Lipoxin A4 exerts protective effects against experimental acute liver failure by inhibiting the NF-κB pathway

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Abstract. Although rare, acute liver failure (ALF) is associated with high levels of mortality, warranting the development of novel therapies. Nuclear factor-κB (NF-κB), tumor necrosis factor-α (TNF-α), and interleukin-6 (IL-6) play roles in ALF. Lipoxin A4 (LXA4) has been shown to alleviate inflammation in non-hepatic tissues. In the present study, we explored whether LXA4 exerted hepatoprotective effects in a rat model of ALF. A rat model of ALF was generated by intraperitoneal injections of D-galactosamine (300 mg/kg) and lipopolysaccharide (50 µg/kg). Animals were randomly assigned to: control group (no ALF); model group (ALF); and the groups treated with a low dose (0.5 µg/kg), medium dose (1 µg/kg), and high dose (2 µg/kg) of LXA4 (all with ALF); and pyrroldidine dithiocarbamate (PDTC)-treated group (ALF and 100 mg/kg PDTC, an inhibitor of NF-κB). Liver histology was measured using H&E staining, serum levels by ELISA, and liver mRNA expression was measured by RT-PCR for the detection of the pro-inflammatory cytokines TNF-α and IL-6. Liver cell apoptosis (as measured using the TUNEL method and examining caspase-3 activity), and Kupffer cell NF-κB activity [using an electrophoretic mobility shift assay (EMSA)] were examined. Serum levels of transaminases, TNF-α and interleukin-6 (IL-6) were substantially higher in the model group compared to controls. In the model group, significant increases in TNF-α and IL-6 mRNA expression, TUNEL-positive cells, and caspase-3 activity in the liver tissue were noted. LXA4 improved liver pathology and significantly decreased the indicators of inflammatory response and apoptosis in a dose-dependent manner. High-dose LXA4 provided better protection than PDTC. LXA4 administration significantly decreased NF-κB expression in hepatocytes and Kupffer cells. These results indicated that LXA4 inhibited NF-κB activation, reduced the secretion of pro-inflammatory cytokines, and inhibited apoptosis of liver cells, thereby exerting protective effects against ALF.

Introduction

Acute liver failure (ALF) is a serious condition resulting from the development of hepatocellular dysfunction over a period of several days or a few weeks (1). ALF is very harmful to patient health, and has a mortality rate of 80% (2). Thus, ALF is associated with a high rate of mortality, and many patients require liver transplantation. Therefore, there is a need to improve our understanding of the underlying mechanisms in order that novel therapies be developed.

The lipoxins comprise a group of arachidonic acid metabolites first discovered by Serhan in 1984 (3). Unlike other arachidonic acid metabolites that are pro-inflammatory, such as prostaglandins and leukotrienes, lipoxins play a role in the resolution of inflammation. During the resolution phase of acute inflammation, the production of prostaglandins and leukotrienes ceases and the production of lipoxins begins; this is known as the ‘lipid mediator conversion’ (4,5). Lipoxins then promote macrophage clearance of the apoptotic polymorphonuclear leukocytes. The lipoxins include lipoxin A4 (LXA4) and lipoxin B4, both of which play an anti-inflammatory role in a number of pathological processes (3).

LXA4 is an important endogenous mediator that promotes the resolution of inflammation, serving as a ‘braking signal’ (3). Previous studies have shown that LXA4 can attenuate airway inflammation following lipopolysaccharide (LPS)-induced lung injury in mice (6), reduce systemic inflammation and improve...
survival rates in a rat model of sepsis (7), suppress inflammation-induced mechanical hypersensitivity in rats (8), and inhibit pulmonary and renal fibrosis in animal models (9,10). In addition, the levels of LXA4 are decreased in patients with asthma (11). The activation of nuclear factor-κB (NF-κB) is necessary for many inflammatory reactions, and LXA4 has been proven to reduce the expression of tumor necrosis factor-α (TNF-α) and the activation of NF-κB in a rabbit model of paracetamol-induced acute hepatic injury (12). LXA4 has also been demonstrated to inhibit NF-κB activation in pulmonary capillary epithelial cells (13), peritoneal macrophages (7), and dorsal root ganglia in the spinal cord (14).

To the best of our knowledge, it has not yet been reported whether or not LXA4 plays a role in ALF. However, LXA4 is known to play protective, anti-inflammatory roles in cases of liver injury induced by a high-fat diet/endotoxin (15), paracetamol (12), or acetaminophen (16), and also in cases of liver fibrosis induced by carbon tetrachloride (17). LXA4 also attenuates the acute rejection of transplanted livers (18), and suppresses hepatocellular carcinoma (19). Given the special role which LXA4 plays in inflammatory processes, in the present study we decided to explore the effects of LXA4 in a rat model of ALF, which was induced by intraperitoneal injection of D-galactosamine (D-GalN) and LPS.

Materials and methods

**Materials.** LXA4 was purchased from the Cayman Chemical Co. (Ann Arbor, MI, USA) as well as D-GalN. Type IV collagenase was from Biosharp (Hefei, China). The Percoll separation solution was from Wuhan Boster Biological Engineering (Wuhan, China). Unless otherwise specified, all other reagents were purchased from Sigma-Aldrich (St. Louis, MO, USA).

**Animal models.** Eight-week old male Wister rats, weighing 180-220 g, were provided by the Experimental Animal Research Center of Hubei, China. The rats were randomly divided into six groups: control group (n=3), model group (n=6), three LXA4-treated groups (low/medium/high doses; n=6 each) and the pyrrolidine dithiocarbamate (PDTC)-treated group (n=6). The animals in the control group received an intraperitoneal injection of phosphate-buffered saline (PBS). Rats in the other experimental groups received an intraperitoneal injection of D-GalN (300 mg/kg) to the lower left abdomen, which was followed 30 min later by an intraperitoneal injection of LPS (50 µg/kg) to the lower right abdomen. The low-, medium- and high-dose LXA4 treatment groups also received intraperitoneal injections of 0.5, 1 and 2 µg/kg LXA4, respectively, 30 min before D-GalN injection. For the PDTC group, PDTC (100 mg/kg; an inhibitor of NF-κB) was administered with D-GalN. The rats were sacrificed 24 h after LPS injection. All procedures and animal experiments were approved by the Animal Care and Use Committee of the Animal Experimental Center, Hubei University of Medicine.

**Liver histology.** The livers were removed from certain rats, fixed with 4% paraformaldehyde, embedded in paraffin, and cut into 8-µm sections. The sections were stained with hematoxylin and eosin (H&E) and examined under an optical microscope (CKX41; Olympus, Tokyo, Japan). Liver injury was assessed by the scoring of five randomly selected fields viewed at x400 magnification: 0, no or minimal damage; 1, mild damage, cell swelling, a limited number of cells showing pyknosis; 2, moderate damage, extensive nuclear pyknosis, enhanced eosin staining in the cytoplasm, the appearance of bridging necrosis; 3, severe necrosis, disappearance of hepatic cords, bleeding, massive inflammatory cell infiltration. Liver tissues and blood samples were taken from certain rats, while others were used for the extraction of liver cells.

**Measuring alanine transaminase (ALT) and aspartate transaminase (AST) levels.** Whole blood samples (totaling 33) were taken 24 h after rats were injected with LPS. These were kept at room temperature for 2 h, and then centrifuged at 3,000 x g for 5 min. The supernatant was collected, and ALT and AST levels were determined without delay using an automatic biochemical analyzer (Hitachi 7020; Hitachi, Tokyo, Japan).

**Detection of TNF-α and interleukin-6 (IL-6).** In the present study, serum TNF-α and IL-6 were both detected using an enzyme-linked immunosorbent assay (ELISA) kit (purchased from R&D Systems, Minneapolis, MN, USA) according to the manufacturer’s instructions. Briefly, 50 µl assay diluent was added to each well. After adding 50 µl standard, control and samples, these were mixed and incubated for 2 h at room temperature. After aspiration and washing, 100 µl rat TNF-α conjugate was added to each well and incubated. Aspiration and washing was repeated, 100 µl Substrate Solution was added, and this was followed by incubation for 30 min; 100 µl Stop Solution was then added to each well. A microplate reader (450 nm) was used to determine the OD. The OD values were then used to determine the concentration of each sample.

**Detection of TNF-α mRNA and IL-6 mRNA in liver tissues.** Hepatic tissues were placed in liquid nitrogen and ground to a powder. Total RNA was extracted using TRIzol (Invitrogen, Carlsbad, CA, USA) and cDNA was produced by reverse transcription and amplification. The primers used were as follows: IL-6, 5'-TTG CCT TCT TGG GAC TGA TGT-3' (sense) and 5'-TAC TGG TCT GTT GTG GGT GGT-3' (antisense); TNF-α, 5'-GCC ACC ACG CTC TTC TGT C-3' (sense) and 5'-TAG B activation in pulmonary capillary epithelial cells (13), peritoneal macrophages (7), and dorsal root ganglia in the spinal cord (14).
mixture or Label Solution (for the control) were added. DNase I was added for the positive control. These were incubated for 60 min in a humidified atmosphere in the dark and then rinsed with PBS. After adding 50 µl Converter-POD, slides were again incubated and washed, and this was followed by the addition of 100 µl DAB substrate, incubation and rinsing with PBS. Samples were counterstained with hematoxylin and analyzed using a light microscope.

**Measurement of caspase-3 activity in liver tissues.** The activity of caspase-3 in the liver tissues was determined using a commercial kit (Beyotime Institute of Biotechnology, Shanghai, China) in strict accordance with the manufacturer’s instructions. Briefly, PNA was added (concentrations 0, 10, 20, 50, 100 and 200 µM) and a microplate reader was used to read standard absorbance (405 nm; A405). PNA absorbance and the standard curve were calculated, 100 µl lysis buffer/10 mg liver tissue was added. After homogenization on ice, centrifugation was undertaken (15 min at 4°C; 16,000 rpm). The supernatant was transferred and put on ice. The reaction system was prepared, and Ac-DEVD-PNA (2 mM) was added. After incubation for 90 min at 37°C, absorbance was measured using a microplate reader (A405). PNA absorbances of samples catalyzed by caspase-3 were subtracted (A405 of blank, free PNA, from A450 of standards). The amount of PNA catalyzed by samples was then calculated according to the standard curve.

**Isolation of hepatocytes and Kupffer cells.** Cell isolation was carried out using a modified version of the method described by Knook (20-22). Briefly, rats were anesthetized with an intraperitoneal injection of sodium pentobarbital. Pre-warmed (37°C) calcium- and magnesium-free Hank’s perfusion solution was infused through a catheter inserted into the portal vein. After 15 min, the solution was changed to one containing 0.05% collagenase IV (BioSharp) and the infusion was continued for a further 20 min. Rats were sacrificed by cervical dislocation. These livers were then removed, placed in 0.05% collagenase IV (Biosharp) and the infusion was undertaken (15 min at 4°C; 16,000 rpm). The supernatant was considered to indicate a statistically significant difference.

**Electrophoretic mobility shift assay (EMSA) for detection of NF-κB.** An EMSA (Pierce Chemical Co., Rockford, IL, USA) for the detection of NF-κB in nucleoprotein from liver tissue and Kupffer cells was carried out using a nucleoprotein extraction kit (Biovision, Milpitas, CA, USA) to extract nucleoprotein from liver tissues and Kupffer cells, together with a commercial NF-κB probe (Beyotime Institute of Biotechnology) according to the instructions provided.

**Statistical analysis.** Data are expressed as the means ± standard deviation (SD). Comparisons between groups were studied using analysis of variance (ANOVA) followed by the SNK (Student-Newman-Keuls) post hoc test. A P-value <0.05 was considered to indicate a statistically significant difference.

**Results**

**LXA4 improves liver function.** In the control group, the enzymatic activity of ALT and AST was below the level of detection. The enzymatic activity of ALT and AST in the model group was substantially elevated, indicating liver damage; this suggests that the model was valid. Administration of LXA4 significantly lowered the enzymatic activity of ALT and AST in a dose-dependent manner. In the PDTC group, the enzymatic activity of ALT and AST was lower than in the model group, but higher than in the medium- and high-dose LXA4 groups (Fig. 1).

![Figure 1. Lipoxin A4 (LXA4) improves liver function in our model of acute liver failure (ALF). Intraperitoneal injections of D-galactosamine (D-GalN, 300 mg/kg) and lipopolysaccharide (LPS, 50 µg/kg) were used to generate the rat model of ALF. Animals were intraperitoneally injected with LXA4 (low-dose, 0.5 µg/kg; medium-dose, 1 µg/kg; or high-dose, 2 µg/kg) or pyrrolidine dithiocarbamate (PDTC, 100 mg/kg). Serum aspartate transaminase (AST) and alanine transaminase (ALT) were measured (U/L). *P<0.05 compared to the model group.](image-url)
LXA4 improves the histological appearance of the liver. In the H&E-stained liver tissues from the model group, we observed liver cell degeneration, severe necrosis, the disappearance of hepatic cords, bleeding and also infiltration of numerous inflammatory cells in the portal area (Fig. 2). Administration of LXA4 was associated with dose-dependent improvements in tissue appearance and a decrease in liver injury scores, as compared to the model group (Fig. 2). The liver injury score in the PDTC group was significantly lower than that of the model group, but higher than that of the high-dose LXA4 group (Fig. 2).

LXA4 attenuates the elevation of serum TNF-α and IL-6 levels. Compared to the control group, the serum levels of IL-6 and TNF-α were significantly higher in the model group (Fig. 3). LXA4 caused dose-dependent reductions in IL-6 and TNF-α levels compared to the model group (Fig. 4). In the PDTC-treated group, IL-6 and TNF-α mRNA levels were significantly decreased compared to the model group, and were comparable to those in the high-dose LXA4 group (Fig. 4).

LXA4 attenuates the increased hepatic expression of TNF-α and IL-6 mRNA. Compared to the control group, the mRNA levels of IL-6 and TNF-α were significantly increased in the model group (Fig. 4). mRNA expression levels of IL-6 and TNF-α were significantly lower in all three LXA4-treated groups than in the model group. In the PDTC-treated group, IL-6 and TNF-α mRNA levels were significantly decreased compared to the model group, and were comparable to those in the high-dose LXA4 group (Fig. 4).

LXA4 decreases the number of TUNEL-positive hepatic cells. The number of TUNEL-positive cells (used as a measure of apoptosis) in the model group was significantly higher than in the control group (Fig. 5). All three LXA4 groups had significantly lower numbers of TUNEL-positive cells than the model group, with a dose-dependent decrease also evident. In the PDTC-treated group, the number of TUNEL-positive cells was significantly decreased compared to the model group, and was comparable to the high-dose LXA4 group (Fig. 5).

LXA4 attenuates the elevation of hepatic caspase-3 activity. Caspase-3 activity in liver tissues was used as an additional measure of apoptosis. Caspase-3 activity was significantly higher in the model group compared to the control group, and was significantly lower in all three LXA4 groups compared to
the model group, and we also observed a dose-dependent reduction (Fig. 6). In the PDTC-treated group, caspase-3 activity was significantly decreased compared to the model group, and was similar to the medium-dose LXA4 group (Fig. 6). These data were in agreement with those which were obtained using the TUNEL method.

**LXA4 attenuates the elevation of hepatic NF-κB activity.** The activity of NF-κB (examined by EMSA) was significantly higher in the model group than in the control group, and significantly lower in the LXA4-treated groups than in the model group (Fig. 7). In the PDTC group, NF-κB activity was significantly decreased compared to the model group, and significantly increased compared to the high-dose LXA4 group (Fig. 7).

**LXA4 attenuates the elevation of NF-κB activity in purified Kupffer cells.** NF-κB activation (assessed using EMSA) in the model group was significantly higher than in the control group (Fig. 8). In the LXA4 groups, NF-κB activity was significantly reduced compared to the model group (Fig. 8). In the PDTC-treated group, NF-κB activity was significantly lower than that in the model group, and was comparable to that in the high-dose LXA4 group (Fig. 8).

**Discussion**

The combined use of D-GalN and LPS has been shown to result in injury to the liver (23), and this combination has...
been used in previous research to generate an animal model of ALF (24). In the present study, the model group exhibited significantly elevated liver transaminase activity (with serum AST >8,000 U/l) as well as disorders of liver structure, including severe inflammatory necrosis and hemorrhage. In addition, the mortality rate in the model group was 50% (data not shown). These results indicate that a model of ALF had been generated successfully.

In this study, LXA4 administration significantly alleviated the signs of ALF, demonstrated by significantly reduced levels of serum ALT and AST, reduced liver tissue inflammation and necrosis, and improved survival (data not shown). These findings suggest that a model of ALF had been generated successfully.

In this study, LXA4 administration significantly alleviated the signs of ALF, demonstrated by significantly reduced levels of serum ALT and AST, reduced liver tissue inflammation and necrosis, and improved survival (data not shown). These findings suggest that LXA4 plays a protective role against ALF, a statement which is supported by a previous study on rabbits (12).

The induction of TNF-α is one of the earliest events that occur during inflammation of the liver. TNF-α initiates a cascade of circulating pro-inflammatory cytokines, leading to the necrosis of liver cells. Depending on the micro-environment, TNF-α is capable of either inducing the proliferation of liver cells or promoting apoptosis and necrosis (25). The anti-apoptotic effects of TNF-α are mediated via activation of NF-κB, whereas the pro-apoptotic actions are mediated via caspases (26). Elevated TNF-α levels have been demonstrated in the serum and liver tissues of patients with acute and chronic hepatitis B and C (27-31). Serum TNF-α levels are also increased significantly in patients with ALF (32). The finding that mice deficient in the TNF-p55 receptor for TNF-α were resistant to fatal liver injury induced by co-administration of D-GlaN/LPS demonstrates the central role of TNF-α in this model (33). A previous study has shown that LXA4 suppressed the effect of LPS on NF-κB (34). In the present study, we noted that LXA4 significantly reduced the serum TNF-α level, and decreased the mRNA expression of TNF-α, the number of TUNEL-positive cells and caspase-3 activity in liver tissue. This strongly suggests that the hepatoprotective effect of LXA4 is effected through inhibition of the actions of TNF-α.

It is known that, in the liver, IL-6 is secreted by Kupffer cells and hepatocytes (35,36). IL-6 induces the production of acute-phase proteins during acute inflammation in the liver, and serum levels of IL-6 correlate positively with the severity of the disease (26). Blockade of the IL-6 signaling pathway significantly increases the sensitivity of mouse hepatocytes to LPS-induced liver failure (26), indicating that IL-6 causes damage to liver cells. The results of the present study showed that LXA4 significantly reduced the serum IL-6 level and IL-6 mRNA expression in liver tissues, implying that the protective effect exerted by LXA4 on the liver is partially related...
to a reduction in IL-6 levels. A previous study has shown that LXA4 protected against obesity-induced systemic diseases, which are mostly inflammatory in nature (37), and this supports the results of the present study.

TNF-α is produced by Kupffer cells in the liver; inhibition of NF-κB activation in Kupffer cells has been shown to block LPS-induced TNF-α generation, thereby inhibiting the death of liver cells sensitized with Propionibacterium acnes (38).

Previous research on rats has demonstrated that the inhibition of NF-κB activation in Kupffer cells reduces the production of pro-inflammatory cytokines, attenuates D-GlaN/LPS-induced liver damage, and improves survival, without affecting anti-apoptotic proteins in liver tissues (39). This raises the possibility that the hepatoprotective effect exerted by LXA4 observed in the present study is due to the inhibition of NF-κB in Kupffer cells. We found that NF-κB activity in Kupffer cells was significantly increased in cases of ALF, and that this was accompanied by elevations in serum TNF-α and IL-6 levels, as well as enhanced mRNA expression of TNF-α and IL-6 in liver tissues. Administration of LXA4 significantly attenuated these increases, suggesting that LXA4 inhibits excessive activation of Kupffer cells in cases of ALF and reduces the generation of pro-inflammatory cytokines.

The aim of the present study was to probe the association between NF-κB activation and Kupffer cells in a rat model of ALF. PDTC acts as a membrane-permeable inhibitor of NF-κB activation in a variety of cells, and it has been reported that PDTC exerts protective effects against LPS-induced acute liver injury (40). Our results revealed that the high dose of LXA4 was superior to PDTC at alleviating ALF, raising the possibility that the protective effects exerted by LXA4 include mechanisms other than NF-κB inhibition. In the present study, LXA4 and PDTC inhibited the expression of mRNA levels of IL-6 and TNF-α in the liver. However, PDTC did not inhibit the expression of TNF-α in the serum as significantly as medium and high doses of LXA4 did. This indicates that LXA4 regulates TNF-α expression through various signaling pathways besides the NF-κB pathway. However, additional studies are necessary to address this issue.

This study has shown that LXA4 reduces serum levels of TNF-α and IL-6 and alleviates liver cell apoptosis and necrosis in a D-GlaN/LPS-induced rat model of ALF. One possible mechanism is that LXA4 inhibits NF-κB activation in Kupffer cells. In addition, we suggest that LXA4 promotes homeostasis of the internal environment of liver cells during acute liver dysfunction, reducing the secretion of inflammatory cytokines, and decreasing the excessive activation of NF-κB in hepatocytes. This prevents large-scale necrosis of liver tissue, and longer term would likely reduce the possibility of genetic mutations and thus malignancy during regeneration of the liver. Our results have also demonstrated that LXA4 exerts a better protective effect than PDTC during ALF, suggesting that LXA4 acts via mechanisms which do not only involve NF-κB inhibition.

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