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**Original Paper**

# Alkannin Inhibited Hepatic Inflammation in Diabetic Db/Db Mice

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**Key Words**
Alkannin • Liver injury • Inflammation • Rho-kinase pathway

**Abstract**
***Background/Aims:*** The current study was designed to investigate the protective role of alkannin (ALK) on liver injury in diabetic C57BL/KsJ-db/db mice and explore its potential mechanisms. ***Methods:*** An oral glucose tolerance test (OGTT) was performed. The levels of insulin, alanine aminotransferase (ALT), aspartate aminotransaminase (AST), total cholesterol (TC) and triglyceride (TG) were determined by commercial kits. The pro-inflammatory cytokines interleukin (IL)-1β, IL-6 and tumour necrosis factor (TNF)-α were determined by ELISA. The levels of the ROCK/NF-κB pathway were determined by Western blotting. ***Results:*** The contents of pro-inflammatory cytokines interleukin (IL)-1β, IL-6 and tumour necrosis factor (TNF)-α were inhibited by ALK, metformin or fasudil in diabetic db/db mice. Further, Western blotting analysis showed that the expression of Rho, ROCK1, ROCK2, p-NF-κBp65, and p-IκBα was significantly reversed by ALK treatment. In human hepatic HepG2 cells, the hepatoprotective effects of ALK were further characterized. With response to palmitic acid-challenge, increased amounts of insulin, ALT, AST, TG, and TC were observed, whereas ALK pretreatment significantly inhibited their leakage in HepG2 cells without appreciable cytotoxic effects. The inflammation condition was recovered with ALK treatment as shown by changes of IL-1β, IL-6 and TNF-α. Further, Western blotting analysis also suggested that ALK improves hepatic inflammation in a Rho-kinase pathway. ***Conclusion:*** The present study successfully investigated the role of Rho-kinase signalling in diabetic liver injury. ALK exhibited hepatoprotective effects in diabetic db/db mice, and it might act through improving hepatic inflammation through the Rho-kinase pathway.



W. Xue, Z. Fan and Y. Li contributed equally to this work.

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## Introduction

Diabetes mellitus, a metabolic disease with multiple aetiologies, is characterized by chronic hyperglycaemia resulting from disturbances of insulin secretion, insulin action or both, which has been considered a major global public health challenge [1]. Approximately 382 million people were reported as living with diabetes in 2013 worldwide, and this number is estimated to reach 592 million by 2035 [2]. Diabetes mellitus causes dysregulation in carbohydrate, fat and protein metabolism, which may lead to serious complication, including blindness, renal failure, liver injury, nerve damage, and atherosclerosis [3, 4]. Diabetes-induced liver injury has received much attention, which has been illustrated from inflammatory responses, liver fibrosis and lipid accumulation [5, 6]. Among these responses, liver inflammation is a critical mechanism, and several studies have reported that anti-inflammation agents showed efficacy in reducing blood glucose level, enhanced insulin activity and protected diabetes caused by liver injury, and it may be a potential therapeutic target for this disease [7].

The Rho protein is a member of the Ras superfamily of small monomeric GTPases, which drives several of the downstream effectors proteins, including Rho-kinase. The Rho-kinase pathway has been implicated in the pathological process of many diseases, including hypertension, heart failure and myocardial hypertrophy [8, 9]. We previously found that Rho/Rho-kinase regulates the activation of NF-κB pathways [10]. Indeed, Rho-associated kinases converge a spectrum of pathophysiological signals triggered by the diabetic milieu and have been reported as promising molecular targets for nephroprotective treatment in diabetes [11]. To our knowledge, the role of Rho-kinase signalling in the diabetic liver injury has not previously been investigated. Natural-derived medicines are generally considered less toxic and free from side effects compared with synthetic medicines. Alkannin is an active constituent isolated from the root extract of *Alkanna tinctoria*, family Boraginaceae. The Boraginaceae species, including *Arnebia euchroma, Lithospermium erythrorhizon* and *Arnebia guttata*, are widely distributed plants in China. Alkannin has been used for centuries as a natural red dye and is used in Chinese popular folk medicine for its anti-inflammatory and antitumour activities [12]. Therefore, in the present study, we investigated the hepatoprotective effects of ALK underlying the diabetic liver injury by investigating Rho-kinase signalling.

## Materials and Methods

### *Materials*

ALT was purchased from Jiangsu Youke Pharmaceutical Technology (Jiangsu, China). ALK was purchased from the Dalian Meilun Biotechnology Co., Ltd. (Dalian, China). Fasudil was obtained from the Tianjin Chase Sun Pharmaceutical. Co., Ltd. (Tianjin, China). Enzyme-linked immunosorbent assay (ELISA) kits for the determination of IL-1β, IL-6 and TNF-α were purchased from R&D. All the antibodies were provided by Cell Signalling Technology (Danvers, USA).

### *Animals*

C57BL/KsJ-db/db mice were purchased from Model Animal Research Center, Nanjing University (Nanjing, China), at 5 weeks of age and housed at 23 ± 2 °C with a 12-h light/dark cycle. Water and food were provided *ad libitum*. All the experimental procedures were performed strictly according to the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals and approved by China Pharmaceutical University (CPU.2012-003). After a 2-week adaptation period, the animals were divided into the following five groups: the wild-type mice group (WY), C57BL/KsJ-db/db (dbdb) mice group, C57BL/KsJ-db/db mice + Metformin (20 mg/kg) group, C57BL/KsJ-db/db mice + ALK (20, 40 mg/kg) group, and C57BL/KsJ-db/db mice + Fasudil (5 mg/kg) group. ALK and metformin were dissolved in distilled water and administered once daily for 14 weeks by gavage; simultaneously, fasudil was dissolved in normal saline and administered by gavage. Blood samples were collected from the carotid artery and centrifuged. The

supernatant was stored at −80 °C for biochemical indicators analysis. Next, the mice were sacrificed, and the livers were collected for the following analysis.

*Oral glucose tolerance test*

C57BL/KsJ-db/db mice were fasted overnight and orally administered with glucose at a dosage of 2 g/kg at 08:00 AM. Blood samples were obtained from the tail vein at 0, 30, 60, 90, and 120 min after glucose load. The contents of blood glucose were evaluated by using a glucose analyser (SureStep, Lifescan, Inc., Milpitas, CA).

*Measurement of biomarkers in serum and cell supernatant*

The levels of alanine aminotransferase (ALT), aspartate aminotransaminase (AST), total cholesterol (TC) and triglyceride (TG) in animal serum and HepG2 cell supernatant challenged with palmitic acid were determined by using commercial kits. The concentration of insulin in the serum and HepG2 cell supernatant induced by palmitic acid were measured with an insulin ELISA kit. All other chemicals used were of analytical grade.

*Measurement of inflammatory cytokines in serum, liver and cell supernatant*

The contents of IL-6, IL-1β and TNF-α in serum, liver tissue, and HepG2 cell supernatant induced by palmitic acid were measured using ELISA kits from R&D according to the manufacturer's instructions. The concentrations of these cytokines were quantified by reference to the standard curves. Next, the optical density (OD) of each well was read at 450 nm.

*The Histologic examination*

The studied animals were euthanized, and the livers were carefully removed, fixed with 10% neutral-buffered formalin and embedded in paraffin. Then, the samples were sectioned into 3-μm-thick slices and stained with haematoxylin–eosin. Immunostaining of in the liver sections was performed using a rabbit anti-insulin polyclonal antibody (Cell Signaling, Danvers, MA), followed by avidin–biotin peroxidase complex visualization (DAKO, Carpinteria, CA).

*Cell Culture*

HepG2 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) plus 10% foetal bovine serum and 1% antibiotics (penicillin/streptomycin). HepG2 cells were incubated in a humidified incubator consisting of 95% air and 5% CO2 at 37 °C. Three generations of HepG2 cells were passed, and then the cells were used for the experiments.

*Measurement of cell viability*

Cell viability was performed by MTT experiments. HepG2 cells were exposed to different concentrations of ALK (0, 5, 10, 20, 40, and 80 μM) for 2 h. Then, the HepG2 cells were exposed to 0.4 mM of palmitic acid (Sigma-Aldrich Corp.) for 6 h, and the cells were incubated with MTT (5 mg/ml, Sigma) solution for 4 h. After the interaction, 150 μl of dimethyl sulphoxide (DMSO) was added, and the absorbance values were determined under a 570 nm wavelength by using a microplate spectrophotometer. The data were expressed as Cell viability (%) = (A Treated/A Control) × 100%.

*Cell culture and treatment*

HepG2 cells were exposed to ALK (5, 10, and 20 μM) or fasudil (10 μM) for 2 h, and HepG2 cells were exposed to 0.4 mM of palmitic acid (Sigma-Aldrich Corp.) for 6 h, and then the cells and the cell supernatants were collected for other experiments.

*Western blotting*

The liver tissues and HepG2 cell cultures were homogenized, washed with PBS and lysed in a commercial RIPA buffer (Beyotime, Nanjing, China). After centrifugation at 12 000 rpm for 20 min, the dissolved proteins were obtained from the supernatant. The protein concentrations of different groups were measured by a BCA protein assay (Beyotime, Nanjing, China). Equal amounts of protein were loaded by 10% sodium dodecyl sulphate polyacrylamide gels (SDS-PAGE) and electrotransferred onto nitrocellulose membranes. The nitrocellulose membranes were further blocked with 5% skim milk. Next, the nitrocellulose membranes were incubated with the separate antibodies against Rho (1:1 000), ROCK1 (1:1 000), ROCK2 (1:1 000),

p-IκBα (1:1 000), IκBα (1:1 000), p-NF-κBp65 (1:1 000), NF-κBp65 (1:1 000) and GAPDH (1:1 000). After washing with TBST, the blots were incubated with horseradish peroxidase-conjugated secondary antibodies (1:10 000). The immunoreactive bands were interacted with enhanced chemiluminescence detection reagents and visualized by a gel imaging system (Tanon Science & Technology Co., Ltd., China).

*Statistical Analysis*

Data are expressed as the means ± SDs of at least three separate experiments. Statistical comparisons between the experimental groups were performed by one-way analysis of variance (ANOVA) with Tukey's multiple comparison test. A value of $P < 0.05$ was considered significant.

## Results

*Effects of ALK on oral glucose tolerance test (OGTT)*

As revealed in Fig. 1, the levels of blood glucose in the C57BL/KsJ-db/db mice group were significantly increased than those in the wild-type mice group at all time points after oral administration. The C57BL/KsJ-db/db mice treated with ALK (20 and 40 mg/kg) showed a significant elevation in blood glucose concentrations at 30 min but returned to basal levels within 2 h after the oral glucose administration, and similar results were also observed for C57BL/KsJ-db/db mice treated with metformin (20 mg/kg) or fasudil (5 mg/kg).

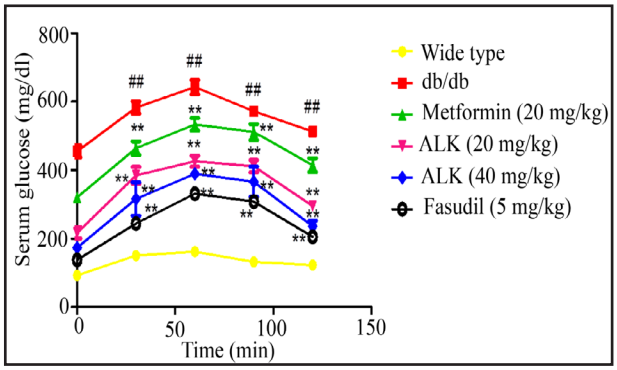


**Fig. 1.** Effects of ALK on oral glucose tolerance test (OGTT). C57BL/KsJ-db/db mice were fasted overnight and orally administered with glucose at a dosage of 2 g/kg at 08:00 AM. Blood samples were obtained from the tail vein at 0, 30, 60, 90, and 120 min after glucose load. The data are expressed as the mean values ± SDs. ##p<0.01, #P<0.05 compared with the wild-type group. **p<0.01, *P<0.05 compared with the dbdb group.

*Effects of ALK on histological examination*

Our histological examination exhibited macro vesicular steatosis in the liver tissues of the C57BL/KsJ-db/db mice group (Fig. 2), while ALK (20 and 40 mg/kg), metformin (20 mg/kg) or fasudil (5 mg/kg) treatment strikingly attenuated the extent of steatosis as observed in Fig. 2. These lipid droplets were notably reduced both in size and number in the livers of C57BL/KsJ-db/db mice treated with ALK (20 and 40 g/kg), metformin (20 mg/kg) or fasudil (5 mg/kg), suggesting that these treatments effectively inhibit lipid accumulation in the liver and showed a significant hepatoprotective effect.

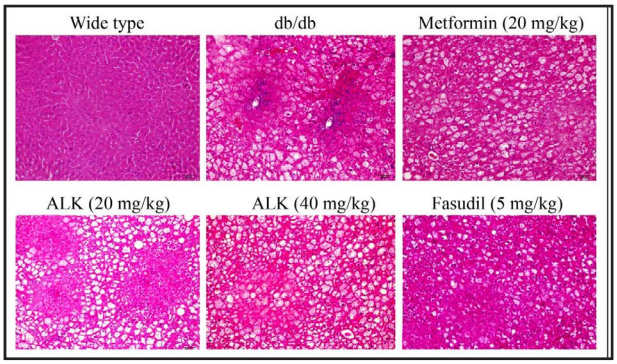


**Fig. 2.** Effects of ALK on histological examination (x200). The animals were euthanized, and the livers were carefully removed, fixed with 10% neutral-buffered formalin, embedded in paraffin and then stained with haematoxylin–eosin.

*Effect of ALK on Measurement of cell viability*

As shown in Fig. 3, palmitic acid inhibited HepG2 viability, and ALK (5, 10, and 20 μM) effectively increased HepG2 viability. Fasudil (10 μM) also effectively increased HepG2 viability.

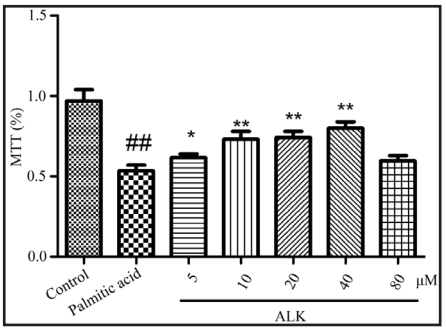


**Fig. 3.** Effect of ALK on the measurement of cell viability. HepG2 cells were exposed to different concentrations of ALK (0, 5, 10, 20, 40, and 80 μM) for 2 h. Then, the HepG2 cells were incubated with MTT (5 mg/ml, Sigma) solution for 4 h. After the interaction, 150 μl of dimethyl sulphoxide (DMSO) was added, and the absorbance values were determined. The data are expressed as the mean values ± SDs. ##p<0.01, #P<0.05 compared with the control group. **p<0.01, *P<0.05 compared with the palmitic acid group.

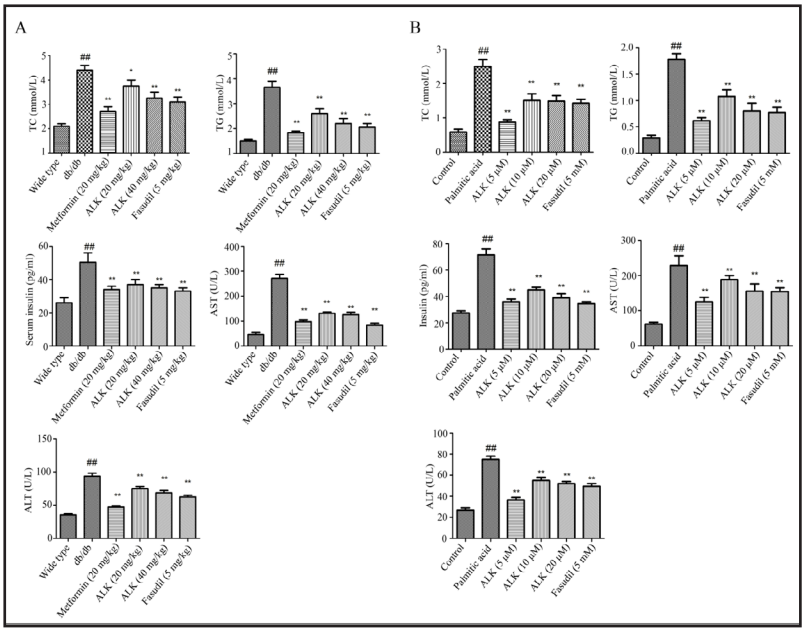


**Fig. 4.** Effect of ALK on biomarkers in serum (A) and cell supernatant (B). The levels of alanine aminotransferase (ALT), aspartate aminotransaminase (AST), total cholesterol (TC) and triglyceride (TG) in animal serum and HepG2 cell supernatant challenged by palmitic acid were determined with commercial kits. The data are expressed as the mean values ± SDs. ##p<0.01, #P<0.05 compared with the wild-type group or control group. **p<0.01, *P<0.05 compared with the dbdb group or palmitic acid group.

*Effect of ALK on biomarkers in serum and cell supernatant*

As illustrated in Fig. 4, the C57BL/KsJ-db/db mice group exhibited significantly higher serum TG and TC compared to wild-type mice. However, ALK (20 and 40 mg/kg), metformin (20 mg/kg) or fasudil (5 mg/kg) administration led to the reversal of the abovementioned biomarkers to levels similar to those of the wild-type group. The serum levels of insulin, AST and ALT in the C57BL/KsJ-db/db mice group were significantly higher than those in the control group, and ALK (20 and 40 mg/kg), metformin (20 mg/kg) or fasudil (5 mg/kg) treatment significantly reduced the serum insulin, AST and ALT compared to the model group. In HepG2 cells, in response to palmitic acid-challenge, increased amounts of insulin, ALT, AST, TG, and TC were observed, whereas ALK pretreatment significantly inhibited the leakage of these compounds from HepG2 cells (Fig. 4).

*Effects of ALK on inflammatory cytokines in serum and cell supernatant*

The inflammatory reaction is one of the major features in diabetic liver injury. To determine whether ALK could inhibit inflammatory responses during diabetic liver injury, serum levels of TNF-α, IL-6 and IL-1β were assessed. Significant increases in the serum levels of TNF-α, IL-6 and IL-1β were observed in the C57BL/KsJ-db/db mice group. In this regard, the serum levels of TNF-α, IL-6 and IL-1β contents were effectively decreased in the ALK (20, 40 mg/kg), metformin (20 mg/kg) or fasudil (5 mg/kg) treatment groups compared with those in model mice. In palmitic acid-treated HepG2 cells, the levels of TNF-α, IL-6 and

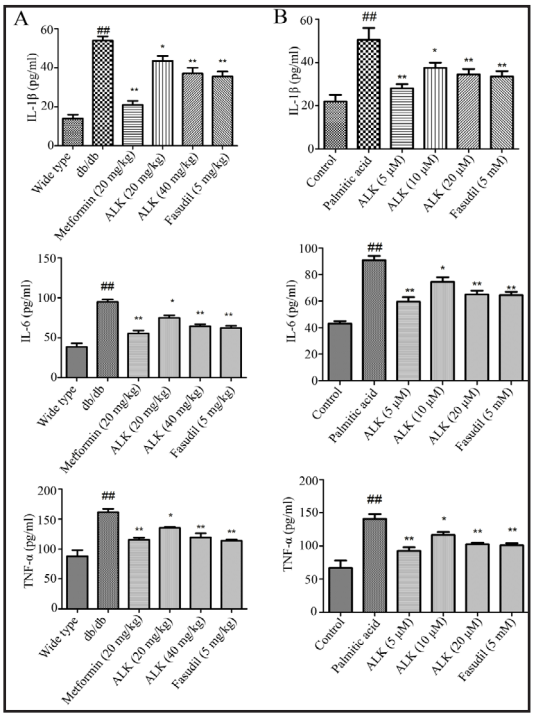


**Fig. 5.** Effects of ALK on inflammatory cytokines in serum (A) and cell supernatant (B). The contents of IL-6 IL-1β and TNF-α in serum, liver tissue, and HepG2 cell supernatant induced by palmitic acid were measured using ELISA kits according to the manufacturer's instructions. The data are expressed as the mean values ± SDs. ##p<0.01, #P<0.05 compared with the wild-type group or control group. **p<0.01, *P<0.05 compared with the dbdb group or palmitic acid group.

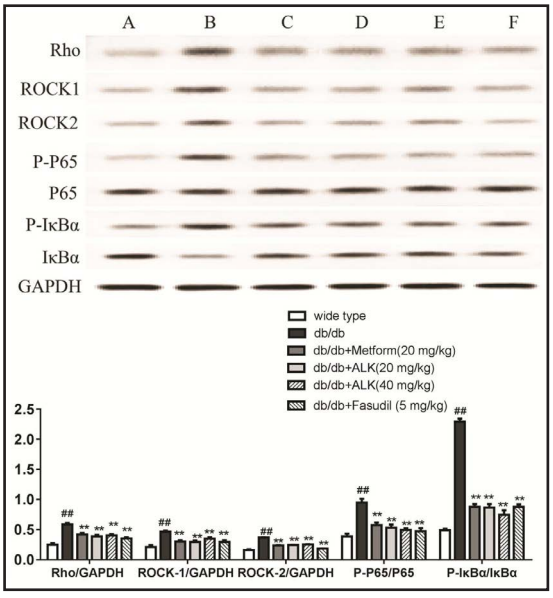


**Fig. 6.** Effects of ALK on the Rho Kinase pathway in mice. The levels of Rho Kinase pathway proteins in liver tissue were measured using Western blotting according to the manufacturer's instructions. A: wild type; B: dbdb; C: Metformin (20 mg/kg); D: ALK (20 mg/kg); E:ALK (40 mg/kg) F: Fasudil (5 mg/kg).

IL-1β were obviously increased compared with the control. The ALK and ALK + fasudil treatments led to a reversal of these inflammatory cytokines to levels similar to those of the control group (Fig. 5).

*Effects of ALK on Rho Kinase Pathway*

As shown in Figs. 6 and 7, the Rho kinase pathway participates in regulating the inflammatory cascades. The expression of Rho, ROCK1 and ROCK2 was significantly up-regulated in liver tissues of C57BL/KsJ-db/db mice. However, treatment with ALK (20 and 40 mg/kg), metformin (20 mg/kg) or fasudil (5 mg/kg) obviously ameliorated these situations. The obtained results demonstrated that ALK (20 and 40 mg/kg) successfully blocked the expression of Rho, ROCK1 and ROCK2. To elucidate the downstream mechanism of ALK in diabetic liver injury in C57BL/KsJ-db/db mice, the phosphorylation of IκB and NF-κB and the expression of Ikkα and Ikkβ were detected. The protein levels of p-IκB and p-NF-κB were unregulated in the C57BL/KsJ-db/db mice group compared with those in the wild-type control group, while ALK (20 and 40 mg/kg) exerted an obviously suppressive effect on diabet-

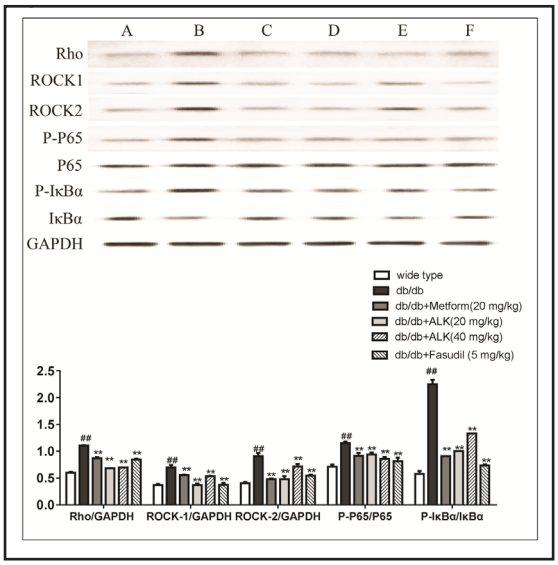


**Fig. 7.** Effects of ALK on the Rho Kinase pathway in HepG2 cells. The levels of Rho Kinase pathway proteins in HepG2 were measured using Western blotting according to the manufacturer's instructions. A: Control; B: palmitic acid; C: ALK (5 μM); D: ALK (10 μM); E:ALK (20 μM) F: Fasudil (10 μM).

ic-induced phosphorylated IκB and NF-κB. Similarly, metformin (20 mg/kg) or fasudil (5 mg/kg) administration was also demonstrated to have similar inhibitory effects on NF-κB signalling. These results showed that the inhibition of ROCK1 prevented the activation of NF-κB activation. *In vitro* experiments showed that the expression of p-IκB, p-NF-κB, p-Ikkα and p-Ikkβ to B, and NF-κB were significantly increased in palmitic acid-treated HepG2 cells compared with in the control group. However, the ALK and ALK + fasudil treatment strikingly reversed these alterations (Figs. 6 and 7).

## Discussion

Diabetes mellitus is characterized by hyperglycaemia, a leakage of insulin action, insulin resistance, and the progression of diabetic pathology in the retina, renal glomerulus, and nerve [13]. Diabetes also contributed to the accelerated atherosclerotic diseases affecting arteries supporting the heart, brain, and lower extremities [14]. In addition, diabetic liver injury is also a serious diabetic complication in our modern-day society. Diabetes and insulin resistance were also identified as important factors in patients with diabetic liver injury. In recent years, there has been an increasing amount of literature on diabetic complications, including metabolism of lipids. Several studies have revealed that diabetes with hyperlipidaemia may induce atherosclerosis, coronary heart disease and cerebrovascular disease. Total cholesterol (TC) and triglyceride (TG) are synthesized by the liver and are closely related to lipid metabolism. In the present study, oral glucose tolerance test performance, the level of insulin, ALT, AST, TC and TG were abnormal in diabetic C57BL/KsJ-db/db mice.

Epidemiological studies showed that diabetic patients are at increased risk of chronic liver disease and hepatocellular carcinoma [15]. In addition, type 1 diabetes is related to higher risk of chronic liver injury, but the underlying mechanisms remain largely unknown [16]. During the development of type 2 diabetes, the insulin resistance-associated inflammation is an important mechanism associated with the pathogenesis of chronic liver disease [17, 18]. In the present study, we investigated the precise mechanism in hepatic inflammation by applying diabetic C57BL/KsJ-db/db mice, a well-established type 2 diabetic animal model, through the Rho-kinase pathway.

The elevated levels of blood glucose and insulin confirmed that the diabetic model has been well established, while the ALK treatment decreased the contents of blood glucose and insulin, indicating that ALK exerted the protective effect against diabetes. Additionally, the histopathological observation of liver sections revealed liver inflammation, whereas ALK obviously attenuated these alterations. Taken together, the present results suggested that ALK could prevent diabetes.

Inflammatory cytokines, including IL-6, IL-1β and TNF-α, have been involved in the development of diabetes nephropathy. IL-1β is also implicated in the progression of irregularities in intraglomerular haemodynamics related to prostaglandin synthesis [19].. IL-6 increases fibronectin level, which hastens mesangial cell proliferation, disturbs extracellular matrix dynamics and increases endothelial permeability. TNF-α is cytotoxic to liver cells. TNF-α also induces direct liver damage through the generation of reactive free radicals. Moreover, several studies have described the role of ALK in inhibiting the reaction of inflammation, such as decreased the level of TNF-α and IL-1β on microglial inflammatory [20] and significantly reduced inflammation (mouse paw oedema induced by FCA) by ALK [21]. The data suggested that ALK significantly reduced the contents of inflammatory cytokines in the serum and liver tissues of diabetic mice.

Rho-kinase (ROCK), a serine/threonine protein kinase, is present in two isoforms, including ROCK1 and ROCK2. Rho-kinase is stimulated by RhoA, belonging to the Rho family of small G-proteins [19]. The Rho-kinase pathway has been implicated in the pathological process of many diseases, including hypertension, heart failure and myocardial hypertrophy [22, 23]. Indeed, Rho-associated kinases converge a spectrum of pathophysiological signals triggered by the diabetic milieu and have been reported as promising molecular targets for nephroprotective treatment in diabetes [11]. Previous studies have suggested that the expression of Rho-kinase can be selectively blocked by its competitive inhibitors. Treatment with ROCK inhibitors improved type 2 diabetes in animal models [24]. The up-regulated Rho-kinase pathway was also reported in diabetic db/db mice, and the ROCK inhibitor was suggested to improve the diabetic complications [25]. To our knowledge, the role of Rho-kinase signalling in the diabetic liver injury has not previously been investigated. Thus, in the present study, we investigated the role of Rho-kinase signalling in diabetic liver injury by applying type 2 diabetic db/db mice. The results showed that ALK (20 and 40 mg/kg), metformin (20 mg/kg), and fasudil (5 mg/kg) successfully blocked the expression of Rho, ROCK1 and ROCK2 and inhibited Rho kinase signalling. A similar situation was also observed in a HepG2 cell line challenged with palmitic acid.

As the downstream molecule of ROCK, NF-κB signalling plays a major role during the mediation of inflammatory progression in type 2 diabetes. The activation of NF-κB is triggered by the phosphorylation and degradation of the IκBα. As inhibitors of NF-κB, the activation of IκBα was regulated by IκB kinases (IKKs). IKK complex consisted of IKK-α and IKK-β [26]. NF-κB governs the mediation of inflammatory cytokine production and affects the production of IL-1β, IL-6 and TNF-α. Evidence has indicated that the Rho/ROCK/NF-κB pathway was involved in experimental diabetic animal models [27]. We previously found that Rho/Rho-kinase regulates the activation of NF-κB pathways. In the present study, ALK (20 and 40 mg/kg), metformin (20 mg/kg), and fasudil (5 mg/kg) significantly inhibited the activation of Rho/ROCK/NF-κB signalling in C57BL/KsJ-db/db mice. Thus, the present results confirmed that ALK (20 and 40 mg/kg), metformin (20 mg/kg), and fasudil (5 mg/kg) successfully blocked the expression of ROCK and the inhibition of ROCK1 and ROCK2, which might be conducive to the suppression of NF-κB activation.

In conclusion, the present study successfully investigated the role of Rho-kinase signalling in diabetic liver injury and characterized the potential mechanism of Rho/ROCK/NF-κB induced inflammatory pathogenesis. ALK (20 and 40 mg/kg) exhibited hepatoprotective effects in diabetic db/db mice, and this chemical might act through decreasing hepatic inflammation through inhibition of the Rho-kinase pathway.

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## Disclosure Statement

All authors have no conflicts of interest to disclose.

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