Original Article

BML-111 ameliorates endotoxin-induced liver injury through increasing prostaglandin J2 production via ERK/COX2 pathway

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Abstract: Endotoxemia-induced fulminant hepatic failure causes an extremely poor prognosis and high mortality due to lack of effective therapy. BML-111, a commercial available lipoxin A4 receptor agonist, has been found to anti-inflammatory and hepatoprotective actions. Here we showed that pretreatment with BML111 protected mice against Lipopolysaccharide (LPS)/D-galactosamine (D-GalN)-induced liver injury, as indicated by the alleviation of mortality and hepatic pathological damage, the reduction of serum aminotransferases activities, and suppression of tumor necrosis factor (TNF)-α production. Notably, BML-111 dose-dependently enhanced prostaglandin (PG) J2 production, cyclooxygenase (COX)-2 expression, and extracellular signal-regulated kinase (ERK) activation in the liver tissues of LPS/D-GalN-primed mice. Further, exogenous PGJ2 treatment attenuated LPS/D-GalN-induced liver injury and inflammatory response, but administration of NS398 (a COX-2 specific inhibitor) or ERK inhibitor PD98059 reversed the protective effect of BML-111 against LPS/D-GalN-induced liver injury as well as PGJ2 production. These results suggest that BML-111 can effectively prevent LPS/D-GalN-induced liver injury by inhibition of TNF-α and the underlying mechanism may be related to enhancement of ERK activation, up-regulation of COX-2 expression and PGJ2 production.

Keywords: BML-111, fulminant hepatic failure, lipopolysaccharide, cyclooxygenase-2, prostaglandin J2, extracellular signal-regulated kinase

Introduction

A strong inflammatory response to virus or bacteria is often involved in the fulminant hepatic failure, which remains an extremely poor prognosis and high mortality due to lack of effective therapies [1-3]. Lipopolysaccharide (LPS)/D-galactosamine (D-GalN)-induced acute liver injury in mice has been used as a typical animal model to mimic the consequences of events in human hepatitis [4, 5]. In this model, LPS directly activates Kupffer cells and macrophages residing in the liver to produce excessive proinflammatory cytokines such as tumor necrosis factor (TNF)-α, which leads to hepatocyte apoptosis and necrosis in D-GalN-sensitized mice [6-9]. The advantage of D-GalN can potentiate the specific hepatotoxic effects of TNF-α and provoke fulminant hepatitis within a few hours [10, 11]. Therefore, inhibition of excessive inflammatory response may become a pharmacological target in the resolution of fulminant hepatic failure.

BML-111, 5(S), 6(R), 7-trihydroxyheptanoic acid methyl ester (Figure 1), is a commercial available lipoxin A4 receptor agonist that has been confirmed equiactive as lipoxin A4 in inhibiting LTB4-induced PMN migration [12]. Previously, we have found that BML-111 could modulate immune response and reduce the severity of collagen-induced arthritis in DBA mice [13], effectively protect from carbon tetrachloride (CCl4)-induced liver injury and the dampened liver injury accompanied by decreased concentration of TNF-α in the serum [14]. Moreover, other studies showed that BML-111 exhibited potent anti-inflammatory activities in various
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models such as sepsis, pancreatitis, and pneumonia [15-17]. In fact, BML-111 has been found to exert protective effect in CCl4 and acetaminophen-induced liver injury [18, 19]. Although BML-111 can modulate immune and inflammatory response, its effects and underlying molecular mechanisms on fulminant hepatitis have not been completely elucidated.

In this study, we found that BML-111 exerted a protective effect against LPS/D-GalN-induced liver injury. Meantime, we also showed BML-111 was capable of enhancing the production of prostaglandin (PG) J2, the expression of cyclooxygenase (COX)-2, and the activation of extracellular signal-regulated kinase (ERK) in the liver tissues of LPS/D-GalN-administered mice. Further, we confirmed that enhanced PGJ2 and COX-2, ERK activation might involve in the protective effect of BML-111 on LPS/D-GalN-induced liver injury.

Materials and methods

Animals

Balb/c-mice (6-8 weeks old; weight range 18-22 g) were obtained from the Laboratory Animal Center of the Chongqing Medical University (Chongqing, China). All mice received human care according to the guidelines of the Local Institutes of Health guide for the care and use of laboratory animals. They were maintained under controlled conditions (22°C, 55% humidity and 12 h day/night rhythm) and fed standard laboratory chow.

Reagents

LPS (Escherichia coli, 0111:B4), D-GalN and NS398 were purchased from Sigma (St. Louis, MO, USA). BML-111 (purity 99%) was obtained from Biomol (Plymouth Meeting, PA, USA). Rabbit anti-mouse COX-2 antibody was purchased from Abcam (Cambridge, UK), PD98059, Rabbit anti-mouse β-actin, p-ERK, p-JNK and p-p38 antibody were purchased from Cell Signaling Technology (Boston, MA, USA), and horseradish peroxidase-conjugated goat anti-rabbit antibody, biocinchonic acid (BCA) protein assay kit and enhancer chemiluminescent (ECL) reagent were obtained from Pierce Biotechnology (Rockford, IL), alanine aminotransferase (ALT) and aspartate aminotransferase (AST) detection kits were purchased from Nanjing Jiancheng Bioengineering Institute (Nanjing, China). Mouse TNF-α enzyme-linked immunosorbent assay (ELISA) kit was purchased from Bender MedSystems (Vienna, Austria). Prostaglandin J2 and Prostaglandin J2 Express EIA kit were purchased from Cayman Chemical Company (Ann Arbor, MI, USA).

Experimental protocols

Mice were injected i.p. with BML-111 (0.3, 1, 3 mg/kg), PGJ2 (0.1, 1 mg/kg), NS398 (3, 10 mg/kg) or PD98059 (5, 10 mg/kg) dissolved in 250 µl DMSO 30 min prior to challenge. Mice were challenged i.p. with a total volume of 250 ml LPS (50 mg/kg) and D-GalN (800 mg/kg) dissolved in PBS. The doses of BML-111 alone did not induce liver injury as determined by evaluating liver enzymes, cytokines, and liver histology (data not shown). Survival rates were evaluated within 48 h after LPS/D-GalN administration. Liver samples for MAPK (p-ERK, p-JNK, pp38) and COX-2 (mRNA and protein expression) analysis were obtained at 0.5 and 6 h after LPS/D-GalN administration.

Analysis of liver enzymes

Hepatocyte damage was assessed 6 h after LPS/D-GalN administration by measuring serum enzyme activities of ALT and AST using corresponding detection kits according to the manufacturer’s instructions.

Measurement of TNF-α levels

Mouse serum samples were assayed for murine TNF-α by enzyme-linked immunosorbent assay as described by the manufacturer. Serum levels of TNF-α were measured at 1.5 h after the LPS/D-GalN challenge [20, 21].

Histological analysis

Liver samples were obtained at 6 h after LPS/D-GalN administration. Liver tissue was fixed in 10% neutral-buffered formalin and subsequently embedded in paraffin. Sections were

Figure 1. The chemical structure of BML-111.
stained with hematoxylin and eosin (HE) using a standard protocol and analyzed by light microscopy.

**RT-PCR analysis**

Total RNA in the liver tissues was extracted with TRIzol Reagent. 1 µg of total RNA was assessed by RT-PCR. The primers used were as follows: mouse COX-2 cDNA (sense, 5’-GCATTCTTTGCACAGCACTT-3’, and antisense, 5’-AGACCAG-CGCCACCAAGA-3’); mouse GAPDH cDNA (sense, 5’-CCTGCACCACCAACTGCTTA-3’, and antisense, 5’-TCATGAGCCCTTCCAATG-3’). Optimal reaction conditions were 40 cycles of PCR (95°C for 15 s, 60°C for 60 s and 72°C for 60 s) after an initial denaturation step (95°C for 10 min). PCR products were electrophoresed in 1.5% agarose gel containing ethidium bromide. The gel was then analyzed on a Bio-Rad Image System.

Figure 2. Effect of BML-111 on LPS/D-GalN-induced liver injury. Balb/c mice were pretreated i.p. with BML-111 (0.5, 1, or 3 mg/kg) or phosphate-buffered saline (PBS) 30 min before LPS/D-GalN administration. A. The mortality was evaluated within 48 h after LPS/D-GalN administration (n=10). B. Serum ALT and AST activities were measured at 6 h after LPS/D-GalN administration. C. The histology of liver sections was analyzed at 6 h after LPS/D-GalN administration. D. Serum levels of TNF-α were measured by ELISA at 1.5 h after LPS/D-GalN administration. Data represent mean ± SD, n=6. *P<0.05, **P<0.01 compared with LPS/D-GalN administration group.
Western blotting

The proteins of hepatic samples were prepared by the protein extract kit (20 mM Tris, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% TritonX-100, 2.5 mM sodium pyrophosphate, 1 mM β-Glycerolphosphate, 1 mM Na3VO4, 1 µg/ml leupeptin and aprotinin). Protein concentrations were determined by the BCA protein assay kit. 40 µg protein extracts were fractionated on 12% polyacrylamide-sodium dodecyl sulfate (SDS) gel and then transferred to nitrocellulose membrane. The membrane was blocked with 5% (w/v) fat-free milk in Tris-buffered saline (TBS) containing 0.05% tween-20, followed by incubation with a rabbit primary antibody or anti-β-actin antibody (1:2000) at 4°C overnight. Then the membrane was treated with horseradish peroxidase-conjugated goat anti-rabbit secondary antibody (1:10000). Antibody binding was visualized with an ECL chemiluminescence system and short exposure of the membrane to X-ray films (Kodak, Japan).

Statistical analysis

Results were analyzed using Student’s t test or by ANOVA where appropriate. All data in this study were expressed as mean ± S.D. P values less than or equal to 0.05 was considered significant.

Results

Effect of BML-111 on LPS/D-GalN-induced liver injury and inflammatory response

LPS/D-GalN administration caused almost all animals death within 48 h, which could be prevented by pretreatment of BML-111 in a dose-dependent manner (Figure 2A). There existed significantly increasing serum activities of ALT and AST 6 h after LPS/D-GalN administration, which were concentration-dependently inhibited by pretreatment with BML-111 (Figure 2B). Histological examination of liver tissue section showed that severe and diffuse necrosis associated with panlobular acute inflammation were observed in mice at 6 h after LPS/D-GalN administration. In contrast, only spotty necrosis of hepatocytes and a slight inflammatory reaction were found in mice pretreated with 3 mg/kg BML-111 before LPS/D-GalN administration (Figure 2C).

Because TNF-α is a critical inflammatory mediator of liver injury induced by LPS/D-GalN, we postulated that the protective effect of BML-
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111 against LPS/D-GalN-induced liver injury was mediated by inhibiting TNF-α production. Therefore, we measured serum TNF-α levels after LPS/D-GalN administration. As shown in Figure 2D, administration of LPS/D-GalN markedly induced elevation of serum TNF-α level. Pretreatment with BML-111 dose-dependently attenuated LPS/D-GalN-mediated promotion of TNF-α levels.

Effect of BML-111 on hepatic PGJ2 production, COX-2 expression, and MAPK activation in mice

As PGJ2 participates in the negative regulation of TNF-α, we attempted to determine whether BML-111 can affect PGJ2 production. As shown in Figure 3A, LPS/D-GalN induced a mild increase of PGJ2 production in liver tissues. Pretreatment with BML-111 further dose-dependently increased PGJ2 production in the liver tissues of LPS/D-GalN-administered mice. Considering the role of COX in the PGJ2 production, we also determined the effect of BML-111 on the expression of COX-1 and COX-2 in the liver tissues of mice. RT-PCR and western blot analysis showed that as expected, LPS/D-GalN induced the expression of hepatic COX-2 mRNA and protein, and this inductive response was additive to BML-111 in a dose-dependent manner, whereas COX-1 was not changed in the liver tissues of LPS/D-GalN-administered mice with or without BML-111 (Figure 3B and 3C).

Finally, we analyzed MAPK signal pathway, as expected, in the liver tissues of LPS/D-GalN-administered mice, phosphorylation of three MAPK subtypes were rapidly upregulated, and however, only ERK activation was further increased by BML-111 in a dose-dependent fashion (Figure 3D).

Inhibition of COX-2 reversed the protective effect of BML-111 on LPS/D-GalN-induced liver injury

Next, to testify whether the increased PGJ2 production and COX-2 expression were associated with the protective effect of BML-111 on LPS/D-GalN-induced liver injury, we respectively used exogenous PGJ2 and the COX-2 specific inhibitor NS398 to interfere with mice. Result shown in Figure 4A and 4B, in line with the effect of BML-111, exogenous addition of PGJ2 also dose-dependently prevented mice from LPS/D-GalN-induce liver injury as well as TNF-α production. However, NS398 dose-dependently abolished the protective effect of BML-111 on LPS/D-GalN-induced liver injury and TNF-α production in mice.

Blockade of ERK activation abrogated BML-111-enhanced COX-2 expression and PGJ2 production, and abolished the protective effect of BML-111 on LPS/D-GalN-induced liver injury

To determine whether ERK activation is necessary for BML-111-enhanced COX-2 expression

Figure 4. Inhibition of COX-2 reversed the protective effect of BML-111 on LPS/D-GalN-induced liver injury. Balb/c mice were pretreated i.p. with BML-111 (0.5, 1, or 3 mg/kg) or PGJ2 (0.1, or 1 mg/kg) 30 min before LPS/D-GalN administration with or without NS398 (3, or 10 mg/kg), serum was collected to measure liver enzymes and cytokines. A. Serum ALT and AST activities. B. Serum TNF-α levels. Data represent mean ± SD, n=6. *P<0.05, **P<0.01 compared with LPS/D-GalN administration group, ***P<0.01 compared with BML-111-LPS/D-GalN group.
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and PGJ_2 production, we examined the role of ERK inhibitor PD98059. Results showed that BML-111-enhanced the expression of COX-2 mRNA and protein in LPS/D-GalN-primed mice was dose-dependently blocked by PD98059 (Figure 5A and 5B). The inhibitory effect of PD98059 on BML-111-enhanced PGJ_2 production was also observed in LPS/D-GalN-primed mice (Figure 5C). Further, we asked whether ERK activation has a role in the protective effect of BML-111, we found that PD98059 blocked the protective effect of BML-111 on LPS/D-GalN-induced liver injury and inflammatory response, as accessed by the rise of serum ALT/AST activities and TNF-α level (Figure 5D and 5E).

Discussion

In the models described here, onset of inflammation is characterized by the release of pro-inflammatory cytokines such as TNF-α [22-24]. Previous many studies have shown that administration of TNF-α was shown to accelerate liver injury; neutralizing antibodies against TNF-α or inhibitor of TNF-α production by agents prevented mortality in this model; knock-out for TNF-α or TNF-α receptors completely abrogated LPS/D-GalN-induced liver injury [24-34]. Thereby, inhibition of TNF-α is associated with alleviation of LPS/D-GalN-induced acute liver injury in mice. Our current report showed that pretreatment with BML-111 dose-dependently inhibited LPS/D-GalN-induced serum TNF-α release in mice. Consistent with the result, BML-111 attenuated LPS/D-GalN-induced liver injury, as indicated by the notable reduction of lethality, ALT/AST activities, and intrahepatic inflammatory response and necrotic tissue injury. These results provide evidence that the protective effect of BML-111 on LPS/D-GalN-induced liver injury is not only due to its ability to attenuate endotoxin-induced liver injury, but also due to its ability to inhibit the expression of pro-inflammatory cytokines such as TNF-α [22-24].

Figure 5. Blockade of ERK activation abrogated BML-111-enhanced COX-2 expression and PGJ_2 production. Balb/c mice were pretreated i.p. with BML-111 (0.5, 1, or 3 mg/kg) 30 min before LPS/D-GalN administration with or without PD98059 (5, or 10 mg/kg). A. Hepatic COX-2 mRNA. B. Hepatic COX-2 protein. C. Hepatic PGJ_2 production. D. Serum ALT and AST activities. E. Serum TNF-α levels. Data represent mean ± SD, n=6. **P<0.01 compared with LPS/D-GalN administration group. *P<0.05, **P<0.01 compared with BML-111-LPS/D-GalN group.
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injury might be interference of TNF-α production or release.

Actually, LPS-induced liver inflammatory process tightly is regulated by not only pro-inflammatory mediators that initiate and exacerbate inflammation but also anti-inflammatory mediators that switch off and resolute inflammation. PGJ2 is an endogenous lipid mediator that has potent anti-inflammatory properties in many inflammatory disorders, including encephalomyelitis, pancreatitis, pleurisy, atopic dermatitis, inflammatory bowel disease [35-39]. It has been found that PGJ2 inhibit neutrophil chemotaxis, NF-κB activation, TNF-α-stimulated expression of the adhesion molecules, and LPS-induced the production of iNOS, TNF-α, and IL-1β [40-42]. In the present study, we interestingly found that BML-111 dose-dependently increased PGJ2 production in the liver tissues of LPS/D-GalN-administered mice, and exogenous addition of PGJ2 significantly inhibited LPS/D-GalN-induced ALT/AST activities and TNF-α release, suggesting that PGJ2 might mediate the protective effect of BML-111 on LPS/D-GalN-induced liver injury.

It was known that cyclooxygenases (COXs) are key limited enzymes which catalyze the conversion of arachidonic acid to the formation of prostaglandin J2. There are two COX enzymes, COX-1 is a constitutive enzyme which maintains some physiological function, whereas COX-2 is the inducible isofrom which has generally been implicated to play a key role in inflammation [43, 44]. Here, we also determined the expression of COX-1 and COX-2 in liver tissues of mice. Consistent with the result of PGJ2, the expression of hepatic COX-2 in LPS/D-GalN-primed mice was upregulated by BML-111 in a dose-dependent manner, whereas, COX-1 expression was not changed by BML-111. Hence, we deduced that the increasable effect of BML-111 on PGJ2 production might be up-regulation of COX-2 expression in LPS/D-GalN-primed mice. These results made us hypothesize whether COX-2 and PGJ2 mediated the protective effect of BML-111 on LPS/D-GalN-induced acute liver injury. To clarify the idea, we used the COX-2 specific inhibitor NS398 to interfere to BML-111-pretreated mice primed by LPS/D-GalN. We noticed that administration of NS398 significantly reverted the protective effect of BML-111 on LPS/D-GalN-induced liver injury, including inhibiting serum ALT/AST activities and TNF-α production, indicating that COX-2 probably mediated the protective effect of BML-111 against LPS/D-GalN-induced liver injury.

An issue raised by several previous studies is that COX-2 is also involved in anti-inflammatory properties, although COX-2 is generally thought to have an important role in the induction of inflammatory response and has been as a key target for the treatment of inflammatory disease [45-47]. Gilroy et al. found that in carrageenin-induced pleurisy in rats, COX-2 protein expression were upregulated at the onset and resolution phases of inflammatory, respectively. The selective COX-2 inhibitor NS-398 inhibited inflammation at onset phase but significantly exacerbated inflammation at resolution phase. Further, they found that this exacerbation was associated with reduced exudate PGJ2 concentrations, and was reversed by replacement of PGJ2. Thereby, they proposed that COX-2 might aid inflammation resolution by generating an alternative set of anti-inflammatory PGJ2 [48]. In addition, a report has demonstrated that expression of COX-2-dependent prostaglandins exerted a protection against liver injury [49]. These data is in agreement with our finding that BML-111 enhanced the expression of COX-2 and production of PGJ2, which exerted anti-inflammatory effect through inhibiting of TNF-α. However, whether other types of PGs generated in COX-2 pathway, such as PGE2, also mediated the protective effect of BML-111 on LPS/D-GalN-induced liver injury remains to be further elucidated.

Mitogen-activated protein kinases (MAPKs), which include three subtypes ERK, JNK, and p38, play a critical role in the cell growth and differentiation, inflammatory responses, and stress [50, 51]. Previous studies on the induction of COX-2 by stress stimuli have shown that MAPKs pathways are responsible for the transduction of signals to initiate COX-2 gene expression [52]. To determine whether a similar signal mechanism is responsible for the upregulation of COX-2 gene expression by BML-111, we examined the activation states of ERK, JNK and p38 in the liver tissues of mice. As expected, LPS/D-GalN-induced ERK phosphorylation, but not JNK and p38, was increased by BML-111 in a dose-dependent manner, and then the specific ERK inhibitor led to an abrogation on the protective effect of BML-111 on LPS/D-
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GalN-induced liver injury, along with enhanced COX-2 expression and PGJ\textsubscript{2} production, indicating that inductive phosphorylation of ERK may mediate the protective effect of BML-111 on LPS/D-GalN-induced liver injury by enhancing COX-2 expression and PGJ\textsubscript{2} production. In fact, the role of activation of ERK in the inflammatory response is contrary. Previous studies suggested that ERK activation induced pro-inflammatory mediators release [53, 54]. However, it has been shown that ERK activation is associated with anti-inflammatory effect in other vitro experiments [55, 56]. Our results strongly suggested that BML-111-enhanced ERK activation mediated its protective effect on LPS/D-GalN-induced liver injury.

In summary, the data presented here revealed that BML-111 could effectively protect against LPS/D-GalN-induced liver injury by inhibiting pro-inflammatory mediator TNF-\alpha. The protective mechanisms of BML-111 might be through promoting ERK activation, enhancing the expression of COX-2 and production of PGJ\textsubscript{2}. Although the detailed mechanisms of BML-111 by which COX-2 and PGJ\textsubscript{2} exerts anti-inflammatory effect on LPS/D-GalN-induced liver injury remains to be further determined, the elucidated anti-inflammatory effect and mechanism will no doubt provide a new insight for BML-111 as potential therapeutic drug of liver diseases associated with inflammation.

Disclosure of conflict of interest

None.

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References

[16] Wang YZ, Zhang YC, Cheng JS, Ni Q, Li PW, Han W and Zhang YL. Protective effects of BML-111 on cerulein-induced acute pancreatitis-associated lung injury via activation of Nrf2/ARE sig-
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