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PROTECTIVE EFFECT OF WY14643 IN HEPATIC ISCHEMIA/REPERFUSION INJURY VIA SUPPRESSING THE ACTIVATION OF TLR4/NF-KB P65 SIGNAL PATHWAY.

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Abstract

Background: Hepatic ischemia/reperfusion (I/R) injury is a disaster common critical event which frequently occurs in a variety of clinical scenarios. To investigate the protective effect of Wy14643 (WY) precondition against hepatic ischemia/ reperfusion (I/R) injury in rats and its potential mechanism.

Methods: Thirty Sprague–Dawley male rats weighing 220-250 g were randomly divided into three groups (n=10) including the sham-operated +saline group (Sham), the ischemic-reperfusion+ vehicle (IRI), and the WY14643 preconditioning group (WY). The three groups were pretreated with saline, ethanol and WY, at 1 h before ischemia with a concentration of 10 mg/kg , respectively. Hepatic ischemia-reperfusion (I/R) was induced by clamping blood supply to the left lateral and median lobes of the liver for 90 min. Blood samples and liver tissues were obtained at the end of 4h reperfusion. The serum levels of alanine aminotransferase (ALT), aspartate aminotransferase (AST) and lactate dehydrogenase (LDH) were measured. The histological changes of hepatic tissues were examanied by HE staining. The expression of interleukin-1β (IL-1β), interleukin-6 (IL-6) and tumor necrosis factor alpha (TNF-α) were detected by ELISA and RT-PCR. Moreover, the expression of TLR-4 , IκB-α, Myd88, p65 and PPAR-α were detected by western blotting.

Result: After 4 h reperfusion, compared with the IRI group, the liver dysfunction (ALT, AST and LDH), damage score, the expression of TNF-α , IL-6, and IL-1β, the protein expression level of TLR4, Myd88, the phosphorylation level of IκB-α and p65 in the WY group was significantly decreased (all $P<0.05$); while the protein expression of IκB-α was increased ($P<0.05$) with no difference in the expression of p65 ($P>0.05$) .

Conclusion: Wy14643 precondition could protect liver againest I/R injury and the protective effect is associated with suppressing inflammation by reducing the activation of TLR-4/NF-κB signaling.

Key words: hepatic ischemia reperfusion injury; inflammation; Wy14643; TLR-4/NF-κB

Introduction

Hepatic ischemia/reperfusion (I/R) injury is a disaster common critical event which frequently occurs in a variety of clinical scenarios such as liver resection, transplantation, trauma, shock, and so on (Guan LY , 2014). The pathogenesis of hepatic ischemia reperfusion injury is complicated and studies have shown that unconstrained inflammation response, necrosis and apoptosis were involved in liver injury (Datta G, 2015). Especially, unconstrained inflammation response is generally acknowledged as a key mechanism of liver ischemia-reperfusion injury (Abu-Amara M, 2010). This indicated that inhibiting inflammatory response may be an effective therapeutic intervention to reduce hepatic ischemia/reperfusion (I/R) injury.

The inflammatory response can be regulated by the Toll-like receptors4 (TLR-4) which has a Toll-interleukin 1 receptor (TIR) and can be activated by ischemia stimulation (Schmalz G, 2011; Salomão R, 2008). Upon activation, TLR-4 will enlist TIR domain-containing adaptor proteins such as Myeloid differentiation factor 88 (MyD88), TRIF-related adaptor molecule(TRAM) and TIR-domain-containing

adapter-inducing interferon-β (TRIF), then conducting the immune signals to nuclear factor-κB(NF-κB) (Schmalz G, 2011; Salomão R, 2008). As one of the most important nuclear transcription factors, NF-κB is involved in the regulating inflammatory responses (Porzionato A, 2013) inactivated, NF-κB is interacted with IκB-α proteins and kept in the cytoplasm of cell . However, when cells receive various pathological stimuli, such as TLR4-mediated immune signaling, IκB-α proteins could be activated by phosphorylation and degradation when then lead to NF-κB activation [6] and NF-κB activation is dependent on its subunit p65 activation. The p65 activation will induce the expression of various pro-inflammatory cytokines, such as IL-6, TNF-α and IL-1β (Porzionato A , 2013) . Therefore, suppressing TLR4/NF-κBp65 pathway may be a promising target for decreasing hepatic ischemia reperfusion injury.

Peroxisome proliferator-activated receptor-α (PPAR-α) is one of the three subunit of the nuclear receptor PPAR family and activating PPAR-α have been demonstrated that could attenuate TLR-4 medicating inflammation (Chen T, 2015). In the present study, we explored whether PPAR-α activation by the selective agonist Wy14643 may reduce hepatic I/R injury in rats by suppressing TLR-4/NF-κBp65 mediating signaling or not.

Materials and Methods

Chemicals

Wy14643 was purchased from Sigma (USA) and solved by ethanol (sigma, USA) at a dose of 10 mg/kg.

Animals

Male Sprague-Dawley rats (220-250 g, 6-8 weeks) were purchased from Hua Fukang Experimental Animal Center, Beijing, China, and housed in a laminar flow, specific pathogen free atmosphere. Animal protocols were approved by Sichuan Provincial People's Hospital Animal Care Committee and the experiments were performed in adherence to the guidelines provided by the National Institutes of Health for the use of animals in laboratory experiments. After a minimum 7 days of acclimation, the rats were randomly allocated into three groups with each containing 10 rats: (1) I/R-ethanol group (IRI), in which the rats were subjected to ischemia for 90 min and pretreated with ethanol(10mg/kg, IP) at 1 h prior to I/R induction; (2) I/R- Wy14643 group (WY), in which the rats were administered Wy (10mg/kg, IP) at 1 h prior to I/R induction; and (3) sham-operated group (Sham), the rats were pretreated with saline (10mg/kg, IP) at1 h prior to Sham operation. The dose of Wy14643 was according to previous study (Xu SQ, 2008).

Warm Hepatic I/R Model

Partial (70%) warm ischemia was performed as previously described (Xu SQ, 2008). In brief, a microvascular clamp was used to occlude the portal triad (hepatic artery, portal vein, and bile duct) to the left and median liver lobes for 90 min to introduce partial warm ischemia. Sham group underwent the same procedure without vascular occlusion. Reperfusion was initiated by removal of the clamp for 4h. The rectal temperature was maintained at 37^0C throughout the surgery by a warming pad.

Liver Damage Assessment

To assess hepatic function and cellular injury following liver ischemia, serum aspartate aminotransferase (AST), alanine aminotransferase (ALT) and lactic acid dehydrogenase (LDH) levels were measured in blood samples obtained at predetermined time points 4 h after reperfusion with a standard automatic analyzer.

Histopathology

Following sacrifice, paraffin embedded liver tissue was cut into 5–7 μm sections, stained with hematoxylin and eosin and examined by two independent, blinded investigators using semi-quantitative point counting system. Briefly, 100 adjacent fields were scored per slide at 200×

magnification to determine the percentage of necrosis using an ordinal scale, with grade 0 equaling no evidence of injury, grade 1 indicating mild injury consisting of cytoplasmic vacuolation and focal nuclear pyknosis, grade 2 indicating moderate to severe injury with extensive nuclear pyknosis, and grade 3 indicating severe necrosis with disintegration of hepatic cords, hemorrhage and neutrophil infiltration (Xu SQ, 2008) .

Western Blot Analysis

Samples of 100 mg liver tissue were homogenized in 2 ml lysate (20 mmol/L phosphate buffer, pH 7.5, 10 mmol/L phosphoglyceraldehyde, 2 mmol/L pyrophosphoric acid, 1 mmol/L sodium fluoride, 1 mmol/L PMSF, 1 g/m leupeptin, 1 mmol/L DTT, 1 mmol/L edetic acid) at 4 ^{o}C and centrifuged at 500 rpm for 1 minute to remove cell debris. Some supernatants were taken out and stored at -80 ^{o}CThe total protein concentration of supernatant was assayed by the Lowry method. Seventy micrograms of protein was subjected to SDS-PAGE before its transfer to nitrocellulose membrane. The membranes were blocked with 5% non-fat dried milk and 1% BSA, then incubated with mouse anti-rat monoclonal antibody specific for p-PPAR-α (Abclonal, USA, 1:500), PPAR-α (CST, USA, 1:1000), p-IκB-α(CST, USA, 1:500) , TLR-4(CST, USA, 1:500), Myd88(CST, USA, 1:500), p- p65(CST, USA, 1:500), p65(CST, USA, 1:1000), and β-actin(Abgent, 1□3000) at 4 □ overnight, washed three times in TBST (50 mmol/L Tris HCl, pH 7.5, 150 mmol/l NaCl, 0.01% Tween 20) and probed with the HRP-conjugated goat anti-rabbit IgG(1:3000) antibody. The signals were assessed with the intensive chemoluminescence kit according to the product instructions. The relative density was quantified with Scion Image software.

ELISA Analysis

Levels of the inflammatory mediators (IL-6, TNF-α and IL-1β) in the serum were quantified using specific ELISA kits for rats according to the manufacturer's instructions (Biosource International Inc, USA).

Real-Time PCR Analysis

Total RNA was isolated from liver tissues using Trizol according to the manufacturer's instructions (Takara, Japan). Four micrograms of total RNA were reverse transcribed into cDNA using the PrimeScript RT Master Mix (Takara, Japan) as instructed. Real-time PCR amplifications were carried out using the ABI 7500 system (Applied Biosystems, USA). PCR primers (Invitrogen, USA) for all analyzed genes are shown in Table 1. PCR was conducted at 95°C for 30 sec, followed by 40 cycles at 95°C for 5sec, 60°C for 34 sec and 95°C for 15 sec. The amount of mRNA for each gene was normalized by β-actin, and the relative expression levels were calculated using the $2^{-\Delta\Delta Ct}$ method as reported (Xu Z, 2014; Zhang S, 2012).

Statistical Analysis

All results are expressed as mean ± SEM. Group comparisons were performed using the Student's *t*-test or analysis of variance and Survival analysis of the experiments. In all cases, *P* value < 0.05 was considered with statistical significance.

Results

Wy14643 Pretreatment Attenuated Hepatic Ischemia Perfusion Injury

Liver function was examined by measuring serum levels of ALT, AST and LDH. Compared with the Sham group, I/R resulted in the rapid increase of ALT, AST and LDH at 4 h after reperfusion as manifested higher level of ALT, AST and LDH in IRI group than Sham group (Fig. 1). Pretreatment with Wy14643 can significantly attenuate I/R-induced liver injury as demonstrated less level of ALT, AST and LDH in WY group. This indicated pretreatment with Wy14643 can significantly decrease hepatic ischemia reperfusion injury.

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Table 1: Primers used for real-time PCR analysis

Gene	Species	Sense strand sequence	Anti-sense strand sequence
IL-6	rat	CTGAACTTCGGGGTGATCGG	GGCTTGTCACTCGAATTTTGAGA
TNF-α	rat	AGCTTCCTTGTGCAAGTGTCT	GACAGCCCAGGTCAAAGGTT
IL-1β	rat	CTGCAAGAGACTTCCATCCAG	AGTGGTATAGACAGGTCTGTTGG
β-actin	rat	AGAGGGAAATCGTGCGTGAC	CAATAGTGATGACCTGGCCGT

Wy14643 Pretreatment Alleviated Liver Pathological Changes in Liver I/R Injury

Compared to the sham group, the liver tissue in IRI group has more marked ballooning degeneration, cellular swelling, vacuolar degeneration, multiple inflammatory cell infiltration (Fig. 2A). Pretreatment with Wy14643 resulted in a significant amelioration of hepatic injury as manifested less liver ballooning degeneration, cellular swelling, vacuolar degeneration, multiple inflammatory and inflammatory cell infiltration. Semi-quantitative assessment of the histological lesions showed a significantly higher score in the I/R-treated rats than the Wy14643 -treated rats and saline-treated rats at 4 h of reperfusion (Fig.2B)

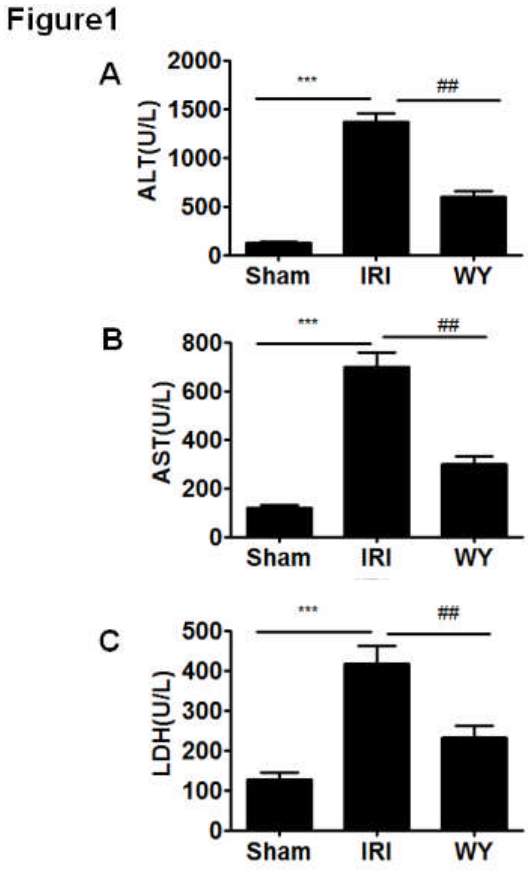

Figure 1: WY14643 precondition ameliorates IR-induced liver injury. A: Serum levels of alanine aminotransferase (ALT) ; B : Serum levels of aspartate aminotransferase (AST) ; C: Serum levels for Lactate dehydrogenase(LDH) Rats preconditioned with WY14643 displayed significantly lower levels for ALT and AST *** $P < 0.001$ (IRI vs. Sham); $^{\#\#}P < 0.01$ (IRI vs. WY).

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Wy14643 further promoted IR-induced PPAR-α activation

As the next step to explore the potential mechanisms of Wy14643 attenuating hepatic ischemia reperfusion injury, we examined the expression of PPAR-α as Wy14643 is a ligand for PPAR-α. Both IR insult and Wy14643 precondition have no influence on the expression of PPAR-α, but have influence on the expression of p-PPAR-α. IR insult can increase the expression of p-PPAR-α and pretreatment with Wy14643 can futher induce the expression of p-PPAR-α. These result indicated that Wy14643 can futher induce the PPAR-α activation in **liver I/R injury** (Figure 3).

Wy14643 decreased liver I/R-induced inflammatory cytokines production

Pro-inflammatory cytokines, such as IL-6, TNF-α and IL-1β play an important role in the I/R induced liver injury (Abu-Amara M, 2010). Compared with the sham group, the IRI group have higher expression level of IL-6 (Fig. 4A, D) TNF-α (Fig. 4B, E), IL-1β (Fig. 4C, F). Howerver, pretreatment with Wy14643 can significantly reduce the expression of IL-6, TNF-α and IL-1β as manifested less level of IL-6, TNF-α and IL-1β in WY group than IRI group.

Wy14643 suppressed the activation of the TLR4/NF-κBp65 pathway in liver I/R injured rats.

It has been demonstrated that TLR4 can activate p65, which then induce the production of downstream-associated pro-inflammatory

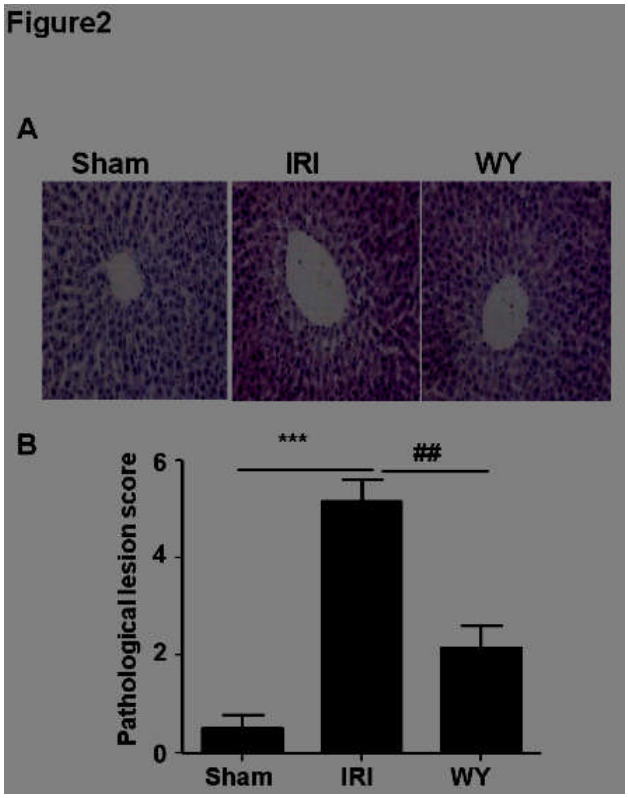

Figure 2: Histological analysis of liver tissues. Representative HE staining results for liver sections originated from: A. Sham rat; ischemia-reperfusion injury-saline treatment (IRI) rat; and IRI- WY14643 rat; B : Semi-quantitative analysis of HE staining for all rats included. Three rats were analyzed in each group, and the images are presented at 200× magnification. $^{***}P < 0.001$ (IRI vs. Sham); $^{\#\#}P < 0.01$ (IRI vs. WY).

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Figure3

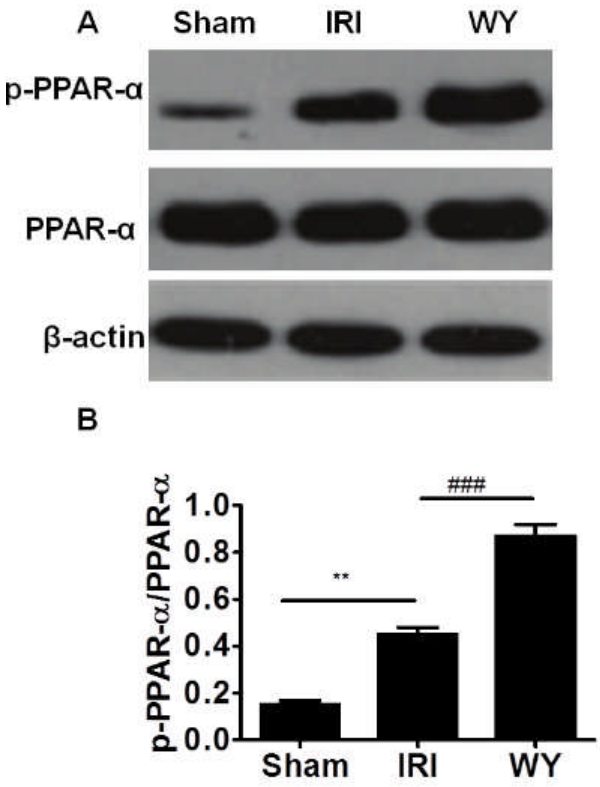

Figure3. Pretreatment with WY14643 promotes PPAR-α activation. Western blot analysis was employed to assess PPAR-α expression and activation by measuring the levels of total PPAR-α and p- PPAR-α. A. A representative result for Western blot analysis. B. Semi-quantitative analysis of ten animals studied in each group. The relative amounts of PPAR-α and p- PPAR-α in each group of rats were normalized by β-actin and presented as a ratio between p- PPAR-α and PPAR-α . $^{**}P < 0.01$ (IRI vs. Sham); $^{###}P < 0.001$ (WY vs. IRI).

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Figure4

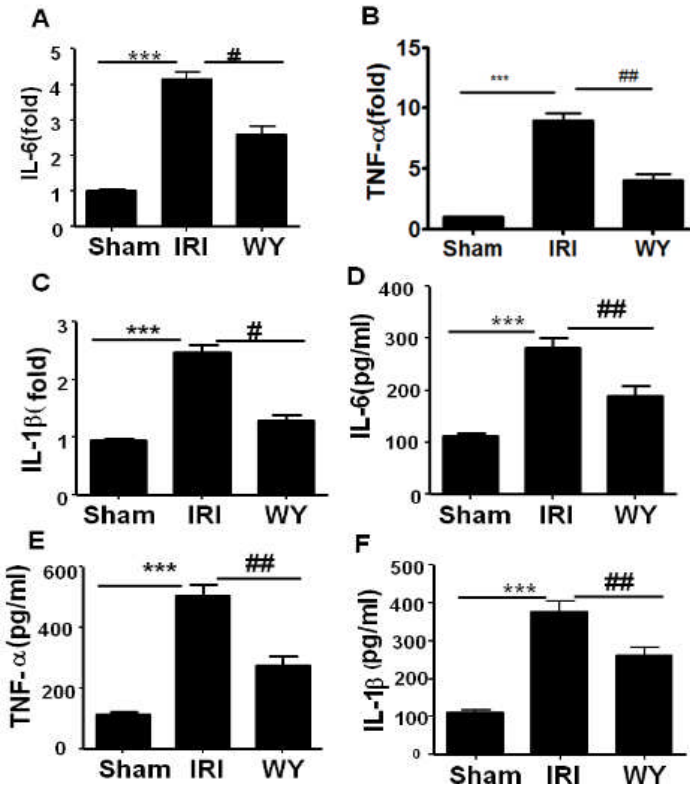

Figure 4: WY14643 treatment suppressed cytokine levels expression in the liver tissue and serum after hepatic ischemia-reperfusion injury (IRI). RT-PCR and ELISA was employed to assess the expression of inflammatory cytokines in the liver tissue and serum, respectively. We selectively analyzed the expression levels for interleukin-6 (IL-6) (A, D) tumor necrosis factor-α (TNF-α), (B, E), and interleukin-1β (IL-1β) (C, F) after IRI. $^{***}P < 0.001$ (IRI vs. Sham); $^{\#\#}P < 0.01$ (IRI vs. WY); $^{\#}P < 0.05$ (IRI vs. WY).

Cytokines (Schmalz G, 2011; Salomão R, 2008; Porzionato A , 2013). To explore the anti-inflammatory mechanism of Wy14643, we examined its effect on the TLR4/NF-κBp65 signaling pathway in ischemic liver tissues. Compared with the Sham group, the expression of TLR4, Myd88, the phosphorylation level of IκB-α and p65 were significantly increased in the IRI group accompanied with the decreasing the expression of IκB-α.However, Wy14643 treatment can significantly attenuate the trend as manifested less level of TLR4, Myd88, p- IκB-α and p-p65 with higher level of IκB-α in the WY group than IRI group (Figure5). Moreover, there is no difference on the expression of p65 among in three groups. These result indicated that the anti-inflammatory effect of Wy14643 precondition is associated with suppressing TLR4/NF-κBp65 signaling in liver I/R injured rats.

Discussion

WY14643 is one of selective agonist of peroxisome proliferator-activated receptor-α and has been demonstrated with anti-infammatory effect in various settings (Chen T, 2015) , but the impact of WY14643 on liver IRI has not been fully investigated. We induced IRI in rats by blocking the portal triad (hepatic artery, portal vein, and bile duct) to the left and median liver lobes for 90 min, and then subjecting the liver to 4 h of reperfusion. Histological analyses demonstrated significant pathological changes in the IRI group manifested by marked ballooning degeneration, cellular swelling, vacuolar degeneration, multiple inflammatory cell infiltration, as well as punctiform, focused and piece-meal necrosis.

Precondition of rats with 10mg /kg of WY14643 can significantly reduce those pathological and serum changes associated with liver IRI. In line with our results, previous studies suggested that treatment with WY14643 significantly decreased pathological and serum changes induced by hepatic IRI (Xu SQ, 2008). Our data indicated that WY14643 might be a new thereapy choice to prevent liver IRI in clinical practice.

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To determine the molecular mechanisms by which WY14643 decrease liver IRI in rats, we first examined the influence of WY14643 on the expression of PPAR-α and p-PPAR-α.The results showed that both IRI and WY14643 have no influence on the expression of PPAR-α, but have influence on the activation of PPAR-α as manifested compared to the Sham group, the expression of p-PPAR-α in IRI group was increased and pretreatment with WY14643 can further increase the expression of p-PPAR-α as shown higher levels of p-PPAR-α in WY group than IRI groups. These results WY14643 provide protection against liver IRI by further activating PPAR-α.

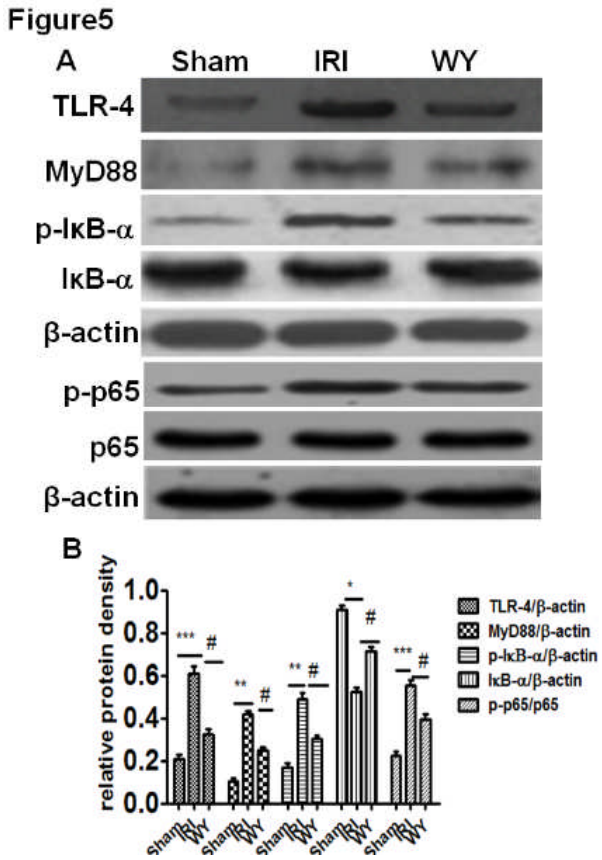

Figure 5: WY14643 suppressed TLR-4/NF-κB signaling activation A: A representative result for Western blot analysis TLR-4, Myd88, p-IκB-α , IκB-α, p-p65 and p65.B. Semi-quantitative analysis of rats studied in each group. B: The relative amount of TLR-4, Myd88, p-IκB-α , IκB-α, p-p65 and p65 was normalized by β-actin. $^{***}P$<0.001(IRI vs. Sham); $^{**}P$<0.01(IRI vs. Sham); $^{*}P$<0.05(IRI vs. Sham);$^{\#}P$<0.05 (WY vs. IRI);

It is reported that PPAR-α activation can reduce IRI in myocardial and cerebral by suppressing inflammation(Yeh CH,2006; Collino M, 2006).Therefore, we exanimed the expression of Pro-inflammatory cytokines, such as IL-6, TNF-α and IL-1β in liver IRI. The result showed that IRI can induce the expression of IL-6, TNF-α and IL-1β as manifested higher level of IL-6, TNF-α and IL-1β in IRI groups than Sham groups. But pretreatment with WY14643 can significantly attenuate the expression of pro-inflammatory cytokines as manifested lower level of IL-6, TNF-α and IL-1β in WY group than IRI group. This implies that PPAR-α activation attenuates IRI in liver though reducing the expression of pro-inflammatory cytokines. In order to asertain the mechanism that PPAR-α activation deceased hepatic IR injury, therefore we explore the effect of PPA-α activation in TLR-4/NF-κBp65 signaling pathway. The reason is that the study showed TLR4 can induce p65 activation, which arouse downstream-associated pro-inflammatory cytokines such as TNF-α, IL-1β and IL-6 that in turn induce inflammation response (Schmalz G, 2011; Salomão R, 2008)and PPAR-α activation can regulate the activation of TLR-4(Yessoufou A, 2009).In ou study, we found that the IR insult can induce TLR-4/NF-κBp65 signaling pathways activation as manifested higher level of TLR4, Myd88, p-IκB-α and p-p65 with the lower level of IκB-α in IRI group than Sham group. However, WY14643 pretreatment can significantly suppress the activation of TLR4/NF-κB signaling induced by liver. I/R injury as manifested lower level of TLR4, Myd88, p-IκB-α and p-p65 with higher level of IκB-α in WY group than IRI group. These results indicated that WY14643 pretreatment can suppress TLR4/NF-κB pathway activation by further promoting PPA-α activation.

It is worth noting that pretreatment with WY14643 decreasing hepatic ischemia may involve in more other pathways than TLR-4/NF-κB. It will be necessary to explore other pathway for fully elucidating the protective mechanism of WY14643 in hepatic I/R injury.

In summary, we demonstrated that pretreatment with Wy14643 can attenuate hepatic IRI as manifested less pathological and serum changes in IRI and the protective mechanism was associated with suppressing TLR-4/NF-κBp65 mediating infammation by further promoting PPAR-α activation. Our study indicated that WY14643 may be an effective therapy for decrase hepatic IRI in clinical practice.

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Disclosure of Conflicts of Interest: The authors declare that they have no competing interests.

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