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Protective effect of low molecular fraction of MGN-3, a modified arabinoxylan from rice bran, on acute liver injury by inhibition of NF-κB and JNK/MAPK expression

Surina Zheng, Shunsuke Sugita, Shizuka Hirai, Yukari Egashira *

Laboratory of Food and Nutrition, Graduate School of Horticulture, Chiba University, 648 Matsudo, Matsudo-shi, Chiba 271-8510, Japan

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ABSTRACT

D-Galactosamine (GalN) induces acute hepatitis in experimental animals; this hepatitis has been shown to be suppressed by oral or intraperitoneal administration of modified arabinoxylan from rice bran (MGN-3), and active low molecular fraction isolated from MGN-3 (LMW). We previously reported that this protective mechanism is mediated in part by downregulation of interleukin-18 (IL-18). The present study shows for the first time that nuclear factor-κB (NF-κB), mitogen-activated protein kinase (MAPK) and CD14 are involved in the suppressive action of LMW on GalN-induced hepatitis. Wistar rats (aged 4 weeks, SLC) were intraperitoneally treated with either MGN-3 or LMW. Then, rats were given GalN at 400 mg/kg at 1 h after the initial treatment. The serum activity of transaminases (ALT and AST) was significantly higher after GalN treatment; these changes were attenuated by MGN-3 and LMW. Furthermore, LMW abrogated inhibitor of κB kinase (IκB) degradation induced by GalN, and this was associated with the inhibition of NF-κB activation. Moreover, phosphorylated stress-activated protein kinase/c-Jun N-terminal kinase (JNK) protein expression in the liver after GalN treatment was significantly higher, and LMW reduced this increase. We also found that GalN treatment induced *TLR4* and *CD14* mRNA expression, and LMW significantly inhibited *CD14* mRNA expression. These results suggest that the suppressive effects of LMW on GalN-induced hepatitis are possibly related to inhibition of NF-κB, JNK phosphorylation and *CD14* expression.

1. Introduction

Hepatitis is a serious health problem worldwide associated with significant morbidity and mortality. A better knowledge of the basic mechanisms governing immune response in the pathogenesis of liver disease has allowed the development of targeted therapies for the management and treatment of hepatitis [1–3]. D-Galactosamine (GalN)-induced hepatitis has been used as an animal model for acute liver injury, since its morphological and pathophysiological characteristics are similar to those of human hepatitis B [4,5]. Hepatitis induced by GalN in rats is considered to be mediated by inhibited macromolecular glycoprotein and RNA biosynthesis through depletion in cellular UTP concentration [6] and elevation of blood levels of tumor necrosis factor-α (TNF-α) caused by increasing absorption of lipopolysaccharide (LPS) endotoxin, from the intestine to the bloodstream [7,8]. However, the precise mechanism for GalN-induced hepatitis has not yet been elucidated.

MGN-3, a modified water-soluble hemicellulose from rice bran has a variety of immune functions. It has been reported that NK cell, T cell, and B cell functions are augmented by MGN-3 both in vitro and in vivo [9–11]. In addition, when MGN-3 is administered in conjunction with conventional chemotherapeutic agents, it has been highly effective in inducing cancer remission in animal models [4]. In our previous study, we showed that GalN-induced hepatitis was suppressed in part by IL-18 reduction following ingestion of BioBran (MGN-3), a modified arabinoxylan from rice bran, or its active fraction (LMW). MGN-3 was hydrolyzed with HCl at 100 °C, and then the hydrolysate treated with cation or anion exchange resin was fractionated by molecular weight (high molecular weight fraction (≥2,000,000 Da), medium molecular weight fraction (2,000,000–400 Da), and low molecular weight fraction (LMW; ≤400 Da)). We concluded that LMW has a stronger hepato-protective effect than MGN-3 [12]. The molecular weight of LMW was measured by ESIMS, and an intense peak at m/z 409 was observed [12]. LMW is a mixture of monosaccharide and oligosaccharides, constituted by glucose as the main component (glucose, 22.8%; mannose, 1.5%; galactose, 0.5%; arabinose, 0.3%; protein, 2.85%) [12]. In our previous study, the results indicate that neutral oligosaccharides and monosaccharides in the LMW seem to be candidates for the effective ingredients for treating GalN-induced liver injury.

IL-18 is a unique activating cytokine belonging to a novel family of inflammatory cytokines that function in the immune response [13–16]. In an animal experimental model, IL-18 is released from murine macrophages or Kupffer cells through Toll-like receptor 4 (TLR4)/CD14-dependent signaling pathways [17].

Abbreviations: NF-κB, nuclear factor-κB; JNK, c-Jun N-terminal kinase; MAPK, mitogen-activated protein kinase.

* Corresponding author. Tel.: +81 47 308 8861; fax: +81 47 308 8720.
E-mail address: egashira@faculty.chiba-u.jp (Y. Egashira).

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TLRs, members of the pattern recognition receptor family, sense pathogen-associated molecular patterns (PAMPs) for host defense; however, endogenous components from necrotic cells, referred to as damage to associate molecular patterns (DAMPs), were recently shown to activate TLR-mediated signals associated with innate immune responses [18]. In mammals, 12 members of the TLR family have been identified [19]. TLR4 is a transmembrane protein, mainly existing in macrophages, which recognize LPS or LPS-CD14 complexes, and mediates macrophage activation and pro-inflammatory cytokine release [12,20]. CD14, a key gene of the innate immune system, functions as a receptor for LPS, a constitutive element of the bacterial cell wall. As a consequence of the CD14-LPS interaction at the level of the membrane, TLR-4 becomes activated. TLR-4 plays an important role in signal transduction in the innate immune response. Importantly, TLR-4 activates a transcription factor known as nuclear factor-κB (NFκB), and members of the mitogen-activated protein kinases (MAPKs) family including p38 kinase (p38), extracellular stress-related kinase 1/2 (ERK) and stress-activated protein kinase/c-Jun N-terminal kinase (JNK) [21]. Activated CD14/TLR4 is associated with hepatic ischemia/reperfusion, regeneration, and alcoholic liver disease [19].

In an animal model, endotoxin stimulation induces activation of procaspase-1 in Kupffer cells, which cleaves preformed IL-18 (proIL-18) into IL-18, resulting in the release of IL-18 [22,23]. IL-18 synergizes with IL-12 for IFN-γ production from lymphocytes, which fully activates the Kupffer cells to produce large amounts of TNF-α, a potent hepatotoxic cytokine [17]. However, the currently available data for the role of TLR4 and TLR4-signaling pathways fulminant hepatic failure are not sufficient.

In the present study, to study the mechanisms underpinning the protective effects of LMW against GalN-induced acute liver injury, we investigated the TLR4/CD14 pathway, NFκB and MAPKs.

2. Materials and methods

2.1. Reagents

D-Galactosamine hydrochloride (GalN) was obtained from Sigma Chemicals (St. Louis, MO, USA); the SV Total RNA isolation system from Promega Corporation (Madison, WI, USA); the first-Strand cDNA synthesis kit for RT-PCR (AMV) from Roche Diagnostics (Mannheim, Germany); the SYBR® Premix Ex Taq™ II kit (perfect real-time PCR) from Takara Bio Inc. (Otsu, Japan); and the reaction mixture for PCR, Absolute™ QPCR SYBR Green mixes from Abgene (Epson, UK). MGN-3 was provided by Daiwa Pharmaceutical Co. (Tokyo, Japan). All the other chemicals were obtained from Wako Pure Chemical Industries, Japan.

2.2. Fractionation of MGN-3 by gel filtration

LMW was prepared from MGN-3 as described previously in Zheng et al. [12]. MGN-3 was hydrolyzed with 1 N HCl, at 100 °C for 1 h, and then the hydrolysate treated with cation or anion exchange resin was fractioned in low molecular weight, riboflavin (M.W. 376.4) as a marker.

2.3. Animal and treatment protocols

Male Wistar rats aged 4 weeks (Japan SLC, Hamamatsu, Japan) and weighing 60–80 g were maintained in an environmentally controlled room at 22 ± 1 °C with a 12 h light/dark cycle (light from 7:00 to 19:00). All the rats were fed with a CE-2 commercial diet (Clea Japan, Japan) for 4 days, and then with a standard AIN-93G diet for 7 days.

The rats in the control and experimental groups (n = 6) were intraperitoneally administered with GalN solution at 400 mg/kg on day 7 of the standard AIN-93G diet feeding, and the standard group (GalN non-treated group, n = 3) was injected with saline solution in the same manner. At 1 h before GalN administration, in experiment 1 (A), the rats in the experimental groups were pretreated with MGN-3 or LMW intraperitoneally at 20 mg/kg and 0.05 mg/kg, respectively. In experiment 1 (B), the rats in the experimental groups (n = 6) were intraperitoneally pretreated with LMW at 0.001, 0.01 and 0.05 mg/kg of body weight. All the rats were fasted for 4 h before and after the GalN treatment (8 h in total).

The rats were anesthetized with pentobarbital at 8 or 24 h after the GalN or saline treatment. Blood was taken from the heart, and the liver was carefully removed and immediately frozen in liquid nitrogen. Serum was obtained from the blood by centrifugation at 3000 ×*g* for 20 min at room temperature. The care and treatment of the rats were carried out in accordance with "The Ethical Guideline for Laboratory Animals" prescribed by Chiba University.

2.4. Transaminase activities

Alanine amino transferase (ALT, C.E.2.6.1.2) and aspartate amino transferase (AST, C.E.2.6.1.1) activities were analyzed by a Transaminase CII-test Wako kit in accordance with the manufacturer's instructions.

2.5. Real-time quantitative PCR

Total RNA was isolated from the liver using the SV Total RNA Isolation system. cDNA was then synthesized from 1 μg of RNA by using a First-strand cDNA Synthesis kit for RT-PCR (AMV). Quantitative real-time PCR (qRT-PCR) was performed for *CD14*, *TLR4* and the housekeeping gene encoding glyceladehyde-3-phosphate-dehydrogenase (*GAPDH*) using the ABI PRISM 7000 sequence detection system (Applied Biosystems, CA, USA). The reaction mixture was composed of Absolute QPCR SYBR Green Mixes (12.5 μL), forward and reverse primers (5 μM, 1 μL of each), nuclease-free water (8 μL), and a cDNA sample (2.5 μL).

The primers used for *CD14* (GenBank Accession no. XM_039364) were as follows: forward, 5′-CAGGAACTTTGGCTTTGCTC-3′; reverse, 5′-ACCGATGGACAACTTTC AGG-3′; Those for *TLR4* (GenBank Accession No. NM_019178) were: forward-5′-CAGGAACTTTGGCTTTGCTC-3′; reverse-5′-TCAAGGCTTTTCCATCCAA C-3′. Those for *GAPDH* (GenBank Accession no. AB017801) were as follows: forward, 5′-TGCCAAGTAT GATGACATCAAGAAG-3′; reverse, 5′-AGCCCAGGATGCCCTTTA GT-3′. The PCR conditions were as follows: 30 s at 95 °C for 1 cycle; 5 s at 95 °C and 31 s at 60 °C for 45 cycles; and finally 15 s at 95 °C, 1 min at 60 °C, and 15 s at 95 °C. The results were analyzed by ABIs sequence detection system software (Applied Biosystems, CA, USA).

2.6. Western blot analysis

Liver tissue was removed and frozen at −83 °C until use. Homogenization was basically performed as previously reported [24]. Samples were homogenized on ice in five volumes of extraction buffer containing 100 mM Tris–HCl (pH 7.5), 200 mM sodium chloride, 100 μM Triton and 100 μM EDTA. All debris was removed by centrifugation at 13,000 ×*g* at 4 °C for 15 min, and the supernatant obtained was used for western blot analysis. Protein concentrations were determined by BCA protein assays. Thirty micrograms of protein extracts was fractionated on 10% polyacrylamide-sodium dodecyl sulfate gel, and then transferred to nitrocellulose membranes. Membranes were blocked with 5% (w/v) fat-free milk in Tris-buffered saline (TBS) containing 0.05% Tween-20, followed by incubation with primary antibodies against inhibitor of κB kinase-α (IκB-α; Santa Cruz Biotechnology, USA; dilution ratio; 1:1000), p-p38, p-JNK, p-ERK, total p38, total JNK, total ERK (Cell Signaling Technology, USA; all at 1:200), and β-actin (Cell Signaling Technology, USA; 1:500), at 4 °C overnight. Then, membranes were treated with anti-rabbit IgG (H+L) HRP-conjugated secondary antibody (Promega, Madison,

Wisconsin, USA; dilution ratio; 1:2000). The immunoblot was examined by Chemiluminescent HRP Substrate (Cat. NO: WBKLS0100; Immobilon™ Western, MA, USA), according to the manufacturer's instructions. Membranes were exposed by a FUJIFILM Luminescent Image Analyzer LAS-1000 (Macintosh™, USA). The intensities of the resulting bands were quantified by Quantity One software on a GS-800 densitometer (BioRad, Hercules, CA, USA).

2.7. Caspase-1 concentration in rat liver

Rat liver samples were analyzed for caspase-1 activity using the FLICA Apoptosis Detection Kit (Caspase-1 FLICA; Immunochemistry Technologies, LLC, Bloomington, USA) in accordance with the manufacturer's instructions.

2.8. Statistical analysis

All the values in the figures and text are expressed as the mean ± SEM. Scheffe's multiple-comparison test was applied when significant differences were obtained by one-way analysis of variance. The level of significance was $P<0.05$.

3. Results

3.1. Hepato-protective effect of MGN-3 and LMW

Rats treated with GalN alone developed hepatocellular damage, as evident from a significant elevation in serum transaminase assays. Pre-treatment with MGN-3 by intraperitoneal injection afforded significant protection against GalN-induced liver injury. Pretreatment with LMW showed the same effect as MGN-3 (Fig. 1A).

LMW was administered to the rats at three different doses to quantitatively study the protective effects of LMW against GalN-induced liver injury. The AST and ALT activities of the LMW-treated groups injected at a dose of 0.05 mg/kg were significantly lower than those of the control groups (Fig. 1B).

3.2. Effect on liver caspase-1 activity

We then measured caspase-1 activity in rat liver and found no significant differences among the three groups (Fig. 2).

3.3. Effect on liver CD14 and TLR4 mRNA expression

This experiment showed that the liver mRNA *CD14* expression levels of the GalN treated group were significantly higher than those of the normal (standard group) 8 h after GalN treatment. There were significant changes when the rats were pretreated with LMW, and we also observed a significant decrease in the liver *CD14* mRNA expression level of the LMW group compared with the control group (Fig. 3B).

Fig. 3A shows the liver *TLR4* mRNA expression levels of the standard group, GalN-treated group and LMW-treated group; there were no significant differences in liver *TLR4* mRNA expression levels among the three groups. However, we found a similar trend of *CD14* mRNA expression levels among the groups compared.

3.4. Effects on IκB expression

The translocation of NFκB to the nucleus is preceded by the phosphorylation and proteolytic degradation of IκBα. To determine whether the inhibitory action of LMW was due to its effect on IκBα degradation, the cytoplasmic levels of IκBα protein were measured by western blot analysis. Eight hours after GalN treatment, IκBα protein was decreased in the GalN-only group, but it had returned to near-normal in the

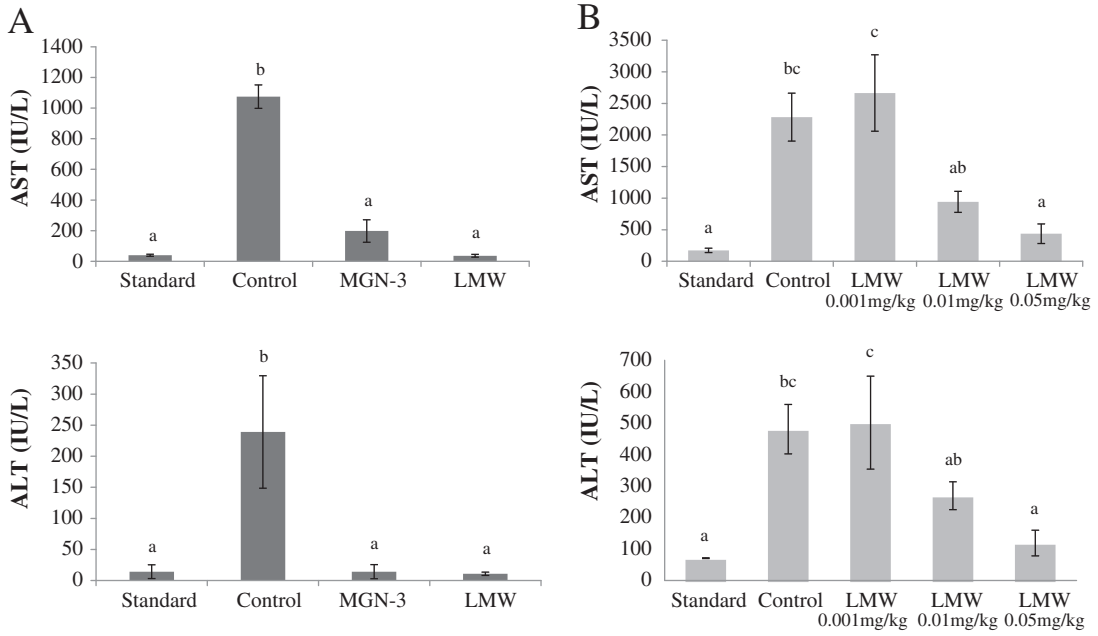

Fig. 1. Effect of MGN-3 and LMW on serum transaminase activity in rats at 24 h after GalN treatment. MGN-3 was hydrolyzed with 1 N HCl, at 100 °C for 1 h, and then the hydrolysate treated with cation or anion exchange resin was fractioned by molecular weight. Low molecular weight fraction (LMW) was used in this experiment. (A) The MGN-3 and LMW were administered i.p. (20 mg/kg B.W.; 0.05 mg/kg B.W.) and saline was administered i.p. into the standard and control groups. (B) LMW (0.001, 0.01, or 0.05 mg/kg B.W.) was administered i.p. All the groups except the standard group received an i.p. injection of GalN (400 mg/kg B.W.) 1 h after the treatment with MGN-3 samples. 24 h after GalN injection, serum was collected for the measurement of aspartate aminotransferase (AST) and alanine aminotransferase (ALT) activities. Values are shown as means ± SEM (n = 6; control and experimental groups (GalN treated groups), n = 3; standard group (GalN non-treated group)). Values indicated by different letters are significantly different at $P<0.05$.

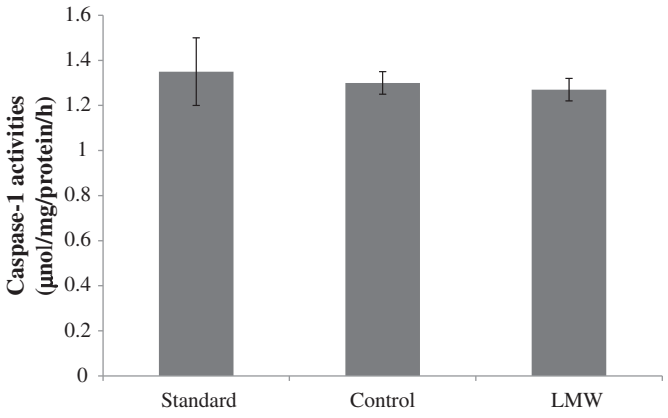

Fig. 2. Effect of LMW on liver caspase-1 activity at 8 h after GalN administration in rats. Values are shown as means ± SEM.

LMW-pretreated group (Fig. 4). LMW had a strong ability to inhibit GalN-induced IκBα degradation.

3.5. Effect on MAPK expression

We next determined whether LMW affected MAPK phosphorylation. GalN treatment significantly increased the phosphorylation of ERK, JNK and p38 in the liver at 8 h. As shown in Fig. 5, pretreatment with LMW significantly inhibited GalN-activated phosphorylation of JNK in the liver tissues of rats, but not p-ERK or p-p38. Also, the levels of ERK, JNK and p38 protein were similar in all groups.

4. Discussion

Acute liver diseases constitute a global concern, and medical treatments for these diseases are often difficult to manage and have limited efficacy. Therefore, there has been considerable interest in the role of complementary and alternative medicines for treatment of liver diseases [25].

GalN is used as an experimental model of severe hepatic damage that closely resembles human viral hepatitis, due to its ability to inhibit RNA and protein synthesis in the liver, resulting in the subsequent release of endotoxin from the intestine [5,26–28]. Endogenously produced endotoxin has been implicated as a cofactor in GalN-induced hepatocellular injury, death [29,30] and TNF hypersensitivity [31]. GalN is known to sensitize animals both to the lethal effects of LPS and to a principal LPS-induced mediator, TNF-α [32]. In our previous experiments, we showed that serum AST and ALT activities in GalN-treated rats were suppressed by preceding intraperitoneal injections of MGN-3 or its active fraction, LMW; moreover, liver *IL-18* mRNA expression and serum IL-18 concentrations were significantly suppressed by the administration [12]. In the present study, we report for the first time that the induction of hepatic injury by GalN injection in the rats was associated with elevated *TLR4*/*CD14* mRNA expression, and IκB and MAPK signaling pathways in the liver. Pretreatment with LMW at 0.05 mg/kg was found to repress the elevation of *CD14* mRNA expression, inhibit I-κB degradation and decrease p-JNK protein levels in the liver after GalN injection.

IL-18 is reported to be related to many kinds of immunological and inflammatory events [33]. For example; serum IL-18 concentration levels were observed to be increased in a murine atopic dermatitis model [34] and in a bronchial asthma model [35]; overexpression of IL-18 caused substantial liver injury in mouse [36]. In addition,

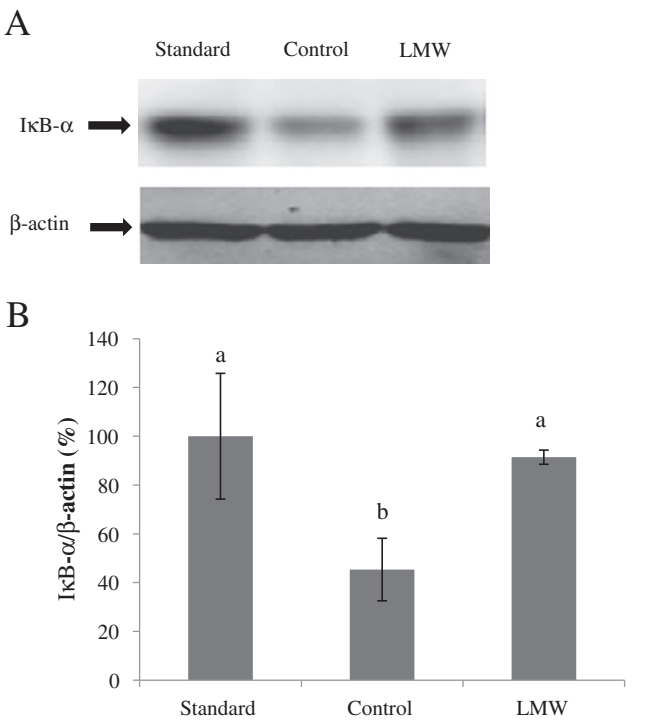

Fig. 4. Effect of LMW on liver IκB-α protein expression in rats at 8 h after GalN treatment. IκB-α protein levels were detected by western blotting. (A) IκB-α and β-actin protein bands. (B) IκB-α protein levels were normalized by β-actin protein levels. The values in (B) are shown as means ± SEM. Values indicated by different letters are significantly different at $P<0.05$.

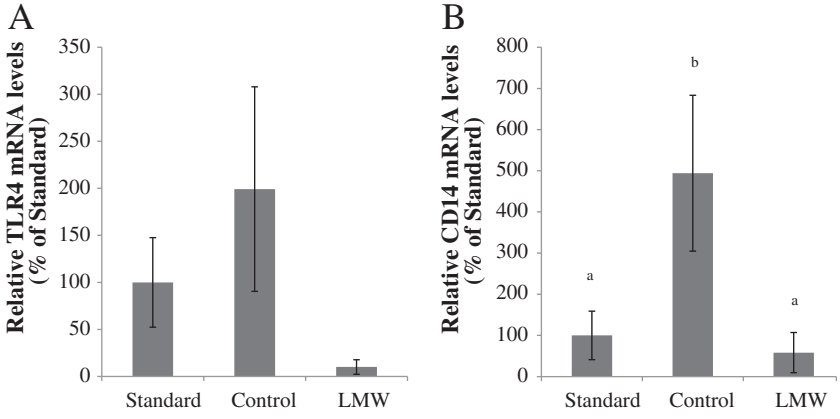

Fig. 3. Effect of LMW on liver *TLR4* (A) and *CD14* (B) mRNA expression levels in rats at 8 h after GalN treatment. Values are shown as means ± SEM. Values indicated by different letters are significantly different at $P<0.05$.

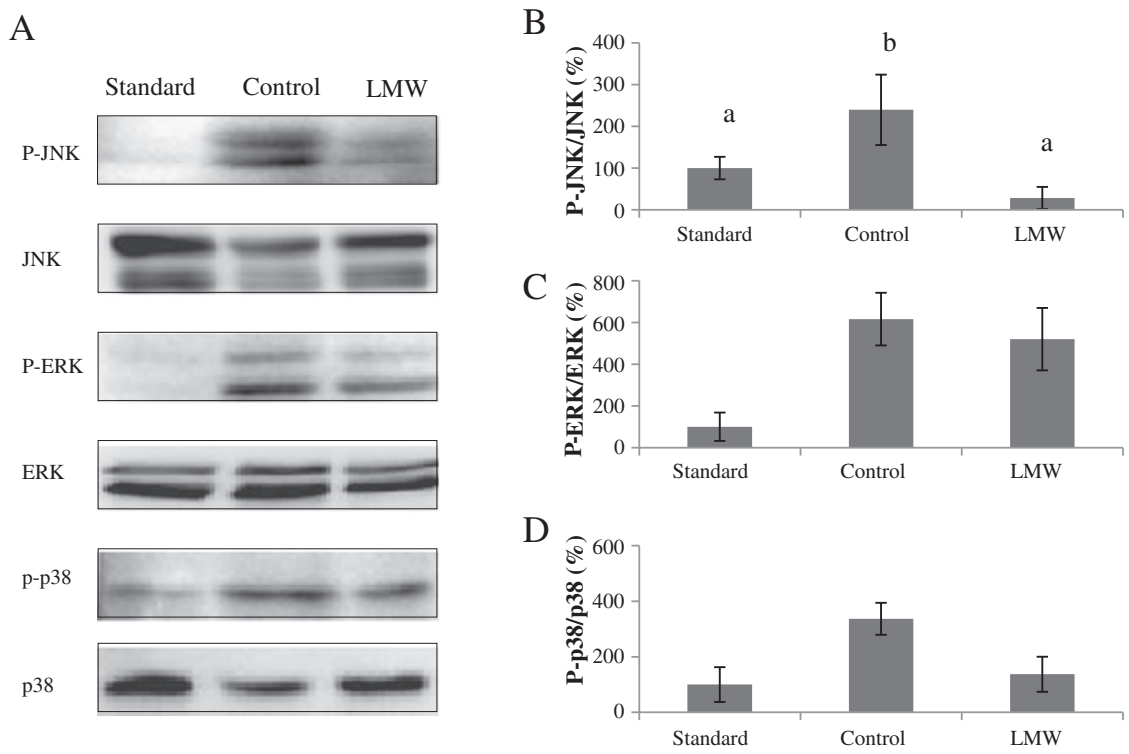

Fig. 5. Effect of LMW on liver MAPK protein expression in rats at 8 h after GalN treatment. The expression levels of phosphorylated and total JNK, ERK and p-38 protein were detected by western blotting. (A) Phosphorylated and total JNK, ERK and p-38 protein bands. (B) Phosphorylated JNK (B), ERK (C) and p-38 (D) were normalized by the value of each total protein. The values in (B–D) are means ± SEM. Values indicated by different letters are significantly different at $P<0.05$.

plasma IL-18 concentration was shown to increase significantly in patients with liver injury caused by hepatitis C virus [37–39]. It requires intracellular processing for its secretion; mature IL-18 (18 kDa) is secreted after cleavage of the inactive precursor (23 kDa) by caspase-1 [40,41]. In the present study, we focused on caspase-1, which was originally designated as IL-1b converting enzyme [41,42]. Kupffer cells from caspase-1-deficient mice did not secrete IL-18 after LPS stimulation, whereas those from wild-type C57BL/6 mice did [41]. LPS challenge induced IL-18 in the serum of *Propionibacterium acnes*-primed wild-type C57BL/6 mice, while IL-18 was not observed in the serum of *P. acnes*-primed caspase-1-deficient mice. To determine whether the inhibitory action of LMW on IL-18 was due to its effect on the decrease of caspase-1, rat liver caspase-1 activity was measured. However, there were no significant differences among the three groups. It suggested that the reduction of IL-18 levels by LMW pretreatment was not associated with caspase-1. A similar caspase-independent processing of IL-1b has also been reported in *P. acnes*-primed Kupffer cells [43]; IL-1b belongs to the same family as IL-18.

In our previous study, LMW significantly suppressed *IL-18* mRNA in rat liver [12]. This change is consistent with the hypothesis that endotoxin binds to CD14, activates the Kupffer cells via TLR4, and elicits the production of NFκB and MAPKs. Activation of the transcription factor NFκB is known to transcriptionally regulate a variety of genes related to inflammatory processes [44]. The regulation of *IL-18* gene expression is also accompanied by effects on NFκB activation [45].

Apoptotic signaling in GalN-induced acute liver injury was observed. The early cellular signal transduction pathways responsible for the activation of NFκB were triggered, and this led to tissue damage [46]. Likewise, we demonstrated that liver damage initiated by GalN led to early activation of nuclear translocation of NFκB, which, in turn, promoted expression of *IL-18* mRNA in the rat liver. This proinflammatory response can be abrogated by LMW, which exerts hepato-protective activity.

The MAPK family plays important roles in the regulation of cell proliferation and cell death in response to various cellular stresses. It has been reported that administration of GalN causes activation of MAPKs [47].

In the present experiments, the phosphorylation of the three MAPKs, JNK, ERK1/2 and p38, in the rat liver were determined. We observed increased levels of activated ERK, JNK, and p38 MAP kinases after GalN administration. Interestingly, LMW attenuated increases in activated JNK, but had no effect on p-ERK1/2 and p38 MAP kinase. It has been reported that the JNK signaling pathway plays an important role in regulating the expression of IL-18 in heat-shocked murine macrophages [48]. We reported that LMW suppressed *IL-18* mRNA. Taken together with Wang's report, our results provide evidence that LMW inhibits activation of p-JNK.

In the liver, parenchymal and non-parenchymal cells express Toll-like receptor (TLR) family members for mediating inflammation under pathological conditions [49]. In recent research, upregulated *TLR4* expression and function was reported in various liver models, such as partial hepatectomy [50], ischemia–reperfusion [20,51], and alcohol loading [52,53].

Recent studies showed that the gut-derived endotoxin activates its cellular receptors CD14 and TLR4, mainly on Kupffer cells but also on hepatocytes. In addition, it was found that the expression of *TLR4* and *CD14* in the liver increased in parallel at 24 h after GalN administration. We measured *TLR4* and *CD14* expression in the rat liver at 8 h after GalN administration to observe the early stage effects. In our observations, *CD14* but not *TLR4* was increased by GalN, and suppressed by pre treatment with LMW. Haziot and colleagues reported that the strong resistance of *CD14*-deficient mice to endotoxin suggested that CD14 plays a predominant role in endotoxin shock [54,55]. Jarvelainen et al. [56] investigated how activated Kupffer cells release proinflammatory cytokines, a process that is regulated by CD14. In another study, wild-type mice showed more severe liver injury caused by chronic ethanol feeding compared

with *CD14* knockouts; this result seems to support the findings showing how endotoxin acting via CD14 plays a major role in the development of alcohol-induced liver injury [57]. CD14 plays a key role in innate immunity through the recognition of bacterial endotoxin. From our results, the protective effects of LMW against GalN-induced hepatitis may be due to inhibition of *CD14* expression.

This study shows for first time that NF-κB, JNK and CD14 are involved in the suppressive action of LMW on GalN-induced hepatitis. Overall, it appears that LMW prevents GalN-induced liver injury by suppression of inflammatory signaling pathways.

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