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ORIGINAL RESEARCH

Knockdown of *GBP5* alleviates lipid accumulation and inflammation in alcoholic fatty liver through inhibition of NF-κB pathway in mice

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Abstract

Alcoholic liver disease (ALD) is a global liver disease that manifests in a variety of forms, including alcoholic hepatitis, liver inflammation, fatty liver, and liver cirrhosis. One of the early stages of ALD is alcoholic fatty liver (AFL) disease, which is primarily characterized by the accumulation of lipids and inflammation in hepatocytes. Guanylate binding protein 5 (*GBP5*) has been investigated for its involvement in disease progression. The regulatory effects of *GBP5* on the progression of AFL disease are still not well understood. This study aims to investigate the impact of *GBP5* on AFL progression and its underlying mechanism of action. To achieve this, gene expression was evaluated using western blot and reverse transcription quantitative polymerase chain reaction (RT-qPCR) methods. Lipid accumulation was confirmed using the Oil Red O staining assay, while the levels of Triglyceride (TG), aspartate aminotransferases (AST), and alanine aminotransferases (ALT) were measured using commercial kits. The levels of inflammatory cytokines were assessed using enzyme-linked immuno sorbent assay (ELISA) assay. Finally, cell apoptosis was evaluated using flow cytometry. Cell apoptosis was evaluated through flow cytometry. Our work demonstrated that *GBP5* expression was up-regulated in the Ethyl Alcohol (EtOH) group. Additionally, lipid accumulation was increased after EtOH inducement, but this change was attenuated by silencing GBP5. Silencing of *GBP5* reduced EtOH-mediated inflammation and cell apoptosis. Finally, it was discovered that knockdown of *GBP5* retarded the EtOH-stimulated nuclear factor kappa-B (NF-κB) pathway. Knockdown of *GBP5* alleviated lipid accumulation and inflammation in AFL through inhibition of NF-κB pathway. This finding suggested that *GBP5* may be a useful bio-target for AFL treatment.

Keywords

GBP5; Alcoholic fatty liver; Lipid accumulation; Inflammation; NF-κB pathway

1. Introduction

Alcohol abuse is a grievous public health problem, and is linked to many diseases such as ALD [1, 2]. ALD is a common hepatic disease due to alcohol consumption and has a high mortality rate [3]. AFL disease is the initial stage of ALD and is characterized by the accumulation of triglycerides (TG) in hepatocytes. If left untreated, AFL can progress to more severe pathologies, such as alcoholic steatohepatitis and alcoholic cirrhosis [4, 5]. The pathogenesis of AFL disease refers to lipid metabolism disorder and oxidative stress [5]. As men drink more alcohol than women, they also have a greater incidence of AFL. Currently, alcohol limitation remains the most effective treatment, and few drugs are available for the direct treatment of AFL. Therefore, the search for more effective biological targets is of essential importance for AFL treatment.

Guanylate binding protein 5 (*GBP5*) belongs to the GBP family and is a guanosine triphosphatase (GTPase) that can be highly stimulated by interferon [6]. *GBP5* has been found to possess pro-inflammatory effects in inflammatory bowel disease [7]. In addition, inhibition of *GBP5* suppresses the NOD-like receptor thermal protein domain associated protein 3 (*NLRP3*) inflammasome to prevent the progression of lupus nephritis [8]. Additionally, *GBP5* affects the NF-κB signaling pathway to polarize macrophages towards the M1 phenotype, thereby aggravating rosacea-like skin inflammation [9]. Moreover, overexpression of *GBP5* exacerbates lipopolysaccharide (LPS)-triggered liver injury in sepsis by facilitating activation of *NLRP3* inflammasome [10]. Besides, *GBP5* knockdown has been confirmed to improve galactosamine (GalN)/LPS-mediated liver injury and inflammation [11]. Nevertheless, the impacts and regulatory mechanism of *GBP5* in AFL disease remain unclear.

This study aims to explore the regulatory function of *GBP5* on the progression of AFL. It is manifested that knockdown of *GBP5* alleviated lipid accumulation and inflammation in AFL

through inhibition of the NF-κB pathway. This work implied that *GBP5* may be a novel and useful biological target for AFL treatment.

2. Materials and methods

2.1 Cell culture

The Alpha Mouse Liver 12 (AML-12) cell line, derived from mouse normal hepatocytes, was obtained from the Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China). The cells were cultured in a humidified incubator (37 °C, 5% carbon dioxide (CO_2)) using Dulbecco's Modified Eagle's Medium (DMEM) medium supplemented with fetal bovine serum (FBS, 10%). To create an *in vitro* AFL model, cells were treated with ethyl alcohol (EtOH, 250 mmol/L) and palmitic acid (PA, 0.25 mmol/L) for 24 hours. This was achieved by treating cells with Sigma-Aldrich product #P0500 from St. Louis, MO, USA.

2.2 Cell transfection

GenePharma, a company located in Shanghai, China, provided the siRNAs used to target *GBP5* (si-GBP5) and a negative control (si-NC). The siRNAs were transfected into AML-12 cells by Lipofectamine 2000 (11668019), which is a product manufactured by Invitrogen located in Carlsbad, CA, USA. Plasmids containing si-GBP5 and si-NC were utilized in the transfection process.

2.3 Western blot

Proteins extracted from AML-12 cells were separated using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) (10%) and transferred onto polyvinylidene difluoride (PVDF) membranes obtained from Beyotime, located in Shanghai, China. After blocking, the membranes were incubated overnight (4 °C) with primary antibodies against *GBP5* (1:1000; ab96119; Abcam, Shanghai, China), B-cell lymphoma-2-associated X (*Bax*) (1:2000; ab182733), B-cell lymphoma-2 (*Bcl-2*) (1:1000; ab196495), phosphorylation-proteinp65 (p-*p65*) (1:1000; ab76302), proteinp65 (*p65*) (0.5 μg/mL; ab16502), phosphorylation-Inhibitory subunit of NF kappa B alpha (p-$I\kappa B\alpha$) (1:10,000; ab133462), Inhibitory subunit of NF kappa B alpha (*$I\kappa B\alpha$*) (1:1000; ab32518), and β-*actin* (1 μg/mL; ab8226; the internal reference). Subsequently, appropriate secondary antibodies (1:2000; ab7090) were added to the membranes for another 2 hours. Finally, the blots were visualized and analyzed.

2.4 Oil Red O staining

To prepare the AML-12 cells for analysis, they were first fixed using paraformaldehyde solution (24 h). Afterward, the cells were washed with phosphate Buffered Saline (PBS) and stained with saturated Oil Red O for a duration of 15 minutes. The cells were then subjected to differentiation in alcohol, which involved treating them with 75% alcohol three times. Images of the cells were captured using an optical microscope-the Nikon Eclipse E100 model (Nikon, Tokyo, Japan).

2.5 TG and aspartate aminotransferases (AST)/alanine aminotransferases (ALT) activity assay

To quantify the levels of TG, AST and ALT, the corresponding assay kits were utilized. These kits were purchased from Jiancheng Biology Institution PeproTech, a company located in Nanjing, Jiangsu, China.

2.6 RT-qPCR

RNA isolation from AML-12 cells was performed using TRIzol reagent (15596018, Invitrogen, Carlsbad, CA, USA) from Invitrogen. Subsequently, cDNA was synthesized using the PrimeScript RT Reagent Kit (RR037A, Takara, Dalian, China). RT-qPCR was performed using the SYBR Premix Ex Taq™ II commercial kit (RR820A, Takara, Dalian, China). The calculation of the relative expressions was performed by the $2^{-\Delta\Delta Ct}$ method, with glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) serving as an internal reference.

Tumor Necrosis Factor-alpha (*TNF-α*),
F: GAA ACA CAC GAG ACG CTG AA;
R: AGG GAG GCC TGA GAC ATC TT.
Interleukin-1 beta (*IL-1β*),
F: TAC AGG CTC CGA GAT GAA CAA C;
R: TTT GAG GCC CAA GGC CAC AG.
Interleukin-6 (*IL-6*),
F: CCA GAA ACC GCT ATG AAG TTC C;
R: GTT GGG AGT GGT ATC CTC TGT GA.
GADPH,
F: CC ACA GTC CAT GCC ATC AC;
R: TCC ACC ACC CTG TTG CTG TA.

2.7 ELISA

To quantify the levels of *IL-6* (ab222503), *IL-1β* (ab197742), and *TNF-α* (ab208348), commercial ELISA kits were employed. The ELISA kits (ab222503, ab197742 and ab208348) used were purchased from Abcam, which is located in Shanghai, China. The protocols provided by the manufacturer were followed in carrying out the assays.

2.8 Flow cytometry

The Annexin V-FITC Apoptosis Detection Kit (C1062S, Beyotime, Shanghai, China), which was obtained from Beyotime located in Shanghai, China, was used to evaluate cell apoptosis. The analysis was conducted using a BD FACS Calibur instrument (BD FACSCalibur, BD Biosciences, San Jose, CA, USA). Prior to analysis, the AML-12 cells were washed with PBS and then stained with Annexin V-FITC and propidium iodide (PI) in a dark environment. Finally, flow cytometry was employed to examine the extent of cell apoptosis using a BD Biosciences instrument located in San Jose, CA, USA.

2.9 Statistical analysis

All experiments were independently performed three times, and the resulting data was presented as means $\pm$ SD. Statistical analysis was performed using IBM Corp.'s SPSS 22.0 statistical software, which is located in Armonk, NY, USA.

To compare the various groups, one-way Analysis of Variance (ANOVA) and Student's *t*-test were used. A *p*-value less than 0.05 was deemed to be statistically significant.

3. Results

3.1 Knockdown of GBP5 suppressed EtOH-induced lipid accumulation

GBP5 protein expression was increased in the EtOH group, but reduced after *GBP5* knockdown (Fig. 1A). Additionally, lipid accumulation was increased after EtOH induction, but this change was attenuated after silencing of *GBP5* (Fig. 1B). Moreover, the levels of TG, AST and ALT were all strengthened after EtOH treatment, but these changes were reversed after *GBP5* inhibition (Fig. 1C). Taken together, knockdown of *GBP5* suppressed EtOH-induced lipid accumulation.

3.2 Silencing of GBP5 reduced EtOH-mediated inflammation

As shown in Fig. 2A, the mRNA expressions of *TNF-α*, *IL-1β* and *IL-6* were all elevated following EtOH treatment, but this effect was attenuated following *GBP5* suppression. In addition, as confirmed by ELISA assay, *TNF-α*, *IL-1β* and *IL-6* levels were also heightened following EtOH treatment, but these changes were attenuated following *GBP5* inhibition (Fig. 2B). These results demonstrated that silencing of *GBP5* reduced EtOH-mediated inflammation.

3.3 Suppression of GBP5 inhibited EtOH-triggered cell apoptosis

Next, it was discovered that cell apoptosis was increased following EtOH induction, but this change was counteracted after *GBP5* suppression (Fig. 3A, B). Furthermore, the enhancement of *Bax* protein expression and the decrease in *Bcl-2* protein expression mediated by EtOH treatment were reversed after *GBP5* knockdown (Fig. 3C). In general, suppression of *GBP5* inhibited EtOH-triggered apoptosis.

3.4 Knockdown of GBP5 retarded the EtOH-stimulated NF-κB pathway

The protein expressions of p-*p65* and p-*IκBα* were enhanced, and *IκBα* was reduced in response to EtOH stimulation, but these effects were rescued following *GBP5* inhibition (Fig. 4). These data illustrated that knockdown of *GBP5* retarded the EtOH-stimulated NF-κB pathway.

4. Discussion

Many proteins have been investigated for their regulation of AFL progression. For example, Myeloid Differentiation Factor 88 (*MyD88*) affects the protein kinase B (AKT) pathway to facilitate the progression of AFL [12]. Additionally, inhibition of miR-181b-5p enhances protein inhibitor of activated STAT, 1 (*PIAS1*) in rats with AFL to suppress inflammatory response and oxidative stress [13]. In addition, Arrb2 modulates the Adenosine 5′-monophosphate (AMP)-activated protein kinase (AMPK) pathway to induce dysfunctional hepatic lipid metabolism in AFL [14]. Moreover, Caveolin-1 activates mitophagy to reduce lipid accumulation in AFL through the Pink-1/Parkin pathway [15]. Besides, Fibronectin type III domain-containing protein 3B (*FNDC3B*) regulates the AMPK pathway to improve steatosis and ferroptosis in AFL [16]. *GBP5* has been shown to be involved in the progression of some diseases [6–11]. However, the regulatory effects of *GBP5* on AFL progression remains obscure. In this study, it was illustrated that *GBP5* expression was up-regulated in the EtOH group. Additionally, lipid accumulation was increased after EtOH induction, but this change was attenuated after silencing of *GBP5*.

Inflammation is a key driver of the process of ALD [4]. Alcohol treatment induces *TNF-α* and *IL-6*, which then affects abnormal metabolism and inflammation, thereby regulating hepatic steatosis [17]. This study showed that silencing of *GBP5* reduced EtOH-mediated inflammation and cell apoptosis. NF-κB pathway has been confirmed to be a vital pathway, which is associated with inflammation [18–20]. During the process of establishing AFL model, NF-κB was found to be consistent with the degree of hepatic steatosis at the level of gene transcription [21]. The NF-κB pathway has been elucidated to be activated in AFL, which thereby triggers disruption of the gut microbiota [22]. In our work, it was discovered that knockdown of *GBP5* retarded the EtOH-stimulated NF-κB pathway.

5. Conclusions

In conclusion, this study revealed for the first time that *GBP5* expression was up-regulated in EtOH-induced AML-12 cells, and that knockdown of *GBP5* alleviated lipid accumulation and inflammation in AFL through inhibition of NF-κB pathway. Nevertheless, the present study also had some limitations in investigating the regulatory role of *GBP5* on AFL progression, such as the lack of human samples, *in vivo* models and other cellular phenotypes. In the future, more experiments are needed to further explore other regulatory functions of *GBP5* in AFL progression.
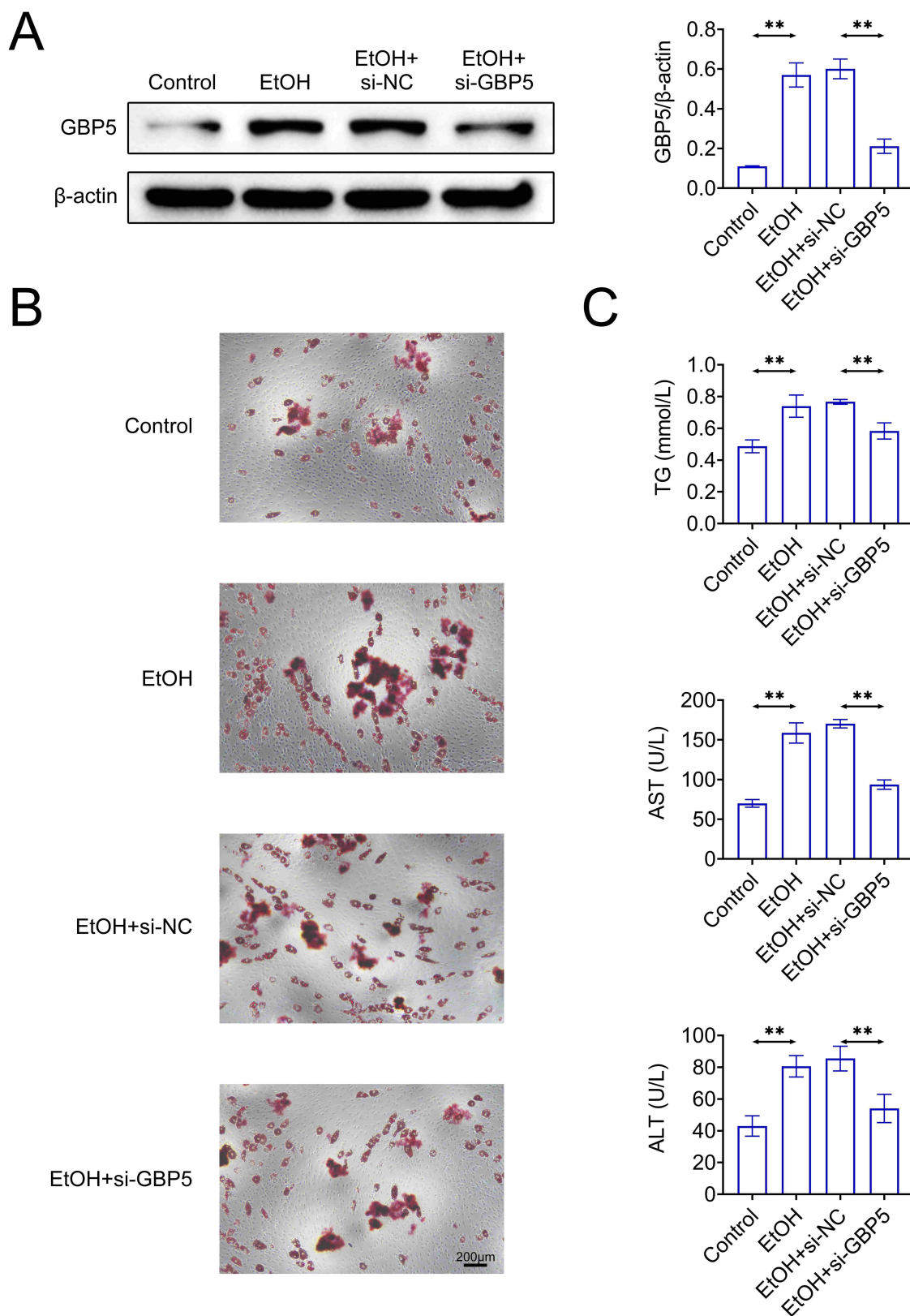
FIGURE 1. Knockdown of Guanylate binding protein 5 (*GBP5*) suppressed Ethyl Alcohol (EtOH)-induced lipid accumulation. Groups were divided into Control, EtOH, EtOH + si-NC and EtOH + si-GBP5 groups. (A) GBP5 protein expression was examined through western blot. (B) The lipid accumulation level was confirmed through Oil Red O staining assay. (C) The Triglyceride (TG), aspartate aminotransferases (AST), and alanine aminotransferases (ALT) levels were measured through the corresponding commercial kits. $**p < 0.01$. si-GBP5: siRNAs used to target *GBP5*; si-NC: siRNAs used to a negative control.
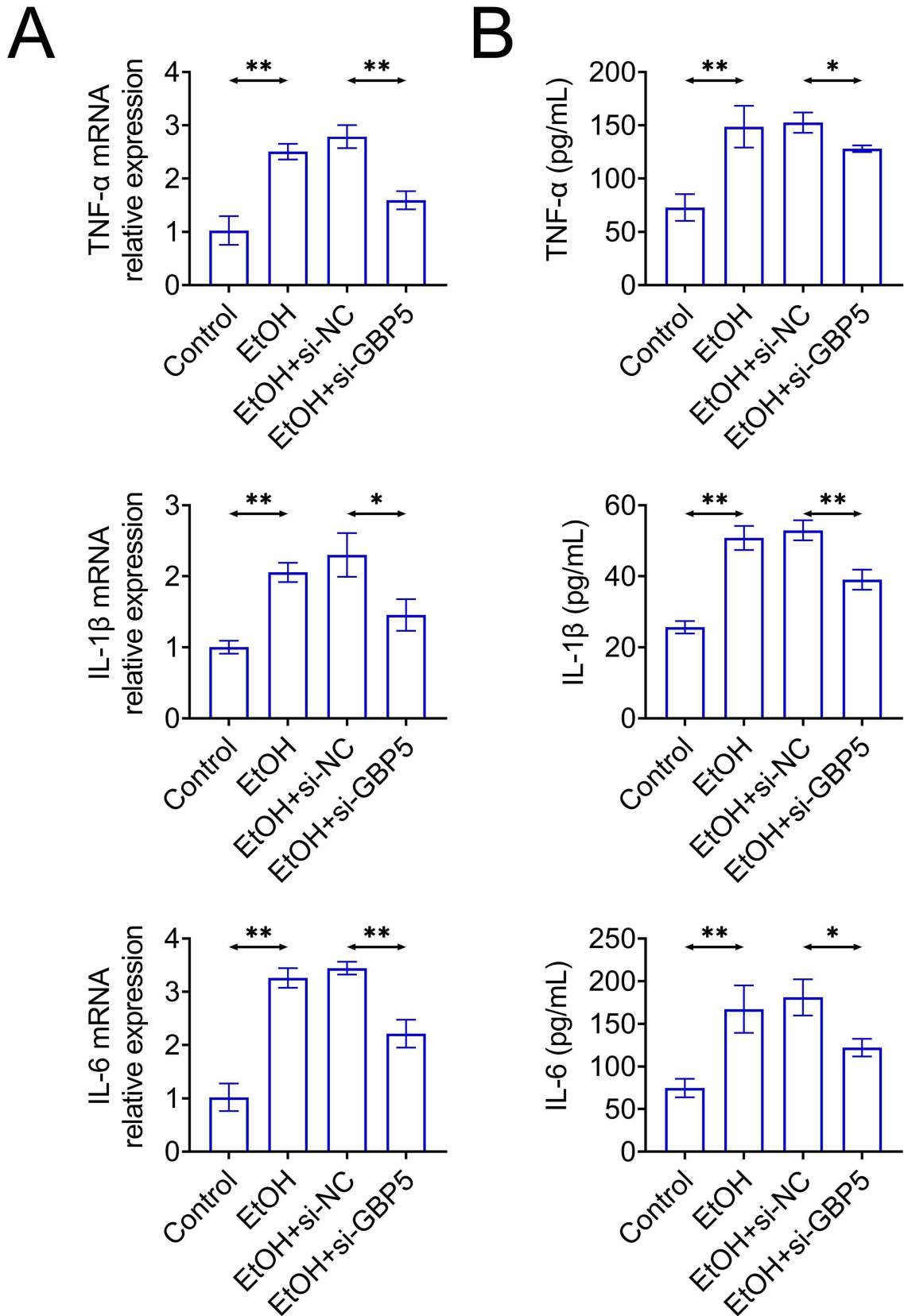
F I G U R E 2. Silencing of *GBP5* reduced EtOH-mediated inflammation. The mRNA expressions (A) and levels (B) of Tumor Necrosis Factor-alpha (*TNF-α*), Interleukin-1 beta (*IL-1β*) and Interleukin-6 (*IL-6*) were confirmed through reverse transcription quantitative polymerase chain reaction (RT-qPCR) and enzyme-linked immuno sorbent assay (ELISA), respectively. $^{*}p < 0.05$, $^{**}p < 0.01$. *GBP5*: Guanylate binding protein 5; EtOH-Ethyl Alcohol; si-GBP5: siRNAs used to target *GBP5*; si-NC: siRNAs used to a negative control.
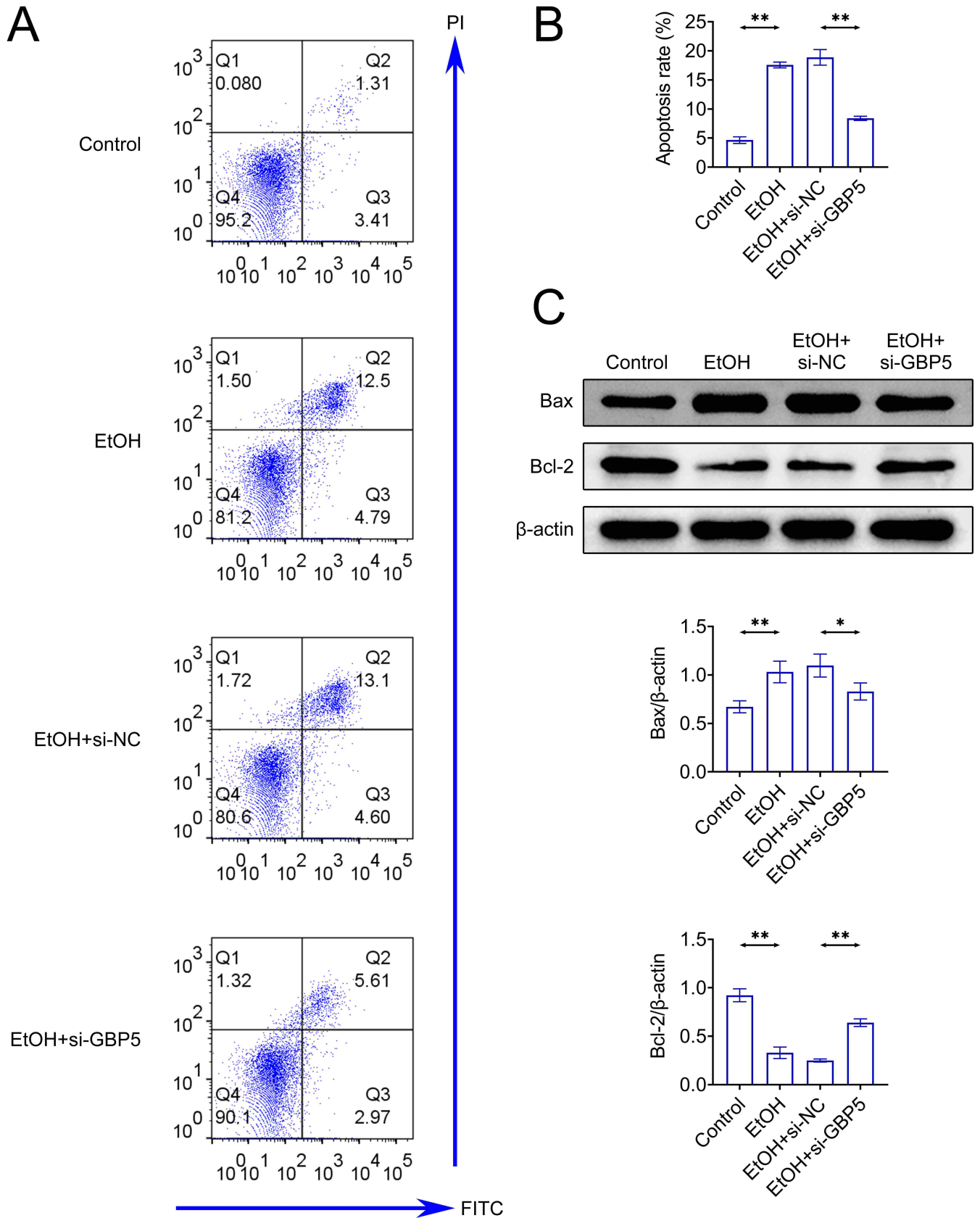
F I G U R E 3. Suppression of *GBP5* inhibited EtOH-triggered apoptosis. (A, B) Cell apoptosis was assessed through flow cytometry. (C) The protein expressions of B-cell lymphoma-2-associated X (*Bax*) and B-cell lymphoma-2 (*Bcl-2*) were evaluated through western blot. $*p < 0.05$, $**p < 0.01$. *GBP5*: Guanylate binding protein 5; EtOH-Ethyl Alcohol; si-GBP5: siRNAs used to target *GBP5*; si-NC: siRNAs used to a negative control.
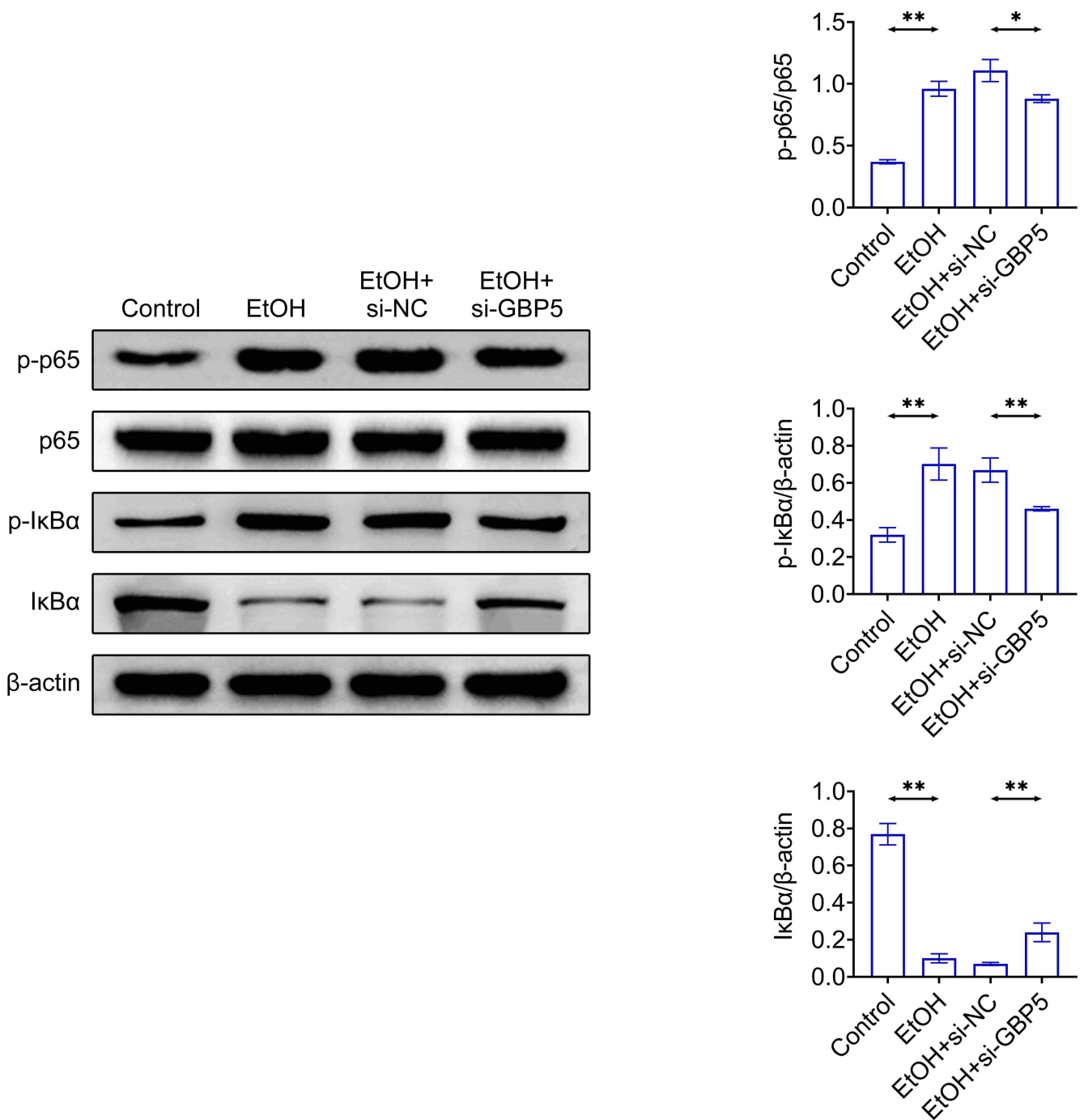
FIGURE 4. Knockdown of *GBP5* retarded the nuclear factor kappa-B (NF-κB) pathway. The protein expressions were examined through western blot. $^*p < 0.05$, $^{**}p < 0.01$. *GBP5*: Guanylate binding protein 5; EtOH-Ethyl Alcohol; si-GBP5: siRNAs used to target *GBP5*; si-NC: siRNAs used to a negative control.

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