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Purinergic P2X7 receptor mediates acetaldehyde-induced hepatic stellate cells activation via PKC-dependent GSK3β pathway

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ABSTRACT

The activation of hepatic stellate cells (HSCs) is an essential part in the development of alcoholic liver fibrosis (ALF). In this study, stimulated HSCs with 200 μM acetaldehyde for 48 h was used to imitate alcoholic liver fibrosis *in vitro*. The western blot and qRT-PCR results showed that P2X7R expression was significantly increased in the activation of HSCs after acetaldehyde treatment. Interestingly, activation of P2X7R by stimulating with P2X7R agonist BzATP significantly promoted acetaldehyde-induced CyclinD1 expression, cell proportion in S phase, inflammatory response, and the protein and mRNA levels of α-SMA, collagen I. In contrast, blockage of P2X7R by stimulating with the inhibitor A438079 or transfecting with specific siRNA dramatically suppressed acetaldehyde-induced HSCs activation. Furthermore, PKC activation treated with PMA could obviously up-regulate the expression of α-SMA and collagen I and the phosphorylation of GSK3β, while inhibition of PKC significantly reduced GSK3β activation. Moreover, GSK3β inhibition harvested a dramatic decrease of the mRNA and protein levels of α-SMA and collagen I by suppressing GSK3β phosphorylation. Taken together, these results suggested that purinergic P2X7R mediated acetaldehyde-induced activation of HSCs via PKC-dependent GSK3β pathway, which maybe a novel target for limiting HSCs activation.

1. Introduction

Based on the global status report, 9% of all deaths worldwide (approximately 3.3 million deaths), happened in adults, were associated with alcohol addictive consumption [1]. The major spectrum of alcoholic liver disease (ALD), which is induced by long-term and heavy alcohol drinking, includes steatosis (fatty liver), steatohepatitis (alcoholic hepatitis), fibrosis, cirrhosis, and hepatocellular carcinoma (HCC) [2,3]. Steatohepatitis is the early phase of ALD and can be further deteriorated into alcoholic liver fibrosis (ALF), which is regarding as a breakthrough point in the development of ALD [4,5]. Unlike previous views that the extensive liver fibrosis is considered to be irreversible, recent studies suggested that even advanced liver fibrosis is reversible [6], however, the mechanisms remain unknown. Therefore, the research on the molecular pathogenesis pathways and potential therapeutic strategies for ALF has captured growing attention in academic circles.

Despite the pathogenesis of ALF is very intricate involving various molecular and biological mechanisms, a multitude of evidence illustrates that acetaldehyde, serving as the major metabolite of ethanol, plays an essential role in the onset and progression of liver fibrogenesis [6]. Hepatic stellate cells (HSCs) is well known to play a key role in the pathogenesis of alcoholic liver fibrosis [7]. Many studies have indicated that acetaldehyde, the principal metabolite of ethanol, promoted type I collagen production [8] and deposition of extracellular matrix (ECM) by activating HSCs, which were the critical cell type involved in the progression of alcoholic liver fibrosis [7,9]. Furthermore, mounting studies reported that HSCs treated with 200 μM acetaldehyde for 48 h were closely simulated with activation of HSCs *in vivo* [10–12]. However, the molecular mechanisms, the complicated association between acetaldehyde, HSCs activation and collagen synthesis, remain unclear and need further exploration.

Recently, purinergic receptors, involved in physiology and pathophysiology of the liver, have attracted lots of attention on its important roles in liver fibrotic diseases [13]. P2X7 receptor is a unique member of extracellular ATP-activated purinergic signal family [14] and activating P2X7R contributes to influx of Ca^{2+} as well as the release of pro-inflammatory cytokines [15]. Huang and colleagues have demonstrated that A438079, the competitive P2X7R antagonist, reduced collagen production and the expression of α-smooth muscle actin (α-SMA) and TGF-β1 in mouse liver fibrosis [16]. Furthermore, Tung HC et al. have also found that the potent P2X7R inhibitor BBG ameliorated liver fibrosis and also significantly decreased hepatic pro-inflammatory cytokines IL-6, IL-1β, TNF-α expression in rats with Bile Duct Ligation-induced liver cirrhosis [17]. These results indicate that P2X7 receptor plays a

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potential role on regulating hepatic fibrosis and targeting P2X7R may be efficacious in the prevention and treatment of hepatic fibrosis. However, the functional role of P2X7R in alcoholic liver fibrosis, involving acetaldehyde-induced HSCs activation, has not been extensively studied yet.

GSK-3β (glycogen synthase kinase-3β), an extensively expressed serine/threonine protein kinase, is previously regarded as a mediator of glycogen metabolism [18,19]. Besides its critical role in glycogen metabolism, recent studies reported that GSK-3β also plays a very important role in the regulation of inflammatory response [20] and fibrotic disease [21,22]. GSK-3β inhibition significantly reduced renal fibrosis by suppressing myofibroblast population, collagen I synthesis and inflammatory cytokines [15]. However, Felipe Ortega and groups have found that the P2X7R selective agonist BzATP provoked GSK3 phosphorylation and regulated neuronal survival via the PKC-dependent GSK3 pathway [23]. Therefore, the relationship between P2X7 receptor and GSK-3β in alcoholic liver fibrosis development progress and acetaldehyde-induced HSCs activation has harvested our group's attention.

In this study, HSCs were cultured and stimulated with 200 μM acetaldehyde [10] for 48 h to establish a vitro model for the study of alcoholic liver fibrosis, which was proposed to simulate the activation process *in vivo*. Finally, we demonstrated that P2X7 receptor mediates acetaldehyde-induced HSCs activation via PKC-GSK3β signaling pathway.

2. Materials and methods

2.1. Antibodies and reagents

Acetaldehyde (40%) was provided by Tianjin DaMao Chemical Reagent (Tianjin, China). BzATP (the selective P2X7R agonist) and dimethyl sulfoxide (DMSO) were purchased from Sigma (USA). A438079 (the competitive P2X7R antagonist) and PMA (the PKC agonist) were obtained from Tocris (Ellisville, USA). TDZD-8 (the selective GSK3β antagonist) was supplied by Selleck (USA). SC-3088 (PKC antagonist) and P2X7R antibody were provided by Santa Cruz (California, USA). Antibodies against β-actin, α-smooth muscle actin (α-SMA), collagen type I, PKC, total GSK3β and IL-1β, IL-6, IL-18 were obtained from Bioss (Beijing, China). Antibodies against pGSK3β, CyclinD1 and TNF-α were purchased from Cell Signaling Technology (USA). Goat anti-rabbit immunoglobulin G (IgG) horse radish peroxidase (HRP) and goat anti-mouse IgG HRP were supplied by ZSGB-BIO (Beijing, China). Trizol reagent was obtained from Invitrogen (California, USA).

2.2. Cell culture and cell treatment with acetaldehyde

The rat HSCs were purchased from KeyGEN Biotechnology (Nanjing, China) and cultured in Dulbecco's modified Eagle's medium (DMEM, KeyGEN, China), which was supplemented with 10% fetal bovine serum (FBS, Kangyuan, China). All cells were incubated in 5% CO2 at 37 °C. HSCs were treated with 200 μM acetaldehyde for 48 h to establish a vitro alcoholic liver fibrosis model.

2.3. RNA interference

Small interfering RNA (siRNA) oligonucleotides against P2X7R gene and negative control sequences were designed and synthesized by the GenePharma (Shanghai, China). The designed sequences were as follows: P2X7R siRNA forward: 5′-CCA GGU AUG AAC AUC UCU UTT-3′ and reverse: 5′-AAG AGA UGU UCA UAC CUG GTT-3′; negative control forward: 5′-UUC UCC GAA CGU GUC ACG UTT-3′ and reverse: 5′-ACG UGA CAC GUU CGG AGA ATT-3′. Cells were transfected with siRNA using Lipofectamin 2000 (Invitrogen, USA) according to the manufacturer's protocol. Western blot analysis were used to identify the efficiency of knockdown.

2.4. Quantitative real-time PCR analysis (qRT-PCR)

Total RNA was extracted from HSCs using Trizol reagent according to the manufacture's protocol. Single-stand cDNA was generated from RNA using a transcripter synthesis kit (Toyobo, Dalian) according to the manufacturer's instructions. Quantitative real-time PCR analyses for the relative level of specific mRNA (P2X7R, collagen I, α-SMA, TNF-α, IL-1β, IL-6, IL-18) were determined by Thermo PIKOREAL real-time PCR detection system using SYBR-Green supermix (Takara). Primer sequences were designed from Sangon Biotech (Shanghai, China) as follows: P2X7R forward: 5′-GTA CAG CTT CCG CCG CCT GG-3′ and reverse: 5′-TGC ACA CAG TGG CCA AGC CA-3′; collagen I forward: 5′-GAT CCT GCC GAT GTC GCT AT-3′ and reverse: 5′-TGT AGG CTA GCT GTT CTT GCA-3′; α-SMA forward: 5′-CGA AGC GCA GAG CAA GAG A-3′ and reverse: 5′-CAT GTC GTC CCA GTT GGT GAT-3′; TNF-α forward: 5′-CCA ACA AGG AGG AGA AGT TCC-3′ and reverse: 5′-CTC TGC TTG GTG GTT TGC TAC-3′; IL-1β forward: 5′-GGA ACC CGT GTC TTC CTA AAG-3′ and reverse: 5′-CTG ACT TGG CAG AGG ACA AAG-3′; IL-6 forward: 5′-TAG TCC TTC CTA CCC CAA CTT CC-3′ and reverse: 5′-TTG GTC CTT AGC CAC TCC TTC-3′; IL-18 forward: 5′-AAA CCC GCC TGT GTT CGA-3′ and reverse: 5′-TCA GTC TGG TCT GGG ATT CGT-3′.

2.5. Cell cycle analysis

In order to analyze cell cycle, we performed Cell Cycle Kit (Beyotime, China). HSCs were harvested after cultured 48 h and washed twice, and then fixed in 70% cold ethanol overnight at −20 °C. After fixation, cells were washed with cold PBS and collected by centrifugation, and then resuspended with 0.5 mL propidium iodide (PI) staining buffer, containing 200 mg/mL RNaseA and 50 μg/mL PI. Then the cell suspensions were incubated at 37 °C for 30 min in the dark. Analyses of the cell cycle phase distributions were performed on Laser eight-color flow cytometry (FACSVERSE, USA).

2.6. Western blot analysis

Cells were washed twice and lysed by RIPA buffer (Beyotime, China), containing the protease inhibitor PMSF (Biosharp, USA) for 30 min. After centrifugation at 4 °C at 12,000 rpm for 30 min, protein concentrations were detected by Nucleic acid quantitative instrument (THERMO Nanodrop-2000, USA) and separated by 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), and then the extracts were transferred onto polyvinylidene difluoride (PVDF) membranes (Millipore, USA). After blocked in 5% non-fat milk, the blots were washed and incubated with specific antibodies against P2X7R (1:200), PKC (1:500), α-SMA (1:300), collagen I (1:300), CyclinD1 (1:500), β-actin (1:300), GSK3β (1:300), pGSK3β (1:1000), TNF-α (1:500), IL-1β (1:300), IL-6 (1:300), IL-18 (1:300) overnight at 4 °C. Then the membranes were washed with TBS/Tween 20 three times, and incubated with corresponding secondary antibodies (1:10,000) for 1 h at room temperature. After repeating the washing, protein bands were visualized with enhanced chemiluminescent kit (ECL, Thermo Scientific, USA). Quantitative analyses of immunoblotting images were performed by ImageJ software.

2.7. Statistical analysis

All data were performed at least three times independently and presented as mean ± SD. One-way ANOVA analysis with SPSS software (version 17.0) was used to estimate the differences between groups. $P < 0.05$ was considered to be statistically significant.

3. Results

3.1. P2X7 receptor are upregulated in acetaldehyde-induced HSCs activation

To investigate whether the expression of P2X7 receptor was changed in acetaldehyde-induced HSCs activation, qRT-PCR and western blotting were performed to detect the mRNA and protein levels of P2X7 receptor. The HSCs treated with 200 μM acetaldehyde for 48 h and then critical markers for HSCs activation including α-SMA and collagen I were detected. Our results showed that P2X7 receptor, α-SMA and collagen I were both significantly up-regulated both in protein (Fig. 1A) and mRNA level (Fig. 1B) compared with the control group, suggesting that P2X7 receptor maybe play an important role in the regulation of acetaldehyde-induced HSCs activation.

3.2. Effect of P2X7 receptor on acetaldehyde-induced HSCs proliferation

To explore the effect of P2X7 receptor on acetaldehyde-induced HSCs proliferation, P2X7R siRNA was used to knock down P2X7 receptor. Transfection efficiency was detected by Western blot. As shown in Fig. 2A, P2X7 receptor was successfully knockdown, and significantly suppressed the protein level of cell cycle regulator CyclinD1, which was correlated with the expression of P2X7 receptor. Moreover, the results of cell cycle analysis showed that silencing of P2X7 receptor significantly decreased the proportion of cells in the G0/G1 phase and increased in the S phase (Fig. 2B) compared with the negative control group, suggesting that knockdown of P2X7 receptor down-regulated HSCs proliferation induced by acetaldehyde.

In addition, HSCs were pretreated with 100 μM [24] BzATP, a P2X7R agonist, and 10 μM A438079 [25], a P2X7R antagonist, for 1 h, and then co-cultured with 200 μM acetaldehyde for 48 h. As shown in Fig. 2C, the expression of CyclinD1 was obviously increased in BzATP group and dramatically decreased in A438079 group compared with the acetaldehyde group. Furthermore, the cell cycle analysis showed that BzATP promoted cell population in S phase and A438079 suppressed (Fig. 2D). In addition, in A438079 add BzATP group, A438079 could relatively reduce the Cyclin D1 expression and cell proportion in S phase compared that of the BzATP group (Fig. 2D), which suggested that inhibition of P2X7R down-regulated acetaldehyde-induced HSCs proliferation.

Taken together, these results revealed that P2X7 receptor may have great effects on mediating acetaldehyde-induced HSCs proliferation.

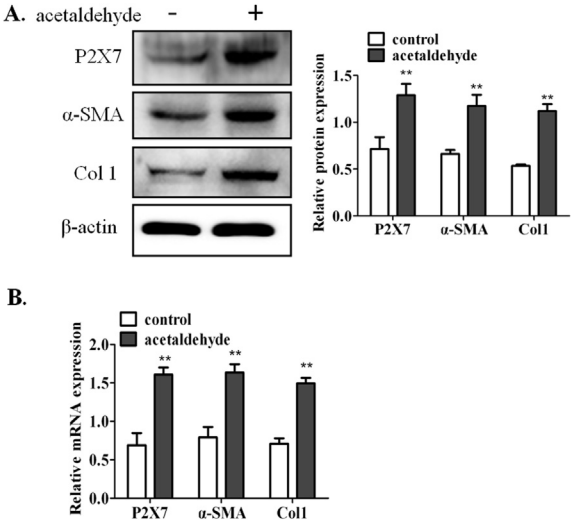

Fig. 1. The protein expression (A) and mRNA level (B) of P2X7 receptor, α-SMA and collagen I in control group and acetaldehyde group. The data were representative of at least three independent experiments. All values were expressed as mean ± SD. $^{**}P < 0.01$ vs. the control group.

3.3. Effect of P2X7 receptor on regulating inflammatory response in acetaldehyde-induced HSCs activation

In order to investigate the association between P2X7 receptor and the inflammatory response in the process of HSCs activation induced by acetaldehyde, we tested the protein and mRNA expression of TNF-α, IL-6, IL-18 and IL-1β. After stimulated with acetaldehyde, TNF-α, IL-6, IL-18 and IL-1β were directly increased in protein (Fig. 3A) and mRNA (Fig. 3B) levels compared with the control group, and in contrast, knockdown of P2X7 receptor significantly suppressed their expression compared with the negative control group. Additionally, both mRNA (Fig. 3D) and protein (Fig. 3C) levels of TNF-α, IL-6, IL-18 and IL-1β harvested a dramatic increase in BzATP (the P2X7R agonist) group and an obvious reduction in A439079 (the P2X7 antagonist) group compared with the acetaldehyde group. And in A438079 add BzATP group, A438079 could relatively decrease the levels of TNF-α, IL-6, IL-18 and IL-1β compared with the BzATP group. Taken together, these findings suggested that P2X7 receptor may up-regulate the inflammatory responses, including TNF-α, IL-6, IL-18 and IL-1β, in acetaldehyde-induced HSCs activation.

3.4. The effect of P2X7 receptor on the synthesis of α-SMA and collagen I in acetaldehyde-induced HSCs activation

To identify the function role of P2X7 receptor in HSCs activation induced by acetaldehyde, we detected the expression of α-SMA and collagen I by western blot and qRT-PCR. Silencing of P2X7 receptor significantly suppressed the protein (Fig. 4A) and mRNA (Fig. 4B) expression of collagen I and α-SMA induced by acetaldehyde compared with the negative control group. Moreover, α-SMA and collagen I were significantly enhanced in BzATP (the P2X7R agonist) group and decreased in A438079 (the P2X7R antagonist) group in protein (Fig. 4C) and mRNA (Fig. 4D) levels compared with the acetaldehyde group. In addition, in A438079 add BzATP group, A438079 relatively reversed the levels of α-SMA and collagen I compared that of the BzATP group. These data suggested that P2X7 receptor up-regulated the expression of collagen I and α-SMA in the activation of HSCs induced by acetaldehyde.

3.5. The effect of P2X7 receptor on PKC-GSK3β pathway in acetaldehyde-induced HSCs activation

To further dissect whether P2X7 receptor mediates HSCs activation through PKC-GSK3β pathway, we detected the protein levels of PKC, pGSK3β and GSK3β. As shown in Fig. 5A and Fig. 5B, after stimulated with acetaldehyde, the protein level of PKC (Fig. 5A) and the ratio of pGSK3β/GSK3β (Fig. 5B) were significantly increased compared with the control group, and reversely, knockdown of P2X7 receptor apparently suppressed their expression compared with the negative control group. Furthermore, compared with the acetaldehyde group, BzATP (the P2X7R agonist) significantly increased the protein level of PKC (Fig. 5C) and the ratio of pGSK3β/GSK3β (Fig. 5D), and A438079 (the P2X7R antagonist) led to a dramatic decrease. And in A438079 add BzATP group, A438079 could relatively reduce PKC expression and GSK3β phosphorylation compared with the BzATP group. Taken together, these findings indicated that P2X7 receptor may enhance the expression of PKC and promote phosphorylation of GSK3β to mediate acetaldehyde-induced HSCs activation.

3.6. The effect of PKC on PKC-GSK3β pathway in acetaldehyde-induced HSCs activation

To further confirm the role of PKC in acetaldehyde-induced HSCs activation, here we examined the influence of the PKC agonist PMA and inhibitor SC-3088 on PKC-GSK3β signaling. HSCs were pretreated with 100 nM PMA and 10 mg/L SC-3088 for 1 h, and then co-cultured with

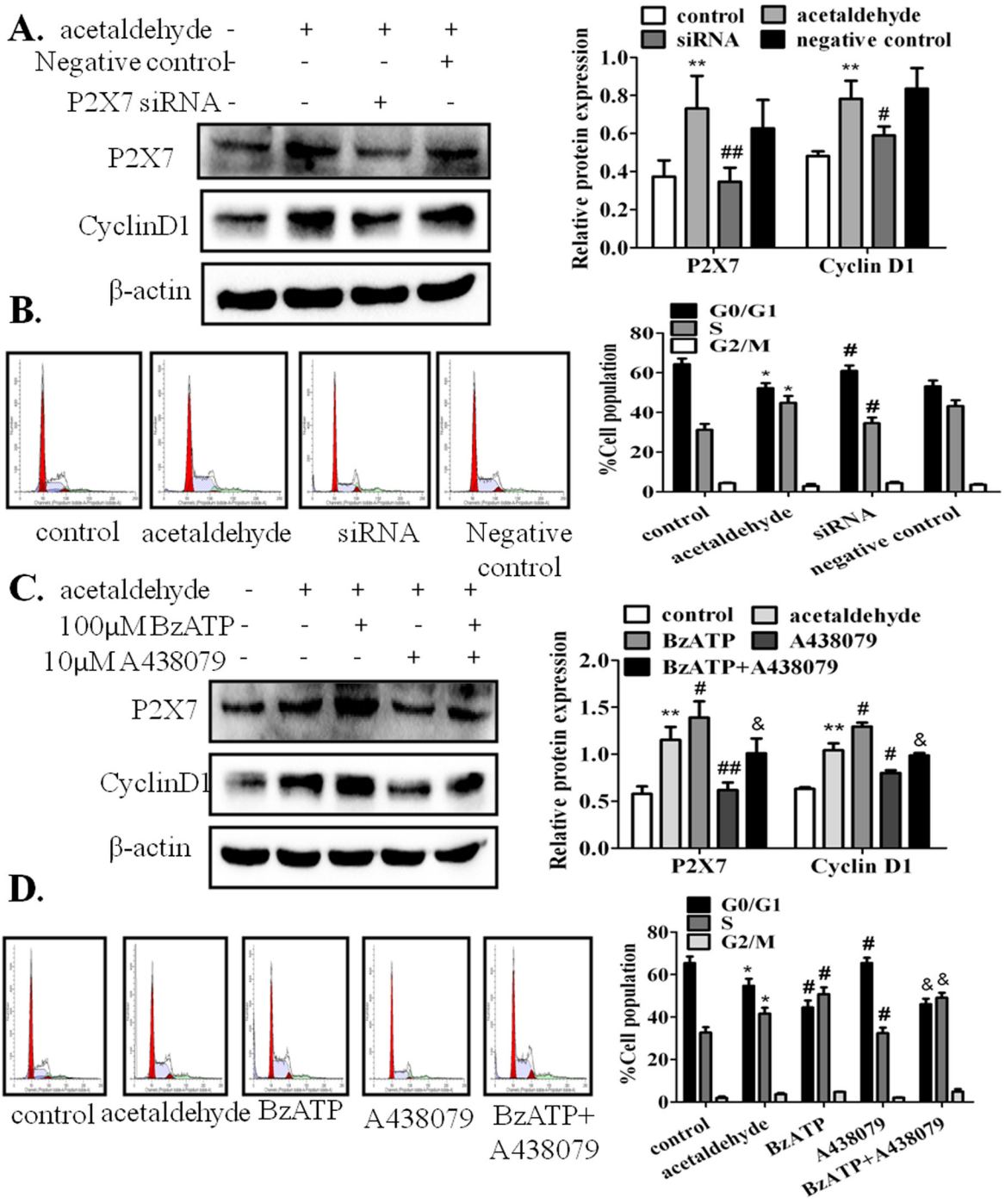

Fig. 2. P2X7 receptor up-regulated the proliferation of HSCs activation induced by acetaldehyde. (A) P2X7R was successfully knockdown and reduced the expression of CyclinD1. The data were representative of at least three independent experiments. All values were expressed as mean ± SD. $^{**}P < 0.01$ vs. the control group, $^{*}P < 0.05$, $^{\#\#}P < 0.01$ vs. the negative control group. (B) Cell cycle analysis for knockdown of P2X7R. The data were representative of at least three independent experiments. All values were expressed as mean ± SD. $^{*}P < 0.05$ vs. the control group, $^{\#}P < 0.05$ vs. the negative control group. (C) The effect of P2X7R agonist BzATP (100 μM) and P2X7R antagonist A438079 (10 μM) on the expression of CyclinD1. The data were representative of at least three independent experiments. All values were expressed as mean ± SD. $^{**}P < 0.01$ vs. the control group, $^{*}P < 0.05$, $^{\#\#}P < 0.01$ vs. the acetaldehyde group, $^{\&}P < 0.05$ vs. the BzATP group. (D) The effect of P2X7R agonist BzATP (100 μM) and P2X7R antagonist A438079 (10 μM) on the cell cycle analysis. The data were representative of at least three independent experiments. All values were expressed as mean ± SD. $^{*}P < 0.05$ vs. the control group, $^{\#}P < 0.05$ vs. the acetaldehyde group, $^{\&}P < 0.05$ vs. the BzATP group.

200 μM acetaldehyde for 48 h. The result showed that PKC was successfully activated and inhibited (Fig. 6A). Then, PMA triggered a significant increase of pGSK3β/GSK3β proportion and SC-3088 reversely caused an apparent reduction compared with the acetaldehyde group (Fig. 6B). Furthermore, α-SMA and collagen I harvested the same alteration as the rate of pGSK3β/GSK3β in mRNA (Fig. 6D) and protein levels (Fig. 6C). These results clearly illustrated that PKC up-regulated acetaldehyde-induced HSCs activation by stimulating the phosphorylation of GSK3β.

3.7. Inhibition of GSK3β down-regulates HSCs activation induced by acetaldehyde

To further explore the influence of GSK3β on acetaldehyde-induced HSCs activation, the selective GSK3β inhibitor TDZD-8 was used. Firstly, HSCs were treated with 10 μM TDZD-8 for 1 h, and then co-cultured with 200 μM acetaldehyde for 48 h. The results in Fig. 7A showed that acetaldehyde up-regulated GSK3β phosphorylation and TDZD-8

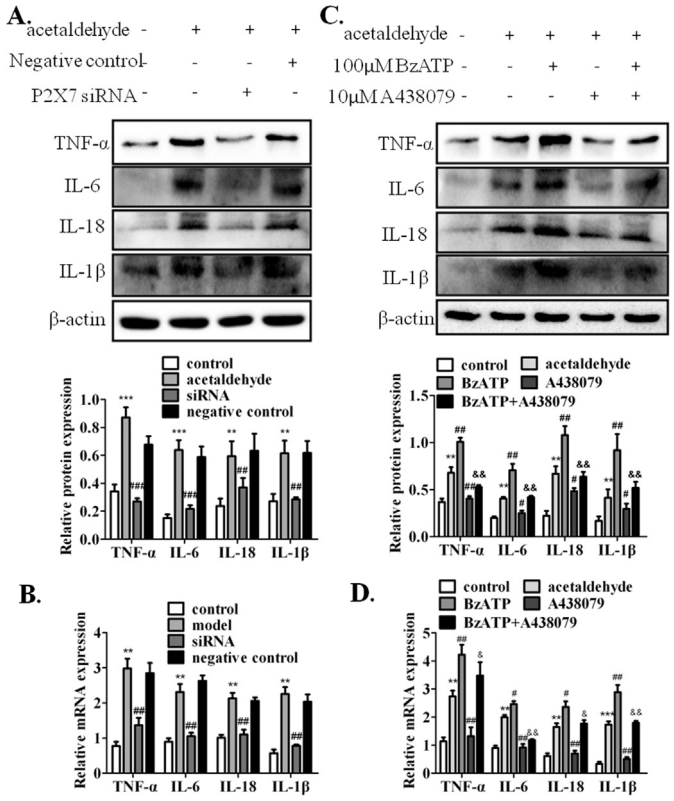

Fig. 3. P2X7 receptor up-regulated the expression of TNF-α, IL-6, IL-18 and IL-1β in acetaldehyde-induced HSCs. Knockdown of P2X7R suppressed the protein expression (A) and mRNA level (B) of TNF-α, IL-6, IL-18 and IL-1β. The data were representative of at least three independent experiments. All values were expressed as mean ± SD. $^{**}P < 0.01$, $^{***}P < 0.001$ vs. the control group, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$ vs. the negative control group. The effect of P2X7R agonist BzATP (100 μM) and P2X7R antagonist A438079 (10 μM) on the protein expression (C) and mRNA level (D) of TNF-α, IL-6, IL-18 and IL-1β. The data were representative of at least three independent experiments. All values were expressed as mean ± SD. $^{**}P < 0.01$ vs. the control group, $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ vs. the acetaldehyde group, $^{\&}P < 0.05$, $^{\&\&}P < 0.01$ vs. the BzATP group.

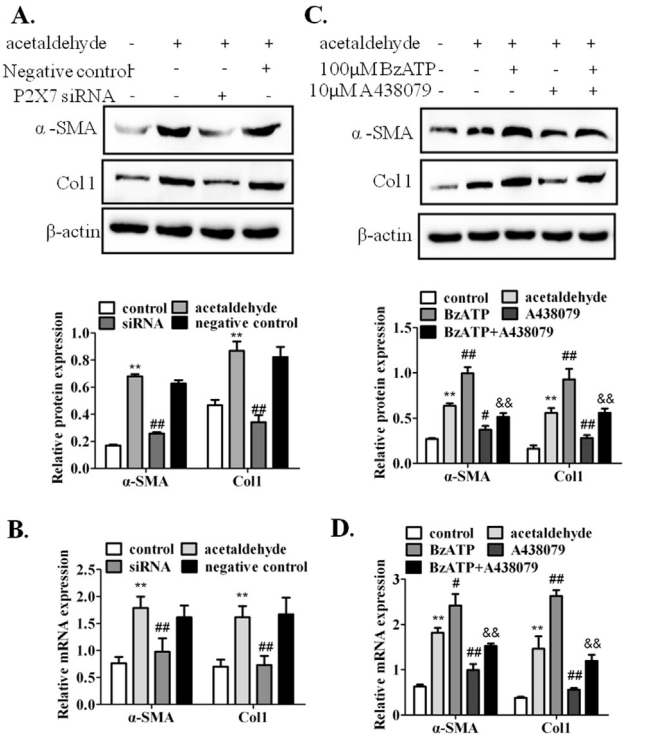

Fig. 4. P2X7 receptor up-regulated the expression of α-SMA and collagen I in acetaldehyde-induced HSCs activation. Knockdown of P2X7R decreased the protein (A) and mRNA (B) level of α-SMA and collagen I. The data were representative of at least three independent experiments. All values were expressed as mean ± SD. $^{**}P < 0.01$ vs. control group, $^{\#\#}P < 0.01$ vs. the negative control group. The protein expression (C) and mRNA level (D) of α-SMA and collagen I were increased and decreased by P2X7R agonist BzATP (100 μM) and P2X7R antagonist A438079 (10 μM). The data were representative of at least three independent experiments. All values were expressed as mean ± SD. $^{**}P < 0.01$ vs. the control group, $^{*}P < 0.05$, $^{\#\#}P < 0.01$ vs. the acetaldehyde group, $^{\&\&}P < 0.01$ vs. the BzATP group.

successfully inhibited the phosphorylation of GSK3β. Moreover, the protein (Fig. 7B) and mRNA (Fig. 7C) levels of α-SMA and collagen I were significantly decreased in TDZD-8 group compared with the acetaldehyde group. Taken together, these results distinctly suggested that inhibition of GSK3β down-regulated HSCs activation induced by acetaldehyde, and further confirmed that P2X7 receptor mediated acetaldehyde-induced activation of HSCs via PKC-GSK3β pathway.

4. Discussion

ALD is a global health problem and ALF is a turning point in ALD progression [26]. In the development of hepatic fibrosis, HSCs are quiescent for storing Vitamin A in normal liver [27], and activated to transform into proliferative myofibroblast cells as the main source of excess ECM production in the injured liver [28]. Therefore, activated HSCs are regarded as a major target for the reversal or attenuation of hepatic fibrosis [29]. Metabolic derivatives of alcohol, acetaldehyde, has been reported to stimulate collagen I synthesis [30] and α-SMA production in HSCs [31]. Moreover, growing evidence showed that activating HSCs with 200 μM acetaldehyde for 48 h could similarly simulate activation of HSCs *in vivo* for the model of alcoholic liver fibrosis [10–12].

The P2X7 receptor, widely distributed in liver, is detectable in various cellular subtypes such as hepatocytes, kupffer cells and endothelial cells [32], it has emerged as a relevant target among purinergic family. In our present study, western blot and qRT-PCR were used to quantify P2X7R, collagen I and α-SMA in mRNA and protein levels in HSCs. We found that P2X7R was significantly increased accompanying with the increase of collagen I and α-SMA in HSCs activation induced by acetaldehyde, which confirmed that P2X7R was also present in rat HSCs and its activation may be largely responsible for HSCs activation induced by acetaldehyde. Recently, experiments have shown that P2X7 receptor up-regulated the proliferation of Schwann cell in injured nerves [33] and P2X7R blockade in different cell types showed different influence on cell proliferation in mesothelioma [34]. However, the effect of P2X7R on HSCs had no available information yet. In our previous research, our results showed that in response to treatment with acetaldehyde, HSCs were activated compared with the control group, and the proliferation was suppressed by incubation with the selective P2X7R antagonist A438079, as well as increased by stimulation in the presence of the competitive P2X7R agonist BzATP compared with the model group. Moreover, knockdown of P2X7R resulted in the suppression of HSCs proliferation. These results suggested that P2X7R up-regulated the proliferation of HSCs induced by acetaldehyde.

According to the recent studies, P2X7R activation can affect various proinflammatory cytokines levels involving IL1β and IL-18 [35,36]. In addition, HSCs are not only the main producer of ECM, but also the target of proinflammatory regulators [37,38]. In our present work, western blot and qRT-PCR were used to determine the expression of TNF-α, IL-6, IL-18 and IL-1β in the process of acetaldehyde-induced HSCs activation. Our results showed that acetaldehyde may have a positive effect on inflammatory response in HSCs, involving not only IL-18 and IL-1β but also TNF-α and IL-6 compared with that of control group. Further results indicated that P2X7R participated in the up-regulation of TNF-α, IL-6, IL-18 and IL-1β levels in HSCs activation induced by acetaldehyde.

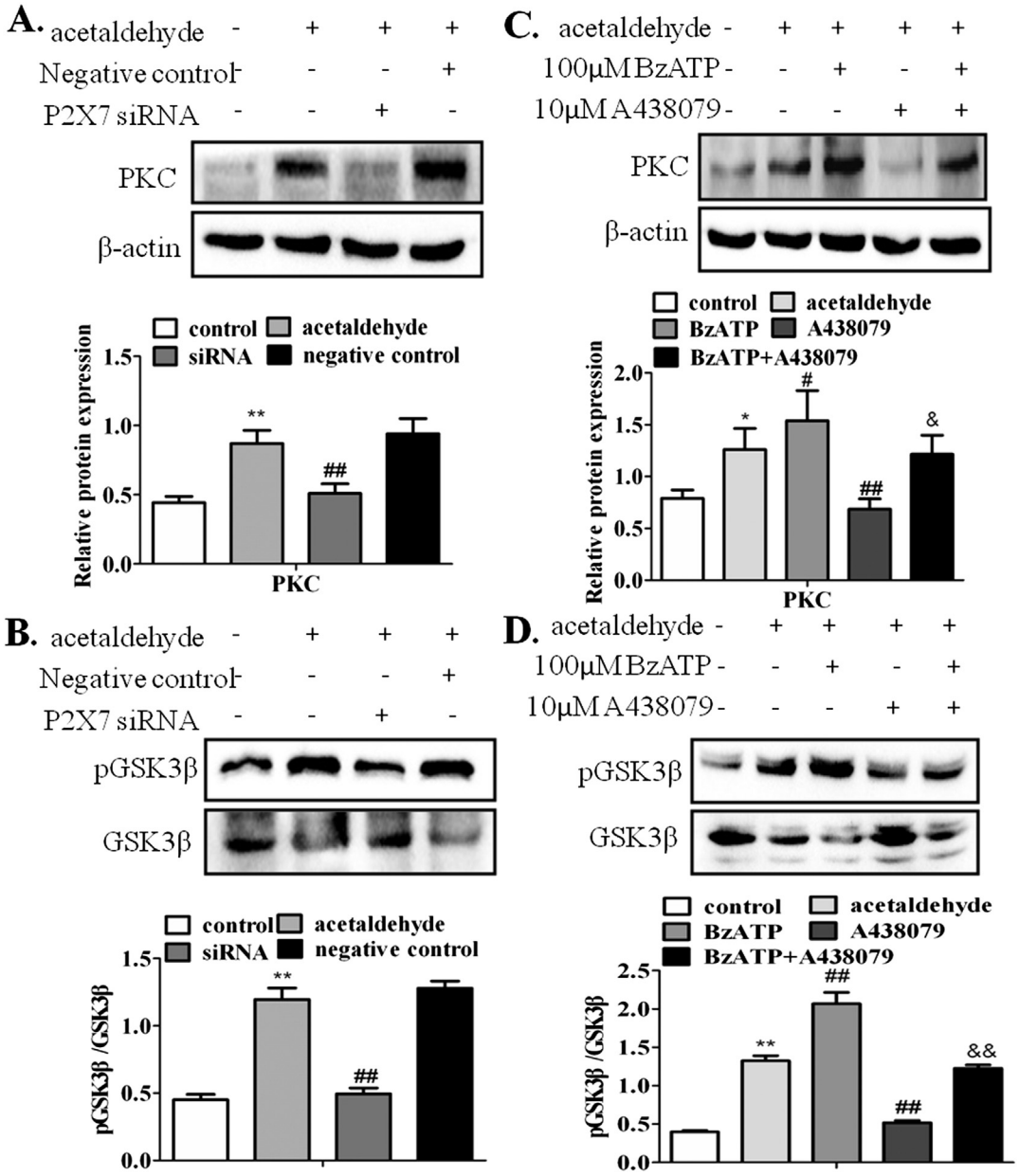

Fig. 5. P2X7 receptor promoted PKC expression and GSK3β phosphorylation in HSCs activation induced by acetaldehyde. The expression of PKC (A) and the phosphorylation of GSK3β (B) were down-regulated by silencing of P2X7R. The data were representative of at least three independent experiments. All values were expressed as mean ± SD. $^{**}P < 0.01$ vs. the control group, $^{\#\#}P < 0.01$ vs. the negative control group. The PKC level (C) and GSK3β phosphorylation (D) were influenced by P2X7R agonist BzATP (100 μM) and P2X7R antagonist A438079 (10 μM). The data were representative of at least three independent experiments. All values were expressed as mean ± SD. $^{*}P < 0.05$, $^{**}P < 0.01$ vs. the control group, $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ vs. the acetaldehyde group, $^{\&}P < 0.05$, $^{\&\&}P < 0.01$ vs. the BzATP group.

Up to now, it is reported that mouse liver fibrosis can be attenuated by block of P2X7R [16] and P2X7R stimulation can increase the danger in pulmonary fibrosis [39]. Our previous results have showed that knockdown or block of P2X7R contributed to the dramatic suppression of collagen I and α-SMA in mRNA and protein levels. In addition, P2X7R activation triggered an obvious over-expression. These results further indicated that P2X7R participated in regulation of HSCs activation induced by acetaldehyde. However, the potential pathway remained no available information yet in HSCs.

Notably, P2X7R stimulation is highly permeable to calcium influx [40], which could induce Ca^{2+}-dependent-PKC activation [41,42] and pGSK3β expression [23]. To gain insight into pathway mediated by P2X7R, the protein levels of PKC, pGSK3β and GSK3β were determined. Data from our previous study showed that stimulating acetaldehyde-induced HSCs with BzATP resulted in a significant increase of PKC and pGSK3β/GSK3β level, as well as the treatment using A438079 led to a remarkable decrease. In addition, silencing of P2X7R provided a negative effect on PKC and GSK3β phosphorylation. These results provided a novel insight into mechanisms that P2X7R may regulate acetaldehyde-induced HSCs activation through PKC/GSK3β pathway.

To further elucidate the involvement of PKC-GSK3β pathway, the PKC agonist PMA and antagonist SC-3088 were used to identify the potential role of PKC, and we found that PMA dramatically stimulated, but SC-3088 obviously suppressed expression of PKC, pGSK3β/GSK3β, collagen I and α-SMA. These data confirmed that PKC stimulated HSCs activation induced by acetaldehyde.

Paradoxically, previous studies have revealed the dramatic expression of pGSK3β protein induced by acetaldehyde in human HSCs but the GSK3β protein was not altered [43]. In our prior study, pGSK3β protein was increased but GSK3β protein was suppressed. Moreover,

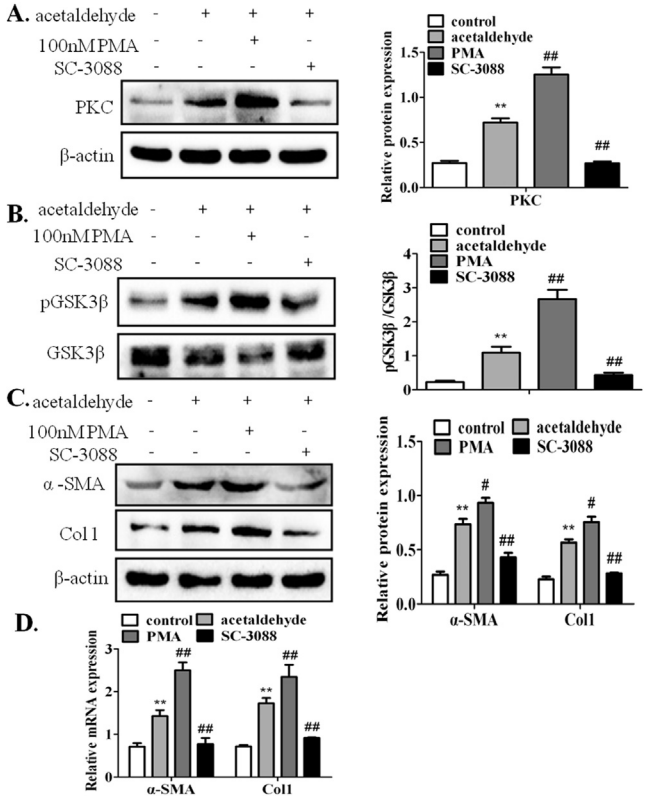

Fig. 6. The effect of PKC agonist PMA (100 nM) and PKC inhibitor SC-3088 (10 mg/L) on the PKC expression (A), GSK3β phosphorylation (B) and the protein expression (C) and mRNA level (D) of α-SMA and collagen I in the activation of HSCs induced by acetaldehyde. The data were representative of at least three independent experiments. All values were expressed as mean ± SD. $^{**}P < 0.01$ vs. the control group, $^{*}P < 0.05$, $^{\#\#}P < 0.01$ vs. the acetaldehyde group.

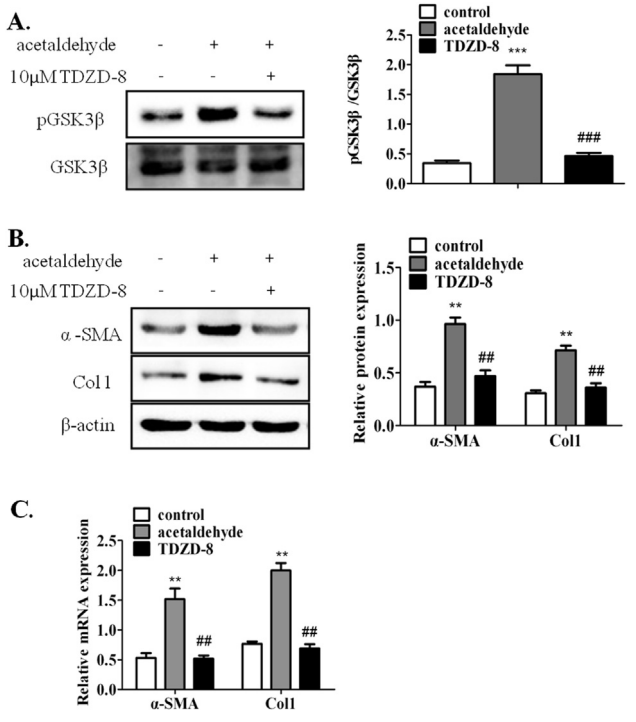

Fig. 7. The impact of GSK3β inhibitor TDZD-8 (10 μM) on the GSK3β phosphorylation (A), the protein expression (B) and mRNA level (C) of α-SMA and collagen I in acetaldehyde-induced HSCs activation. The data were representative of at least three independent experiments. All values were expressed as mean ± SD. $^{**}P < 0.01$, $^{***}P < 0.01$ vs. the control group, $^{*}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.01$ vs. the acetaldehyde group.

compared with the model control, both of BzATP and PMA reduced the expression of GSK3β protein and contrastly facilitated pGSK3β level, as well as treatment with A438079 and SC-3088 and TDZD-8 harvested the promotion of GSK3β and the suppression of pGSK3β. Furthermore, compared with negative control, knockdown of P2X7R stimulated the expression of GSK3β while reduced the level of pGSK3β. But the reason why GSK3β contributed to the same functional influence with differential expression in human HSCs and rat HSCs activation induced by acetaldehyde is not clearly estimated and need further study. In the other words, the ratio of pGSK3β/GSK3β elucidated acetaldehyde can promote the phosphorylation of GSK3β to up-regulate the levels of -SMA and collagen I. Furthermore, inhibition of GSK3β reduced acetaldehyde-induced expression of α-SMA and collagen I by suppressing the phosphorylation of GSK3β as compared that of the model group, which illustrated the close association between P2X7R, PKC and GSK3β in acetaldehyde-induced HSCs activation.

5. Conclusion

In the current study, we demonstrated that P2X7R was existed in rat HSCs and probably exerted positive influence via PKC-dependent GSK3β pathway. At present, we concluded that acetaldehyde may up-regulated the HSCs proliferation, inflammatory response, the synthesis of α-SMA and collagen I and PKC and the phosphorylation of GSK3β, which could be activated by P2X7R agonist and suppressed by P2X7R inhibitor. Moreover, PKC activation had positive effects on the expression of α-SMA and collagen I and GSK3β phosphorylation, and PKC inhibitor had the opposite effects. Furthermore, GSK3β inhibition suppressed the levels of α-SMA and collagen I by reducing GSK3β phosphorylation. Collectively, this study provided the first evidence that P2X7R promoted the progression of alcoholic-induced liver fibrosis through PKC/GSK3β-dependent mechanisms, and P2X7R inhibitor may serve as a novel therapeutic agent for treating liver fibrosis.

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