate innate and adaptive immune responses. Key genes in the TLR4 signaling pathway include TLR4, MyD88, IRAK1, and tumor necrosis factor receptor-associated factor 6 (TRAF6), while the NOD signaling pathway comprises NOD1, NOD2, and RIPK2. Previous research has shown that activation of TLR4 and NOD can stimulate downstream signaling molecules, ultimately activating NF- B and inducing the expression of inflammatory mediators that lead to liver injury [5]. Therefore, we hypothesized that flaxseed oil might regulate inflammatory mediator production through TLR4 and NOD signaling pathways, thereby modulating liver damage. This study investigated the effects of flaxseed oil on the expression of key genes in hepatic TLR4 and NOD signaling pathways and inflammatory mediators in weaned piglets challenged with LPS, aiming to provide preliminary evidence on whether flaxseed oil alleviates inflammatory liver injury by modulating these pathways.

1.1 Experimental Animals and Design

Twenty-four "Duroc × Landrace × Yorkshire" weaned piglets with an average body weight of (6.98$±$0.05) kg were randomly assigned to four groups based on similar body weight: control group (5.0% corn oil), LPS group (5.0% corn oil + LPS), 2.5% flaxseed oil group (2.5% corn oil + 2.5% flaxseed oil + LPS), and 5.0% flaxseed oil group (5.0% flaxseed oil + LPS). Each group consisted of six replicates with one piglet per replicate, and the experimental duration was 21 days. Corn oil was provided by Shandong Xiwang Food Co., Ltd., and flaxseed

oil was provided by Gansu Longyuxiang Grain and Oil Industry Co., Ltd. The fatty acid compositions of corn oil and flaxseed oil are presented in Table 1 . On day 21 of the experiment, piglets in the LPS, 2.5% flaxseed oil, and 5.0% flaxseed oil groups were intraperitoneally injected with 100 g/kg body weight of LPS (Escherichia coli serotype O55:B5, Sigma), while piglets in the control group received an equivalent volume of sterile saline. The LPS dosage and treatment duration were based on previous studies [6-8], which demonstrated that intraperitoneal injection of 100 g/kg LPS induces an inflammatory response and liver injury in piglets within 4 hours. The experimental diets were formulated according to NRC (2012) nutrient requirements, and the composition and nutrient levels of the basal diet are shown in Table 2 .

1.2 Sample Collection

Four hours after LPS or saline injection, piglets were slaughtered and liver samples were immediately collected, snap-frozen in liquid nitrogen, and subsequently stored at -80 °C for total RNA extraction.

1.3 mRNA Expression Analysis

1.3.1 Equipment and Reagents The following equipment was used: 7500 Real-Time PCR System (Applied Biosystems), gradient thermal cycler (TaKaRa), NanoDrop 2000 microspectrophotometer (Thermo), and Tanon-4100 gel imaging system (Shanghai Tanon). RNAiso Plus (Total RNA extraction reagent), PrimeScript® RT reagent kit with gDNA eraser (cDNA synthesis kit), and SYBR® Premix Ex Taq™ (Tli RNaseH Plus) (Real-time PCR kit) were purchased from Takara Bio (Dalian, China).

1.3.2 Methods Total RNA extraction, cDNA synthesis, and real-time PCR were performed according to the methods described by Chen et al. [9]. Real-time PCR data were calculated using the 2-ΔΔCt method of Livak and Schmittgen [10], with glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as the internal reference gene.

1.3.3 Primer Design and Synthesis Based on published porcine gene sequences in GenBank for TLR4, MyD88, IRAK1, TRAF6, NOD1, NOD2, RIPK2, NF- B, TNF-α, COX2, HSP70, and GAPDH, real-time PCR primers were designed using Primer Premier 6.0 software (Table 3). Primers were synthesized by Takara Bio (Dalian, China).

1.4 Statistical Analysis

Data were analyzed by one-way ANOVA and LSD multiple comparisons using SPSS 17.0 software. Results are expressed as means ± standard error. $P<0.10$ indicated a significant trend, and $P<0.05$ indicated a significant difference.

2.1 Effects of Flaxseed Oil on mRNA Relative Expression Levels of COX2, HSP70, and TNF-α in Liver of LPS-Challenged Piglets

As shown in Table 4 , compared with the control group, LPS challenge significantly increased the mRNA relative expression levels of COX2, HSP70, and TNF-α in the liver ($P<0.05$). Dietary supplementation with 2.5% flaxseed oil significantly decreased the mRNA relative expression levels of COX2 and TNF-α compared with the LPS group ($P<0.05$), while 5.0% flaxseed oil significantly reduced the mRNA relative expression level of TNF-α ($P<0.05$).

2.2 Effects of Flaxseed Oil on mRNA Relative Expression Levels of Key Genes in TLR4 and NOD Signaling Pathways in Liver of LPS-Challenged Piglets

As shown in Table 5 , compared with the control group, LPS challenge significantly increased the mRNA relative expression levels of TLR4, MyD88, IRAK1, NOD1, NOD2, RIPK2, and NF- B in the liver ($P<0.05$). Supplementation with 2.5% flaxseed oil significantly decreased the mRNA relative expression levels of NOD1 and NOD2 ($P<0.05$) and showed a tendency to reduce RIPK2 mRNA expression ($P<0.10$) compared with the LPS group. The 5.0% flaxseed oil group exhibited significantly lower NOD2 mRNA relative expression than the LPS group ($P<0.05$).

The classical method for inducing inflammation involves intraperitoneal or intravenous injection of LPS [11], which has been widely applied in studies of liver injury in piglets. Previous research has confirmed that LPS challenge causes histological damage and functional impairment in the liver [6-8]. Kupffer cells in the liver secrete a series of inflammatory mediators (such as TNF-α) upon LPS stimulation, which play important roles in liver injury [1]. Few studies have investigated the regulatory effects of flaxseed oil on liver damage in pigs. Previous reports indicate that flaxseed oil is added at 5%-10% in rodent liver injury studies [3-4] and at 2% in porcine intestinal injury studies [12]. Considering practical swine production (where oil supplementation typically does not exceed 5%), this study selected dietary flaxseed oil levels of 2.5% and 5.0% to investigate whether flaxseed oil could alleviate LPS-induced hepatic inflammation in piglets.

TNF-α and COX2 are typical inflammatory mediators that are normally not expressed or are expressed at low levels [2,6-7]. Research has shown that the expression of these mediators increases dramatically under inflammatory conditions, serving as markers of the inflammatory response, and LPS challenge significantly upregulates their expression [2,6-7]. HSPs are generally considered intracellular molecules with cytoprotective functions [13], and high intracellular levels of HSP70 can reduce inflammation and promote liver regeneration [14]. Studies have demonstrated that intracellular HSP70 can directly interact with NF- B, thereby preventing its activation [15]. The current results showed that LPS challenge significantly increased the mRNA relative expres-

sion levels of TNF-α, COX2, and HSP70 in piglet liver, possibly because LPS enhanced TNF-α expression, which through negative feedback pathways subsequently induced the expression of anti-inflammatory mediators such as HSP70 [16]. Previous studies have confirmed that LPS challenge significantly increases both mRNA and protein expression of HSP70, COX2, and TNF-α in the liver [2,6-7,16]. LPS challenge in pigs at different growth stages consistently induces HSP70 overexpression [17], which can be alleviated by PUFA supplementation. Flaxseed oil has been shown to significantly reduce HSP70 mRNA expression in canine leukocytes [18], and Narayanan et al. [19] found that DHA significantly decreased HSP70 protein expression in prostate cancer cells. Chen et al. [8] reported that fish oil rich in EPA and DHA alleviated the LPS-induced increase in hepatic COX2 and TNF-α mRNA and protein expression. Similarly, fish oil reduced mRNA and protein expression of HSP70, COX2, and TNF-α in a rat alcoholic fatty liver model [20]. Chen et al. [2] demonstrated that DHA alleviated liver injury by inhibiting inflammatory mediator expression. Han et al. [4] found that flaxseed oil significantly reduced the increased hepatic TNF-α mRNA expression induced by high-fat diets in rats with fatty liver. Jangale et al. [3] also observed that dietary supplementation with flaxseed oil or fish oil significantly reduced the increased hepatic NF- B, TNF-α, and IL-6 mRNA expression in diabetic rats. The present study demonstrated that 2.5% flaxseed oil significantly decreased COX2 and TNF-α mRNA expression, while 5.0% flaxseed oil significantly reduced TNF-α mRNA expression. Therefore, flaxseed oil may protect the liver by reducing the production of hepatic inflammatory mediators.

To explore the mechanisms by which flaxseed oil modulates inflammation, this study examined the mRNA expression of key genes in the TLR4 and NOD signaling pathways. TLR4 is an important member of the Toll-like receptor family that primarily recognizes LPS [21]. Upon LPS challenge, LPS binds to TLR4 and transmits signals intracellularly. Within the cell, TLR4 interacts with the carboxyl terminus of MyD88 through its TIR (Toll/IL-1) domain, activating MyD88. Activated MyD88 sequentially stimulates downstream IRAK1 and TRAF6, ultimately activating NF- B. The activated NF- B translocates into the nucleus and induces the expression of inflammatory mediators [21]. NOD1 and NOD2 are the most representative members of the NOD family, primarily recognizing peptidoglycan (PGN). Both NOD1 and NOD2 can bind to the common downstream molecule RIPK2 and further activate NF- B to induce the transcription of inflammatory mediators [5,22-23]. When animals experience stress or infection, excessive production of inflammatory mediators causes liver injury. Therefore, we hypothesized that flaxseed oil might exert hepatoprotective effects by regulating TLR4 and NOD signaling pathways to modulate inflammatory mediator production.

This study found that LPS challenge significantly increased the mRNA expression of key genes in both TLR4 (TLR4, MyD88, IRAK1, NF- B) and NOD (NOD1, NOD2, RIPK2) signaling pathways. Supplementation with 2.5% flaxseed oil significantly reduced NOD1 and NOD2 mRNA expression

and showed a tendency to decrease RIPK2 mRNA expression, while 5.0% flaxseed oil significantly lowered NOD2 mRNA expression. Currently, no studies have reported the effects of flaxseed oil on the expression of key genes in hepatic TLR4 and NOD signaling pathways. Previous research has shown that fish oil (rich in EPA and DHA) can reduce the LPS-induced increase in hepatic NOD1, NOD2, and RIPK2 mRNA expression [8]. Additionally, fish oil significantly attenuated the upregulation of NOD signaling pathway genes in the intestine, muscle, and hypothalamic-pituitary-adrenal axis following LPS challenge [24-26]. The present results suggest that LPS challenge induces the expression of key genes in hepatic TLR4 and NOD signaling pathways, leading to the release of inflammatory mediators and subsequent liver injury, while flaxseed oil may exert hepatoprotective effects by modulating the NOD signaling pathway to reduce the LPS-induced upregulation of inflammatory mediators in piglet liver.

In conclusion, LPS challenge induces the expression of key genes in hepatic TLR4 and NOD signaling pathways, resulting in excessive release of inflammatory mediators (COX2 and TNF-α). Dietary supplementation with 2.5% and 5.0% flaxseed oil can alleviate the excessive expression of inflammatory mediators in LPS-challenged piglet liver by inhibiting the NOD signaling pathway.

References

[1] SCHMÖCKER C, WEYLANDT K H, KAHLKE L, et al. Omega-3 fatty acids alleviate chemically induced acute hepatitis suppression cytokines[J]. Hepatology, 2007, 45(4): 864–869.

[2] CHEN W Y, LIN S Y, PAN H C, et al. Beneficial effect of docosahexaenoic acid on cholestatic liver injury in rats[J]. The Journal of Nutritional Biochemistry, 2012, 23(3): 252–264.

[3] JANGALE N M, DEVARSHI P P, DUBAL A A, et al. Dietary flaxseed oil and fish oil modulates expression of antioxidant and inflammatory genes with alleviation of protein glycation status and inflammation in liver of streptozotocin-nicotinamide induced diabetic rats[J]. Food Chemistry, 2013, 141(1): 187–195.

[4] HAN H, MA H F, RONG S, et al. Flaxseed oil containing flaxseed oil ester of plant sterol attenuates high-fat diet-induced hepatic steatosis in apolipoprotein-E knockout mice[J]. Journal of Functional Foods, 2015, 13: 169–182.

[5] FUKATA M, VAMADEVAN A S, ABREU M T. Toll-like receptors (TLRs) and Nod-like receptors (NLRs) inflammatory disorders[J]. Seminars Immunology, 2009, 21(4): 242–253.

[6] Pi Ding' an. Regulation of asparagine on lipopolysaccharide-induced liver injury and muscle protein degradation in piglets[D]. Master' s Thesis. Wuhan: Wuhan Polytechnic University, 2014.

[7] Leng Weibo. Regulation of aspartic acid on lipopolysaccharide-induced liver

injury and muscle protein degradation in piglets[D]. Master' s Thesis. Wuhan: Wuhan Polytechnic University, 2014.

[8] CHEN F, LIU Y L, ZHU H L, et al. Fish oil attenuates liver injury caused by LPS in weaned pigs associated with inhibition of TLR4 and nucleotide-binding oligomerization domain protein signaling pathways[J]. Innate Immunity, 2013, 19(5): 504-515.

[9] Chen Shaokui, Liu Yulan, Li Quan, et al. Effects of lipopolysaccharide challenge on the expression of key genes in the Toll-like receptor 4 signaling pathway in the hypothalamic-pituitary-adrenal axis of piglets[J]. Chinese Journal of Animal Nutrition, 2014, 26(11): 3356-3361.

[10] LIVAK K J, SCHMITTGEN T D. Analysis of relative gene expression data using real-time quantitative PCR and 2-ΔΔCt method[J]. Methods, 2001, 25(4): 402-408.

[11] JOHNSON R W. Inhibition of growth by pro-inflammatory cytokines: an integrated view[J]. The Journal of Animal Science, 1997, 75(5): 1244-1255.

[12] CHYTILOVÁ M, NEMCOVÁ R, GANCARČÍKOVÁ S, et al. Flax-seed oil and Lactobacillus plantarum supplementation modulates TLR and NF- B gene expression in enterotoxigenic Escherichia challenged gnotobiotic pigs[J]. Acta Veterinaria Hungarica, 2014, 62(4): 463-472.

[13] SHI Q, DONG Z, WEI H. The involvement of heat shock proteins in murine liver regeneration[J]. Cellular & Molecular Immunology, 2007, 4(1): 53-57.

[14] OKA Y, AKAGI Y, KINUGASA T, et al. Heat-shock pre-treatment reduces liver injury and liver recovery after partial hepatectomy mice[J]. Anticancer Research, 2013, 33(7): 2887-2894.

[15] KUBOKI S, SCHUSTER R, BLANCHARD J, et al. Role of heat shock protein 70 in hepatic ischemia-reperfusion injury in mice[J]. American Journal of Physiology Gastrointestinal and Liver Physiology, 2007, 292(4): G1141-G1149.

[16] WU H T, LIU Y L, PI D A, et al. Asparagine attenuates hepatic injury caused by lipopolysaccharide in weaned piglets associated with modulation of Toll-like receptor 4 and nucleotide-binding oligomerisation domain protein signalling and their negative regulators[J]. British Journal of Nutrition, 2015, 114(2): 189-201.

[17] MCCOMB M A, SPURLOCK M E. Expression of stress proteins in porcine tissues: developmental changes and effect of immunological challenge[J]. The Journal of Animal Science, 1997, 75(1): 195-201.

[18] PURUSHOTHAMAN D, BROWN W Y, VANSELOW B A, et al. Flaxseed oil supplementation alters the expression of inflammatory-related genes in dogs[J]. Genetics And Molecular Research, 2014, 13(3): 5322-5332.

[19] NARAYANAN N K, NARAYANAN B A, BOSLAND M, et al. Docosahexaenoic acid in combination with celecoxib modulates HSP70 and p53 proteins

in prostate cancer cells[J]. International Journal of Cancer, 2006, 119(7): 1586-1598.

[20] NANJI A A, GRINIUVIENE B, YACOUB L K. Heat-shock gene expression in alcoholic liver disease related severity of liver injury and lipid peroxidation[J]. Experimental Biology and Medicine, 1995, 210(1): 12-19.

[21] AKIRA S, UEMATSU S, TAKEUCHI O. Pathogen recognition innate immunity[J]. Cell, 2006, 124(4): 783-801.

[22] TAKEUCHI O, AKIRA S. Pattern recognition receptors inflammation[J]. Cell, 2010, 140(6): 805-820.

[23] ECKMANN L. Sensor molecules intestinal innate immunity against bacterial infections[J]. Current Opinion in Gastroenterology, 2006, 22(2): 95-101.

[24] LIU Y L, CHEN F, ODLE J, et al. Fish oil increases muscle protein mass and modulates Akt/FOXO, TLR4 and NOD signaling in weaning piglets after LPS challenge[J]. The Journal of Nutrition, 2013, 143(8): 1331-1339.

[25] LIU Y L, CHEN F, ODLE J, et al. Fish Oil enhances intestinal integrity and inhibits TLR4 and NOD2 signaling pathways in weaned pigs after LPS challenge[J]. The Journal of Nutrition, 2012, 142(11): 2017-2024.

[26] LIU Y L, CHEN F, LI Q, et al. Fish alleviates activation hypothalamic-pituitary-adrenal axis associated with inhibition of TLR4 and NOD signaling pathways in weaned piglets after a lipopolysaccharide challenge[J]. The Journal of Nutrition, 2013, 143(11): 1799-1807.

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