Journal of Functional Foods 46 (2018) 482–489

Contents lists available at ScienceDirect

Journal of Functional Foods

journal homepage: www.elsevier.com/locate/jff

Flaxseed oil improves liver injury and inhibits necroptotic and inflammatory signaling pathways following lipopolysaccharide challenge in a piglet model

Longmei Wang[a], Zhixiao Tu[a], Haibo Wang[a], Shuhui Wang[a], Xiuying Wang[a], Huiling Zhu[a], Chien-An Andy Hu[a,b], Yulan Liu[a,*]

[a] 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
[b] Department of Biochemistry and Molecular Biology, University of New Mexico School of Medicine, Albuquerque, NM 87131, USA

ARTICLE INFO

Keywords:
Flaxseed oil
Liver
Lipopolysaccharide
Necroptosis
Inflammation
Pigs

ABSTRACT

The hepatoprotective effects of flaxseed oil (FO) were tested in a pig model of lipopolysaccharide (LPS)-induced liver injury. Twenty-four piglets were used in a 2 × 2 factorial design including diet and an LPS challenge. After 3 weeks of feeding with 5% FO or 5% corn oil, pigs were challenged with LPS or saline. FO alleviated LPS-induced morphological liver damage, reduced serum alanine aminotransferase, aspartate aminotransferase and alkaline phosphatase activities, and increased claudin-1 protein expression. FO decreased tumor necrosis factor-α, interleukin-6 and cyclooxygenase 2 mRNA expression, and heat shock protein 70 mRNA and protein expression. FO downregulated receptor interacting protein kinase (RIP)1, RIP3, and mixed-lineage kinase domain-like protein (MLKL) mRNA expression, RIP1 and RIP3 protein expression, and MLKL phosphorylation. FO downregulated mRNA expression of toll-like receptor 4, nucleotide-binding oligomerization domain protein 1 and multiple downstream signaling molecules, and decreased p38 and extracellular signal-regulated kinase 1/2 phosphorylation. These results indicate that FO inhibits necroptotic and inflammatory signaling pathways to protect the liver from inflammation-related injury.

1. Introduction

The liver is the largest internal organ, and has essential functions including nutrient and drug metabolism, detoxification, and generation of immune response (Olthof et al., 2017). A wide range of factors such as bacterial, viral and parasitic infections, and toxins, can lead to liver inflammation, parenchymal liver damage, necrosis and liver dysfunction such as acute hepatitis (Chen et al., 2013). Nutritional interventions may exert beneficial effects in attenuating liver damage.

Fish oil, which is rich in long chain n − 3 polyunsaturated fatty acids (PUFAs) such as eicosapentaenoic acid (EPA; 20:5n − 3) and docosahexaenoic acid (DHA; 22:6n − 3), can decrease inflammatory responses and have shown hepatoprotective effects in animal models of liver injury and in human clinical trials (Sorrell et al., 2017; Shang et al., 2017). Nutritional guidelines recommend consumption of fish oil (Krauss et al., 2000), but fish oil can be costly, have an unpleasant fishy taste, and may produce halitosis and flatulence (Parpinello, Meluzzi, Sirri, Tallarico, & Versari, 2006). Therefore, plant sources of n − 3 PUFAs are an attractive alternative. Flaxseed oil (FO) is the richest known plant source of n − 3 PUFAs, α-linolenic acid (ALA; 18:3n − 3) (Xu et al., 2013). ALA is the precursor of longer n − 3 PUFAs, and can be converted to EPA and possibly DHA (Connor, 1999; Stark, Reifen, & Crawford, 2016; Taylor, Noto, Stringer, Froese, & Malcolmson, 2010; Harper, Edwards, DeFilippis, & Jacobson, 2006). Recent evidence from experimental models of alcoholic or nonalcoholic liver disease indicates that FO has hepatoprotective effects (Zhang et al., 2017; Yang, Tseng, Chang, & Chen, 2009). However, unlike fish oil, few studies have evaluated the hepatoprotective effect and mechanism(s) of FO.

Cell death from necrosis, apoptosis, and autophagy is a central event in liver injury (Luedde, Kaplowitz, & Schwabe, 2014). Necroptosis, a

Abbreviations: AKP, alkaline phosphatase; ALA, α-linolenic acid; ALT, alanine aminotransferase; AST, aspartate aminotransferase; BW, body weight; COX2, cyclooxygenase 2; DAMP, damage-associated molecular pattern; ERK1/2, extracellular signal-regulated kinase 1/2; GGT, glutamyl transpeptidase; HSP70, heat shock protein 70; IL-6, interleukin-6; IRAK1, IL-1 receptor-associated kinase 1; LPS, lipopolysaccharide; MLKL, mixed-lineage kinase domain-like protein; MyD88, myeloid differentiation factor 88; NOD, nucleotide-binding oligomerization domain protein; PAMP, pathogen associated molecular pattern; RIP, receptor interacting protein kinase; TLR4, toll-like receptor 4; TNF-α, tumor necrosis factor-α; TRAF6, TNF-α receptor-associated factor 6

* Corresponding author at: 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.
E-mail address: yulanflower@126.com (Y. Liu).

https://doi.org/10.1016/j.jff.2018.05.015
Received 12 March 2018; Received in revised form 9 May 2018; Accepted 9 May 2018
Available online 26 May 2018
1756-4646/

newly discovered form of programmed cell death that combines the features of both apoptosis and necrosis, has been implicated in the development of many liver diseases (Afonso et al., 2016). Necroptosis is driven by receptor interacting protein kinase (RIP)1, RIP3 and mixed-lineage kinase domain-like protein (MLKL), and the cell rupture and necrosis result in the release of cell damage-associated molecular patterns (DAMPs) (Afonso et al., 2016). The DAMPs can initiate and sustain the inflammatory response through activation of inflammatory signaling pathways such as toll-like receptors (TLRs) and nucleotide-binding and oligomerization domain proteins (NODs), which further results in secondary tissue injury (Rani, Nicholson, Zhang, & Schwacha, 2017). Necroptosis and inflammation interact under pathologic conditions (Newton & Manning, 2016). Intracellular contents released from necrotic cells trigger inflammation, and inflammation-related factors such as TLR3 and 4 agonists and tumor necrosis factor-α (TNF-α), can trigger necroptosis (Newton & Manning, 2016). Both necroptosis and inflammation result in tissue injury.

Accordingly, we hypothesized that dietary supplementation of FO would attenuate liver injury by regulating necroptosis and inflammation by influencing RIP1/PIP3/MLKL, and TLRs and NODs signaling pathways. In the current experiment, we administrated *Escherichia coli* lipopolysaccharide (LPS), a potent endotoxin, to establish the model of liver damage (Chen et al., 2013). LPS has been demonstrated to be a key factor that contributes to the pathogenesis of manytypes of liver diseases, such as ischemic liver injury (Colletti et al., 1990), liver cirrhosis (Nielsen, Grøfte, Tygstrup, & Vilstrup, 2006; Tarao et al., 1979), alcoholic or non-alcoholic fatty liver disease (Thurman, 1998; Diehl, 2002), alcoholic or nonalcoholic steatohepatitis (Thurman, 1998; Diehl, 2002). In addition, we used a piglet model, a good animal model for human nutrition research (Puiman & Stoll, 2008; Merrifield et al., 2011). Our aim was to investigate whether FO could mitigate liver damage induced by LPS challenge, and to explore its molecular mechanism(s).

2. Experimental methods

2.1. Animals and diets

The experiment and animal handling procedures complied with Animal Care and Use Committee of Wuhan Polytechnic University. A total of 24 healthy weaned crossbred male piglets (Duroc × Large White × Landrace) with similar body weight (BW) [8.9 ± 0.2 kg initial BW] and age (35 ± 1 d) were used. The piglets were provided by Aodeng Agriculture and Animal Husbandry Technology Co., Ltd (Hubei, China). It was confirmed that the piglets had no enteric diseases and had not been inoculated with any *Escherichia coli* vaccines. The piglets were housed individually in stainless steel metabolic cages (1.80 × 1.10 m^2) and maintained at a controlled ambient temperature (22–25 °C) with free access to feed and water. After 1 week of acclimatization, piglets were randomly divided into two dietary treatments [5% FO diet (n = 12) or 5% corn oil diet (control) (n = 12)]. FO and corn oil were provided by Yulongxiang Grain and Oil Company (Gansu Province, China) and Xiwang Food Company (Shandong Province, China), respectively. The experimental diets (Table S1) were formulated to meet the nutrient requirements of piglets, which were based on recommendations of NRC (1998). The dietary fatty acid composition (Table S2) was measured by gas chromatography according to Nieto, Torres, Ríos, and Gil (2002).

2.2. Experimental design

Piglets were fed 5% FO or 5% corn oil diets for 21 d before LPS treatment. Before piglets were treated by LPS, the experimental design was a randomized complete block. After piglets were treated by LPS, the experiment became a 2 × 2 factorial design. The main factors included diet (5% corn oil or 5% FO diets) and immunological challenge (LPS or saline). On d 21, half of the piglets (n = 6) in each dietary treatment received an intraperitoneal injection with either LPS (*Escherichia coli* serotype 055: B5; purity > 99%; Sigma Chemical Inc., St. Louis, MO, USA) at 100 mg/kg BW or the equal amount of 0.9% (wt: v) NaCl solution. The dose of LPS and the time of injection were chosen according to our previous study (Chen et al., 2013).

2.3. Blood and liver sample collections

The procedures of blood and liver sample collections were the same as previously described (Chen et al., 2013). Briefly, at 2 or 4 h following LPS or saline injection, blood samples were collected and centrifuged to harvest serum. Serum was stored at −80 °C for analysis of serum biochemical parameters. Following blood collection at 4 h, all pigs were humanely killed, and the liver samples were collected. One fragment of liver samples was stored in fresh 4% paraformaldehyde/phosphate-buffered saline at least for 24 h for histological analysis. The remaining portions were immediately frozen in liquid nitrogen and then stored at −80 °C for further analysis of mRNA and protein expressions.

2.4. Serum biochemical parameters

The activities of serum aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (AKP) and glutamyl transpeptidase (GGT) were measured according to the method of Chen et al. (2013).

2.5. Liver histology

Tissue slides (5 mm) for liver histology were dehydrated with graded ethanol solutions, cleared with xylene and embedded in paraffin. Cross-section (4-μm thick) of each sample was stained with haematoxylin and eosin. Histological analysis was carried out in accordance with the method described previously (Chen et al., 2013).

2.6. Hepatic fatty acid composition

Hepatic fatty acid composition was measured by gas chromatography in accordance with Nieto et al. (2002).

2.7. mRNA abundance analysis by real-time PCR

The methods for total RNA isolation, quantification, reverse transcription, and real-time PCR were the same as Chen et al. (2013). The primer pairs used are presented in Table S3 to amplify the target genes. The $2^{-\triangle\triangle CT}$ method was used to analyze the mRNA abundance of the target genes relative to housekeeping gene (GAPDH) (Livak & Schmittgen, 2001). In our study, GAPDH displayed no variation among four treatments. Relative mRNA abundance of each target gene was normalized to the pigs receiving 5% corn oil diet and treated with 0.9% NaCl solution.

2.8. Protein abundance analysis by Western blot

Protein abundance analysis in liver was conducted in accordance with the method of Chen et al. (2013). Specific primary antibodies included rabbit anti-claudin-1 (1:1000) (Invitrogen Technology Inc., Danvers, MA, USA), mouse anti-heat shock protein 70 (HSP70) (1:1000) (Enzo Life Sciences Inc., Farmingdale, NY, USA), rabbit anti-total p38 (t-p38) (1:1000) (Cell Signaling Technology Inc., Danvers, MA, USA), rabbit anti-phosphorylated p38 (p-p38) (1:1000) (Cell Signaling), rabbit anti-total extracellular signal-regulated kinase 1/2 (t-ERK1/2) (1:1000) (Cell Signaling), rabbit anti-phosphorylated ERK1/2 (p-ERK1/2) (1:1000) (Cell Signaling), rabbit anti-RIP1 (1:1000) (Life-Span BioSciences, Inc., Seattle, USA), rabbit anti-RIP3 (1:1000) (Santa Cruz Biotechnology, Inc., CA, USA), rabbit anti-total MLKL (t-MLKL) (1:1000) (Cell Signaling), rabbit anti-phosphorylated MLKL (p-MLKL)