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Pretreatment with wortmannin alleviates lipopolysaccharide/D-galactosamine-induced acute liver injury

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ABSTRACT

Intestinal endotoxemia-induced liver injury is a common clinical disease which leads to liver failure and death. Wortmannin, an inhibitor of phosphatidylinositol 3-kinase, could be used for suppressing autophagy in vitro and in vivo. Autophagy is an evolutionarily conserved and lysosome dependent protein degradation pathway, which participates in various physiological and pathological processes. The present study aims to explore the effect of pretreatment with wortmannin on acute liver injury and the autophagy in acute liver injury. We demonstrated that wortmannin could downregulate the expression of phosphorylated extracellular regulated protein kinase and p65, decrease the production and release of hepatic inflammatory cytokines, and then reduce hepatocytes apoptosis and necrosis. More importantly, we found that autophagy was induced to increase in LPS/D-GalN-induced acute liver injury, and pretreatment with wortmannin could effectively inhibit increased autophagy in acute liver injury. In conclusion, these results indicate that wortmannin plays a protective role in LPS/D-GalN induced hepatocytotoxity maybe by inhibiting autophagy and could be acted as a target for the treatment of acute liver injury.

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1. Introduction

Intestinal endotoxemia induced by severe trauma, burn or scald will lead to acute liver injury, liver failure and death [1,2]. In addition, intestinal endotoxin (lipopolysaccharide, LPS) is also an important cofactor in toxic liver injury by a number of agents such as alcohol [3]. Therefore, the relationship between intestinal endotoxemia and acute liver injury has been increasingly concerned [4,5]. LPS, as a critical component of the cell membrane of gram-negative bacteria, could activate kupffer cells via toll-like receptor 4 (TLR4)-mediated mitogen-activated protein kinases (MAPK) and nuclear factor-kappa B (NF-κB) signal pathways, and stimulate the production and release of inflammatory factors including tumor necrosis factor-α (TNF-α) which will induce the apoptosis and necrosis of hepatocytes [5]. Combining D-galactosamine (D-GalN) with LPS stimulation is a widespread approach to increase the hepatotoxicity of LPS [6,7]. Accordingly, LPS/D-GalN-treated mouse is acted as a well-established model to mimic intestinal endotoxemia-induced liver injury [8–11].

Wortmannin (WM) is a cell-permeable, fungal metabolite which acts as a potent, selective and irreversible inhibitor of phosphatidylinositol 3-kinase (PI3K), and also shows the immunosuppressive effect in rats and mice [12]. Recently, it has been reported that wortmannin could be used for suppressing autophagy in vitro and in vivo [13–15]. Autophagy is a homeostatic process taking place in eukaryotic cells extensively, it can mediate

Abbreviations: ALT, alanine aminotransferase; AST, aspartate aminotransferase; DAB, diaminobenzidine; DAPI, 4,6-diamidino-2-phenylindole; D-GalN, D-galactosamine; ECL, electrochemical luminescence; ERK, extracellular regulated protein kinase; HE, hematoxylin and eosin; IHC, immunohistochemistry; IP, intraperitoneal; JNK, c-Jun N-terminal kinase; LC3, light chain 3; LPS, lipopolysaccharide; MCP-1, monocyte chemoattractant protein-1; MyD88, myeloid differentiation factor 88; NS, normal saline; NF-κB, nuclear factor-kappa B; PI, phosphatase inhibitor; PI3K, phosphatidyl inositol 3-kinase; PVDF, polyvinylidene fluoride; RIP1, receptor-interacting protein 1; RIPA, radio immunoprecipitation assay; TLR4, toll-like receptor 4; TNF-α, tumor necrosis factor-α; TRIF, TIR-domain-containing adapter-inducing interferon-β; TUNEL, terminal-deoxynucleotidyl transferase mediated nick end labeling; MAPK, mitogen-activated protein kinase; UTP, uridine triphosphate; WM, wortmannin.

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damaged or disabled organelles, misfolded or long-lived proteins to enter lysosome, and make them degrade into small metabolites in order to recycle and provide energy for cell [16]. Under normal physiological conditions, most of cells maintain a basic level of autophagy. When they undergo some internal and external environment stimuli such as nutrient depletion and cellular stress, increased autophagy is induced to keep cell alive [17]. However, excessive autophagy could cause autophagic cell death. The previous studies showed that autophagy could be also involved in the pathogenesis of diverse disease states including liver diseases such as liver ischemia reperfusion injury, viral hepatitis, alcoholic liver disease, non alcoholic fatty liver disease, hepatocellular carcinoma [18,19]. Kun Wang et al. reported that LPS/D-GalN induced the increase of microtubule-associated protein 1 light chain 3B (LC3B) expression which is a marker of autophagy, suggesting autophagy may participate in the development of LPS/D-GalN-induced acute liver injury [20].

The present study aims to explore the effect of pretreatment with wortmannin on acute liver injury and the autophagy in acute liver injury. We demonstrated that wortmannin could alleviate LPS/D-GalN-induced hepatocytes apoptosis and necrosis by decreasing the production and release of inflammatory cytokines. More importantly, we found that autophagy was induced to increase in LPS/D-GalN-induced acute liver injury, and pretreatment with wortmannin could effectively inhibit increased autophagy in acute liver injury.

2. Materials and methods

2.1. Reagents and antibodies

LPS from *Escherichia coli* 055:B5, D-GalN and wortmannin were purchased from Sigma Aldrich Shanghai Trading Co Ltd (Shanghai, China). DMSO was purchased from Solarbio (Beijing, China). Rabbit polyclonal antibodies specific for cleaved caspase-3 (Asp175), LC3B, phospho-c-Jun N-terminal kinase (p-JNK, Thr183/Tyr185) and monoclonal antibodies specific for phospho-extracellular regulated protein kinase (p-ERK, Thr202/Tyr204), p-p38 (Thr180/Tyr182) were purchased from Cell Signaling Technology (Boston, MA, USA). Rabbit polyclonal antibody specific for p-p65 (Ser529) was purchased from Abcam (Hong Kong) Ltd (Hong Kong, China) and rabbit polyclonal antibody specific for β-actin was purchased from ImmunoWay (Newark, DE, USA).

2.2. Animals and experimental design

C57BL/6 mice at the age of 6–8 weeks and body weight of 16–18 g were purchased from Vital River Laboratories (Beijing, China), and then fed under specific pathogen-free conditions. Animal experiments were approved by the Animal Care and Use Committee of the Institute of Zoology, Chinese Academy of sciences. Acute liver injury was induced by intraperitoneal (IP) injection of LPS (50 μg/kg) together with D-GalN (800 mg/kg) which can increase the sensitivity of hepatocytes to LPS by depleting uridine triphosphate (UTP). Blood was collected from retro-orbital venous plexus at 3 h and 5 h after the injection of LPS/D-GalN. Then mice were executed by cervical dislocation and liver tissues were removed immediately for subsequent RNA, protein extraction and histological detection. Normal saline (NS) was used in control group mice. Wortmannin (0.5 mg/kg) was dissolved in dimethylsulfoxide (DMSO) and injected intraperitoneally to each treated mouse at 1 h before the injection of LPS/D-GalN. As a control, NS containing the same concentration of DMSO was injected into the matched group mice.

2.3. Serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST)

Blood sample was kept stationary for at least half one hour at 4 °C, then the serum was isolated after centrifugation at 3000 rpm for 10 min at 4 °C. The ALT and AST levels in serum were determined by automatic biochemical analyzer DimensionRLMax (Siemens, Munich, Germany).

2.4. Liver histology

Liver tissues were fixed overnight in 10% formaldehyde solution, dehydrated and embedded with paraffin, and then sliced into 4 μm-thickness sections. Liver tissue sections were deparaffinized, rehydrated and stained with hematoxylin and eosin (HE) to evaluate the liver tissue pathology.

2.5. Immunohistochemistry (IHC) analysis

Liver tissue sections were first deparaffinized by infiltrating in dimethylbenzene twice and rehydrated using graded ethanol. The sections were rinsed with cold phosphate-buffered saline (PBS) for 3 times after antigen microwave retrieval and endogenous peroxide blocking. The slides were incubated overnight at 4 °C with primary antibody against cleaved caspase-3 (1:100) after nonspecific antigen was blocked by 10% goat serum. Next, the slides were incubated for 30 min at 37 °C with secondary antibody conjugated with horseradish peroxidase, and then visualized using diaminobenzidine (DAB) kit (Zsbio, Beijing, China) and nucleuses were counterstained with hematoxylin.

2.6. Immunofluorescence assay

The deparaffinage, rehydration, antigen retrieval, endogenous peroxidase blocking, nonspecific antigen blocking and primary antibody incubation were performed as same as IHC. After washing with PBS for 3 times, the liver tissue sections were incubated with secondary antibody labeled with Alexa Fluor 555 (1:500, Beyotime, Beijing, China) for 1 h at 37 °C. Next, the sections were washed with PBS and incubated in DAPI staining solution (Beyotime, Beijing, China) for 15–30 min and then washed again. The sections were mounted with Antifade Mounting Medium (Beyotime, Beijing, China) and observed under a High Sensitivity Laser Confocal Microscope LSM780 (Carl Zeisis, Oberkochen, Germany) in a week.

2.7. Reverse transcription-polymerase chain reaction (RT-PCR)

Liver tissues were grinded in liquid nitrogen. Total RNA was extracted with Trizol Reagent (TIANGEN, Beijing, China), and then reversely transcribed into cDNA using a FastQuant RT Kit (With gDNase) (TIANGEN, Beijing, China) according to the manufacture's instruction. Polymerase chain reaction was performed using 2× Taq PCR MasterMix (TIANGEN, Beijing, China) and specific primers as previously described [11]. PCR products were subjected to electrophoresis on a 1.5% agarose gel and the results were imaged by GelDoc XR-Quantity One gel-imaging and analysis system (Bio-Rad, Hercules, CA, USA), and then quantified with gray analysis software Gel-Pro Analyzer 4.0.

2.8. Western blot analysis

Liver tissue samples were lysed in Radio Immunoprecipitation Assay (RIPA) buffer containing 1% Phenylmethanesulfonyl fluoride

(PMSF) and 0.5% phosphatase inhibitor (PI). Protein concentrations were measured using the bicinchoninic acid protein assay kit (Thermo Scientific, Rockford, IL, USA). Then 40 μg of protein was run on 10% or 12% sodium dodecyl sulfate–polyacrylamide gel and transferred onto a polyvinylidene fluoride (PVDF) membrane. The membrane was blocked with 5% bovine serum albumin (BSA) and incubated overnight at 4 °C with 1:1000 dilution of primary antibodies. Then the membrane was incubated in a 1:2000 dilution of secondary antibodies conjugated to horseradish peroxidase (Zsbio, Beijing, China) at room temperature for 1 h and visualized by a ECL western blotting detection system. The intensity of the target protein bands was normalized to the loading control β-actin.

2.9. Terminal-deoxynucleotidyl transferase mediated nick end labeling (TUNEL)

Liver tissue sections were deparaffinized, rehydrated, retrieved, and blocked in a mixed buffer containing 0.1 M Tris–HCl (pH 7.5), 3% BSA and 20% normal bovine serum for 30 min at 15–25 °C. Then the sections were incubated in TUNEL reaction mixture from the In Situ Cell Death Detection Kit, Fluorescein (Roche, Basel, Switzerland) for 60 min at 37 °C according to the manufacturer's instruction, and observed under an Inverted Fluorescence Microscopy. The number of TUNEL-positive cells in 5 randomly selected high power fields (200×) was counted,

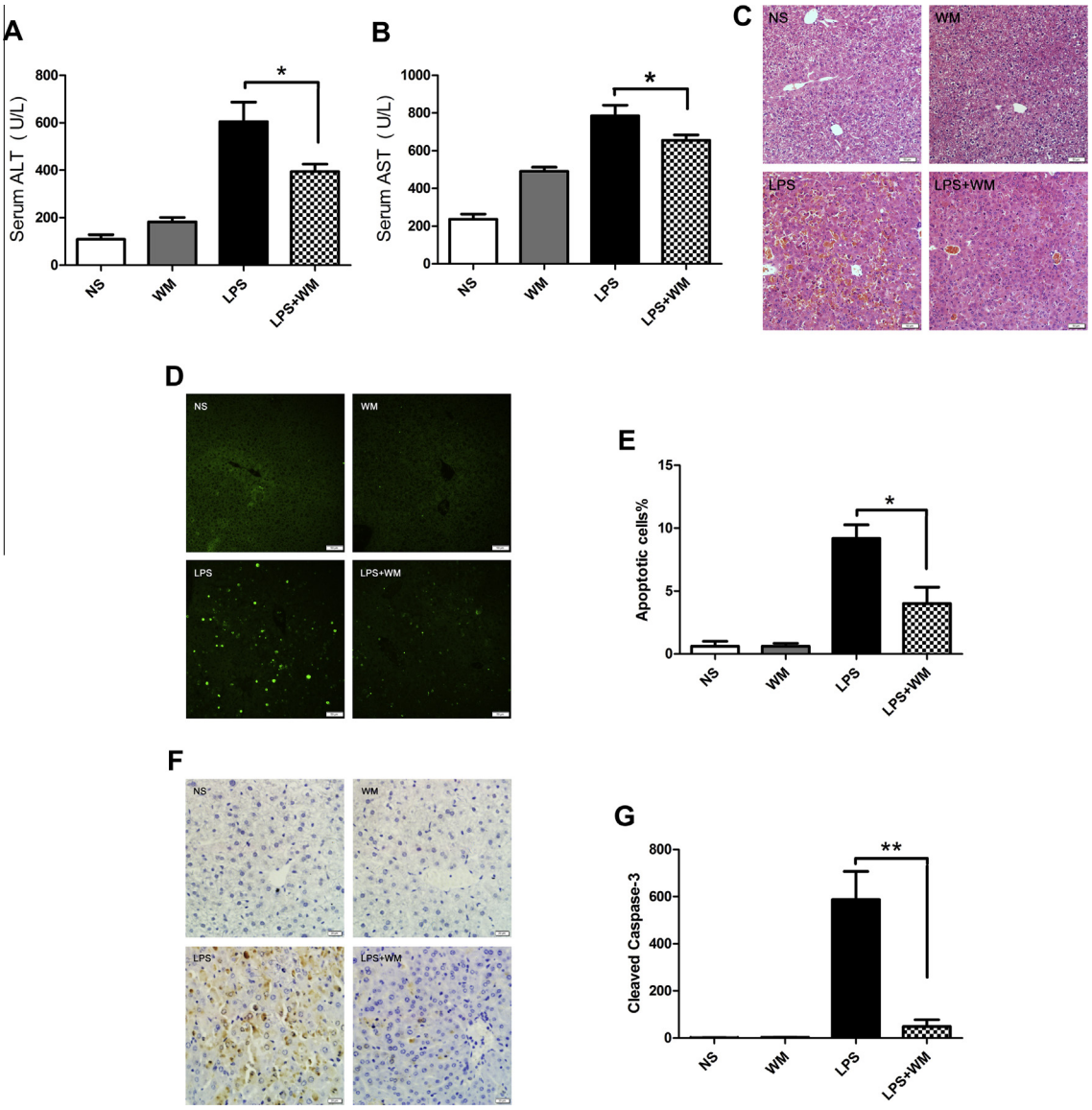

Fig. 1. Pretreatment with wortmannin could alleviate hepatocyte damage and apoptosis in lipopolysaccharide/D-galactosamine induced acute injury. Mice were intraperitoneally injected with wortmannin (WM) at 1 h before lipopolysaccharide (LPS)/D-galactosamine (D-GalN) (n = 11/group/time point). Blood and liver tissues were collected at 5 h after LPS/D-GalN injection. (A and B) The serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST) levels were obviously decreased in lipopolysaccharide (LPS)/D-galactosamine (D-GalN) and wortmannin (WM) co-treatment group. (C) Hematoxylin eosin staining showed that LPS/D-GalN and WM co-treatment group displayed less damage hepatocytes and hemorrhage than LPS/D-GalN group (×200). (D and E) The results from TUNEL showed that LPS/D-GalN and WM co-treatment group displayed fewer apoptotic hepatocytes than LPS/D-GalN group (fluorescein isothiocyanate (FITC) labeling, green highlight spots, ×200). (F and G) The result from immunohistochemical staining showed that the expression of cleaved caspase-3 was reduced in LPS/D-GalN and WM co-treatment group compared with LPS/D-GalN group (×400). NS: normal saline. $^{*}P < 0.05$, $^{**}P < 0.01$.

and the percentages of TUNEL-positive cells (Apoptotic cells %) were calculated.

2.10. Statistical analysis

All data were expressed as mean ± SD. The statistical analysis was performed by the unpaired Student's *t*-test using the GraphPad Prism 5.0 software. Statistical significance was considered as $P < 0.05$.

3. Results

3.1. Pretreatment with wortmannin could alleviate hepatocytes damage in LPS/D-GalN induced acute injury

To explore the effect of pretreatment with wortmannin on LPS/D-GalN induced acute injury, we firstly detected the serum ALT and AST levels. The results showed that the serum ALT and AST levels were obviously decreased in LPS/D-GalN and WM co-treatment group compared with LPS/D-GalN group ($P < 0.05$) (Fig. 1A and B). In addition, HE staining results also showed that LPS/D-GalN and WM co-treatment group displayed less damage hepatocytes and hemorrhage than LPS/D-GalN group (Fig. 1C). These results suggest pretreatment with wortmannin could alleviate the liver injury induced by LPS/D-GalN.

3.2. Pretreatment with wortmannin could mitigate hepatocyte apoptosis

To further explore the effect of pretreatment with wortmannin on hepatocyte apoptosis, we next performed TUNEL assay. LPS/D-GalN and WM co-treatment group displayed significantly fewer apoptotic hepatocytes than LPS/D-GalN group ($P < 0.05$) (Fig. 1D and E). The results from IHC also showed that the expression of

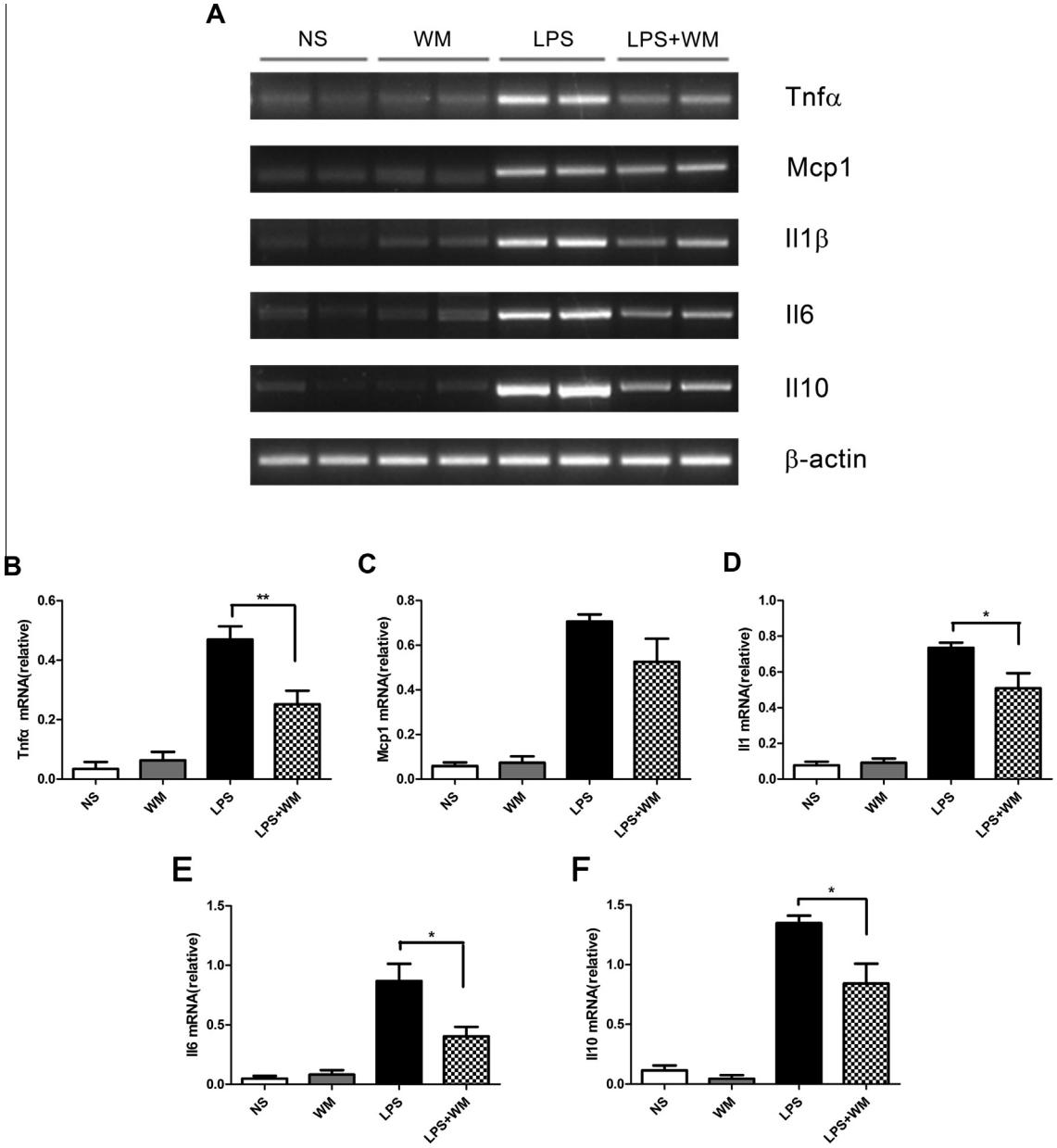

Fig. 2. Pretreatment with wortmannin could decrease hepatic cytokine levels. (A) The mRNA expression of inflammatory cytokines was detected by RT-PCR after pretreatment with wortmannin (WM). The mRNA levels of pro-inflammatory cytokines *Tnfα* (B), *Il-1β* (D), *Il-6* (E) except for *Mcp1* (C) and anti-inflammatory cytokine *Il-10* (F) were decreased in the liver tissues of lipopolysaccharide (LPS)/D-galactosamine (D-GalN) and WM co-treatment group compared with LPS/D-GalN group. NS: normal saline. $^{*}P < 0.05$, $^{**}P < 0.01$.

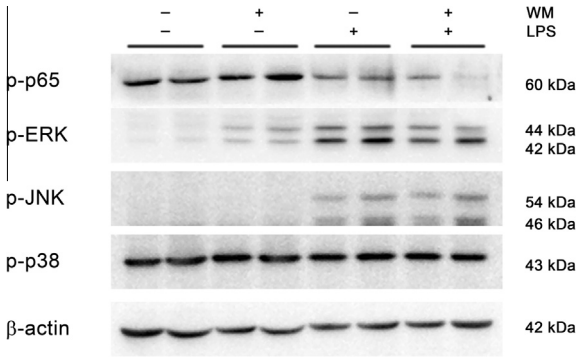

Fig. 3. Pretreatment with wortmannin could affect the activation of MAPK and NF-κB signal pathways in lipopolysaccharide/D-galactosamine induced acute liver injury. Western blot analysis showed that phosphorylated ERK and p65 were decreased in lipopolysaccharide (LPS)/D-galactosamine (D-GalN) and wortmannin (WM) co-treatment group compared with LPS/D-GalN group, however, there was no difference in the expression of phosphorylated JNK and p38 between the above two groups.

cleaved caspase-3 was obviously decreased in LPS/D-GalN and WM co-treatment group compared with LPS/D-GalN group ($P < 0.01$) (Fig. 1F and G), suggesting that increased hepatocyte apoptosis induced by LPS/D-GalN could be alleviated after pretreatment with wortmannin.

3.3. Pretreatment with wortmannin could decrease hepatic cytokine levels

We further investigate whether pretreatment with wortmannin could affect LPS-induced cytokine secretion. The results showed that the mRNA levels of pro-inflammatory cytokines *Tnfα*, *Il-1β*, *Il-6* except for *Mcp-1* and anti-inflammatory cytokine *Il-10* were decreased in the liver tissues of LPS/D-GalN and WM co-treatment group compared with LPS/D-GalN group (Fig. 2A–F). Therefore, we speculate that pretreatment with wortmannin could reduce hepatic cytokine secretion.

3.4. Pretreatment with wortmannin could affect the activation of MAPK and NF-κB signal pathways in LPS/D-GalN induced acute liver injury

We also detected the expression of phosphorylated ERK, JNK, p38 and p65. As shown as Fig. 3, phosphorylated ERK and p65 were decreased in LPS/D-GalN and WM co-treatment group compared with LPS/D-GalN group. However, there was no difference in the expression of phosphorylated JNK and p38 between the above two groups. This indicates that the expression of phosphorylated ERK and p65 could be down-regulated because of pretreatment with wortmannin, which could reduce the levels of hepatic cytokines and then decrease hepatocyte necrosis and apoptosis.

3.5. Pretreatment with wortmannin could effectively inhibit increased autophagy in LPS/D-GalN induced acute liver injury

To assess the effect of pretreatment with wortmannin on autophagy in LPS/D-GalN induced acute liver injury, western blot analysis were performed. As showed in Fig. 4A and B, the expression of LC3B-II was significantly decreased in the LPS/D-GalN and WM co-treatment group compared with LPS/D-GalN group ($P < 0.05$). The results of immunofluorescent assay also showed that the expression of LC3B was lower in LPS/D-GalN and WM co-treatment group than that in LPS/D-GalN group (Fig. 4C). This suggests that increased autophagy in LPS/D-GalN induced liver injury could effectively inhibited by pretreatment with wortmannin.

4. Discussion

The current study investigated the effect of pretreatment with wortmannin on acute liver injury and the autophagy in acute liver injury. We demonstrated that pretreatment with wortmannin may attenuate acute liver injury, which may be related to down-regulated phosphorylated ERK and p65. Furthermore, we found that autophagy was induced to increase after LPS/D-GalN injection and pretreatment with wortmannin could effectively inhibit increased autophagy in LPS/D-GalN induced acute liver injury.

Autophagy is an evolutionarily conserved and lysosome dependent protein degradation pathway, which participates in various physiological and pathological processes [21]. It has been reported that autophagy plays protective roles in ethanol, acetaminophen and TNF-induced liver injury [22–24]. But it is also reported autophagy suppression could improve both liver damage and the survival rate of recipient rats in the cold ischemia-warm reperfusion (CI/WR) injury which is a kind of the liver transplantation model [25]. In addition, autophagy was increased in concanavalin A (ConA) induced acute hepatitis in SCID/NOD mice [26], and

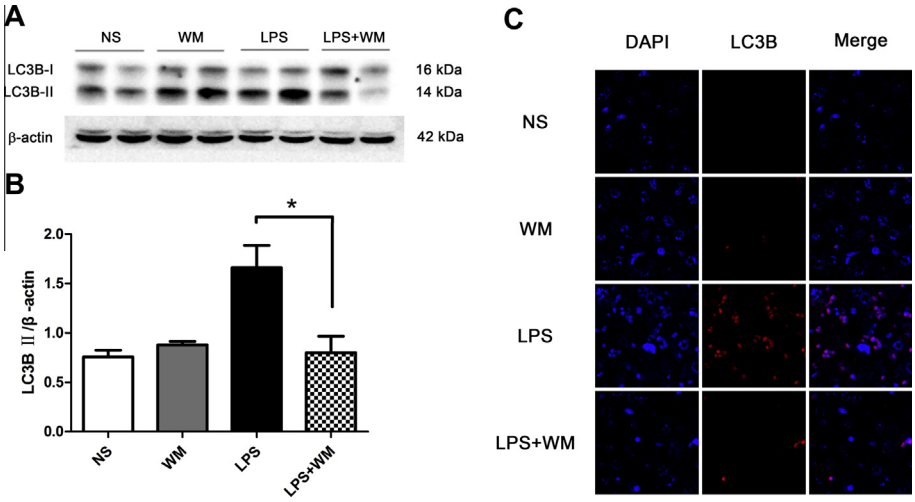

Fig. 4. Pretreatment with wortmannin could effectively inhibit increased autophagy in LPS/D-GalN induced acute liver injury. (A and B) Western blot analysis showed that the expression of microtubule-associated protein 1 light chain 3B (LC3B)-II was significantly decreased in the LPS/D-GalN and WM co-treatment group compared with LPS/D-GalN group. (C) Immunofluorescent assay also showed that the expression of LC3B was lower in LPS/D-GalN and WM co-treatment group than that in LPS/D-GalN group (×500). NS: normal saline. $^{*}P < 0.05$, $^{**}P < 0.01$.

induction of autophagy promoted the death of hepatocytes and endothelial cells [27]. These results indicate that induction of autophagy may also aggravate liver damage.

Wortmannin is a highly cell permeable antifungal antibiotic isolated from penicillium funiculosum [28]. It can affect various signal pathways by interacting with the catalytic subunits of phosphatidylinositol 3-kinases (PI3Ks) and inhibiting its activity [28,29]. Previous work by Blommaart et al. found that PI3K could be involved in autophagic sequestration and its inhibitor wortmannin could inhibit autophagy in isolated rat hepatocytes [13]. Others also found that wortmannin could suppress hepatocyte autophagy induced by nutrient deprivation or endoplasmic reticulum stress [30].

In this research, we found that pretreatment with wortmannin may attenuate hepatocyte apoptosis and necrosis. To elucidate if the molecular basis of wortmannin-mediated protection in LPS/D-GalN induced injury is related to autophagy inhibition, we established LPS/D-GalN induced acute liver injury model in C57 mice and explored the change of autophagy at different time point. At 3 h after LPS/D-GalN treatment, the mRNA expression of pro-inflammatory cytokines *TNF-α*, *IL-1β*, *IL-6*, *MCP-1* and anti-inflammatory factor *IL-10* was tremendously increased (see Fig. S2 in the supplemental material). However, there were no obvious apoptosis, necrosis of hepatocytes and autophagy induction at this time point (Fig. S1 and S3). At 5 h after injection, hepatocytes apoptosis and necrosis were also obviously elevated (Fig. S1) along with the increase of the cytokines (Fig. S2), and autophagy was also induced to increase (Fig. S3). Furthermore, pretreatment with wortmannin could effectively inhibit increased autophagy in LPS/D-GalN induced acute liver injury (Fig. 4A–C).

LPS/D-GalN induced hepatic injury is associated with the levels of inflammatory cytokines. LPS stimulates kupffer cells to produce and release inflammatory factors. The inflammatory factors, especially TNF-α, can further induce hepatocyte apoptosis and necrosis. It has been reported that autophagy could be involved in the regulation of TNF-α secretion by macrophages and dendritic cells [31,32]. Treatment of human PBMC with 3-methyladenine (3-MA), a blocker of the Beclin-1 complex that regulates the initiation of autophagy, strongly inhibits TLR-dependent TNF-α secretion [33]. We also detected the expression of inflammatory cytokines in the liver tissues. The results showed that pro-inflammatory cytokines TNF-α, IL-1β, IL-6 and anti-inflammatory factor IL-10 were down-regulated after pretreatment with wortmannin, which indicates wortmannin could reduce the production of some inflammatory cytokines in the liver tissues, and then decrease hepatocyte apoptosis and necrosis maybe by inhibiting autophagy.

LPS activates kupffer cells and induces the production of inflammatory cytokines by TLR4-mediated and MyD88-dependent activation of MAPK (ERK, JNK and p38) and NF-κB pathways [4]. However, LPS induced autophagy is mainly dependent on TLR4-mediated, Toll-interleukin 1 receptor domain-containing adaptor-inducing interferon-β (TRIF)-dependent signal pathway, receptor interacting protein 1 (RIP1) and p38 are downstream components of this pathway [34]. In addition, it is possible that NF-κB activation by the TRIF-RIP1 pathway might be involved in LPS induced autophagy [34]. Pan et al. reported that H5N1pps significantly induced autophagy both in A549 human lung epithelial cells and in mouse lung tissue. Blocking autophagy with 3-MA (an autophagy inhibitor) or siRNA knockdown of autophagy-related genes (*Beclin 1* and *Atg5*) dramatically attenuated H5N1pp-induced pro-inflammatory cytokines and chemokines, such as IL-1β,TNF-α, IL-6, CCL2, and CCL5, both in vitro and in vivo by decreasing the activation of NF-κB and p38 MAPK signaling pathways [35]. These results indicate that autophagy-mediated the inflammatory responses could involve NF-κB and p38 MAPK signaling pathways. In the present study, we detected the change of MAPK and NF-κB signal pathways, and found that the expression phosphorylated ERK and p65 were reduced after pretreatment with wortmannin. However, there was no difference in the expression of phosphorylated p38 and JNK. Our results suggests that pretreatment with wortmannin could affect the activation of MAPK and NF-κB signal pathways, then downregulate the production of inflammatory cytokines and attenuate hepatocyte apoptosis and necrosis maybe by inhibiting autophagy. However, because wortmannin is not a specific autophagy inhibitor, it remains to be further investigated whether wortmannin-mediated protection in LPS/D-GalN induced injury is indeed related to autophagy inhibition by wortmannin.

Taken together, we found that intraperitoneal injection of LPS/D-GalN could induce upregulation of autophagy, while pretreatment with wortmannin could decrease autophagy and alleviate acute liver injury induced by LPS/D-GalN in mice. This result indicates that wortmannin plays a protective role in LPS/D-GalN induced hepatocytotoxity maybe by inhibiting autophagy and could be acted as a target for the treatment of acute liver injury.

Disclosure

The authors declare no conflict of interest, financials and otherwise.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2014.10.152.

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