



Betulin Targets Lipin1/2-Mediated P2X7 Receptor as a Therapeutic Approach to Attenuate Lipid Accumulation and Metaflammation

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Abstract

The present study focused on the potential mechanism of betulin (BT), a pentacyclic triterpenoid isolated from the bark of white birch (*Betula pubescens*), against chronic alcohol-induced lipid accumulation and metaflammation. AML-12 and RAW 264.7 cells were administered ethanol (EtOH), lipopolysaccharide (LPS) or BT. Male C57BL/6 mice were fed Lieber-DeCarli liquid diets containing 5% EtOH for 4 weeks, followed by single EtOH gavage on the last day and simultaneous treatment with BT (20 or 50 mg/kg) by oral gavage once per day. *In vitro*, MTT showed that 0-25 mM EtOH and 0-25 μ M BT had no toxic effect on AML-12 cells. BT could regulate sterolregulatory-element-binding protein 1 (SREBP1), lipin1/2, P2X7 receptor (P2X7r) and NOD-like receptor family, pyrin domains-containing protein 3 (NLRP3) expressions against EtOH-stimulation. Oil Red O staining also indicated that BT significantly reduced lipid accumulation in EtOH-stimulated AML-12 cells. Lipin1/2 deficiency indicated that BT might mediate lipin1/2 to regulate SREBP1 and P2X7r expression and further alleviate lipid accumulation and inflammation. *In vivo*, BT significantly alleviated histopathological changes, reduced serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST) and triglyceride (TG) levels, and regulated lipin1/2, SREBP1, peroxisome proliferator activated receptor α/γ (PPAR α/γ) and PGC-1 α expression compared with the EtOH group. BT reduced the secretion of inflammatory factors and blocked the P2X7r-NLRP3 signaling pathway. Collectively, BT attenuated lipid accumulation and metaflammation by regulating the lipin1/2-mediated P2X7r signaling pathway.

Key Words: Betulin, Adipose metabolic disease, Metaflammation, Lipin1/2, P2X7r

INTRODUCTION

Lipids are fundamental structures in cells and tissues that are involved in regulating intracellular energy metabolism and immune function (Walther and Farese, 2012). However, massive lipids can induce metabolic disorders and inflammatory responses (Ertunc and Hotamisligil, 2016). Alcohol is a unique toxin that may perturb hepatic lipid metabolism, further inducing alcoholic liver disease (ALD) (You and Arteel, 2019). Alcoholic fatty liver disease (AFLD) is the earliest stage of ALD, and persistent excessive alcohol consumption could lead to the development of ALD from steatosis to alcoholic hepatitis, fibrosis and even cirrhosis (Addolorato *et al.*, 2016). Currently, alcohol withdrawal is one of the most effective methods for the treatment of ALD, while the clinical efficacy is not ideal due to the limitation of patient compliance (Liu, 2014). Therefore, it

is urgent to find safe and effective treatment strategies and candidates for AFLD.

During the development of AFLD, sterol regulatory-element-binding protein 1 (SREBP1) and peroxisome proliferator activated receptor α/γ (PPAR α/γ) are important transcriptional regulatory factors that are involved in lipid production and fatty acid oxidation, respectively (Menon *et al.*, 2001; Han *et al.*, 2019). Our previous study indicated that alcohol could down-regulate SREBP1 and PPAR α expression while upregulating PPAR γ expression (Yao *et al.*, 2017a). Moreover, lipin1 is a protein generated by the LPIN1 gene and can interact with other nuclear receptors to regulate lipid metabolism, such as SREBP1 and PPAR α/γ (Koh *et al.*, 2008; Barroso *et al.*, 2011; Song *et al.*, 2018). Lipin2 also functions as a transcriptional coactivator for PPAR γ , similar to lipin1 (Donkor *et al.*, 2009). These studies confirmed the critical role of SREBP1, PPAR α/γ

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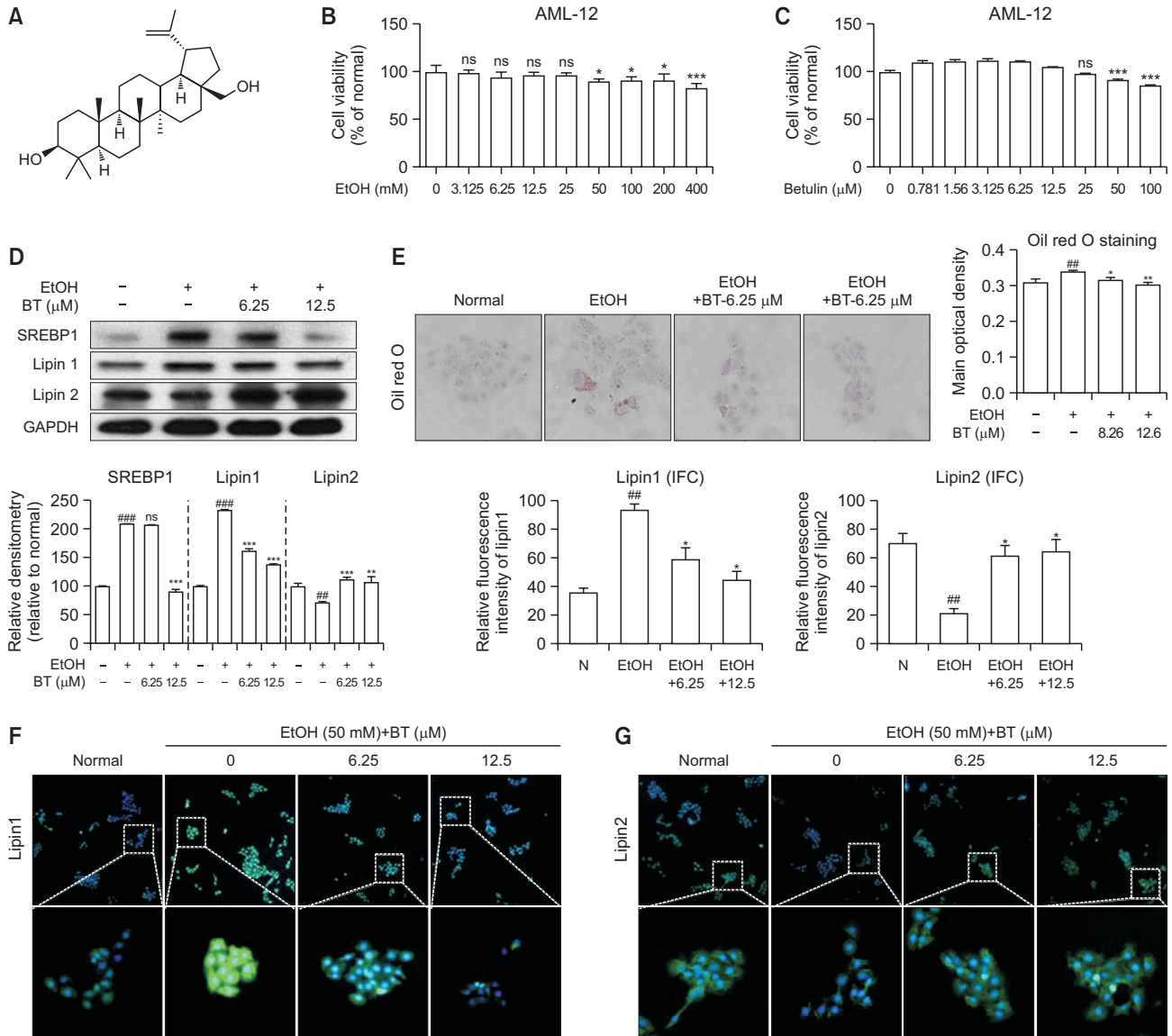


Fig. 1. BT regulates lipid deposition in EtOH-stimulated AML-12 cells. (A) Chemical structure of BT. (B) Cell viability of EtOH on AML-12 cells. (C) Cell viability of BT on AML-12 cells. (D) Representative Western blotting analysis for expressions of SREBP1, lipin1 and lipin2. (E) Oil Red O staining present in 400 \times magnification. (F) Immunofluorescence staining of lipin1 expression in AML-12 cells (100 \times and 600 \times). (G) Immunofluorescence staining of lipin2 expression in AML-12 cells (100 \times and 600 \times). The GAPDH was used as internal reference to normalize the data. ### p <0.001, ## p <0.01 vs normal group, * p <0.05, ** p <0.01, *** p <0.001 vs EtOH group, ns, not significant. All histograms represent the mean \pm SD of at least three independent assays.

and lipin1/2 in the regulation of lipogenesis.

Alcohol intake-induced excess lipid accumulation is accompanied by an inflammatory response. The P2X7 receptor (P2X7r) is an ATP-gated channel and can activate the NOD-like receptor family pyrin domain-containing protein 3 (NLRP3) inflammasome (Adinolfi *et al.*, 2018). Activation of the NLRP3 inflammasome complex can induce the maturation and release of pro-IL-1 β , pro-IL-18, and pro-caspase-1, further promoting the inflammatory response (Shao *et al.*, 2015; Del Campo *et al.*, 2018). Previously, we reported that the inhibition of the P2X7r-NLRP3 inflammasome could ameliorate extracellular matrix (ECM) deposition and liver fibrosis (Hou *et al.*, 2020). In addition, NLRP3 is involved in metabolic diseases, such as

type 2 diabetes and obesity-induced inflammation (Haneklaus and O'Neill, 2015). Therefore, thorough inquiry into the relationship between inflammation and metabolism might be a new entry point for the treatment of metabolic diseases.

Betulin (BT, lup-20(29)-ene-3 β , 28-diol) is a naturally occurring pentacyclic triterpene and mainly isolated from the bark of white birch (*Betula pubescens*) (Fig. 1) (Grymel *et al.*, 2019). Numerous studies have demonstrated that BT has various pharmacological effects, including anti-HIV, anti-malarial, anti-cancer activities (Alakurtti *et al.*, 2006). Previously, we indicated that BT could ameliorate acute alcohol induced liver injury via activating SIRT1-AMPK signaling pathways (Bai *et al.*, 2016; Yao *et al.*, 2017b). Acute alcohol injury is mainly

characterized by oxidative stress and inflammation without significant lipid accumulation. However, many alcoholic liver disease patients have a long history of chronic drinking and an addiction to alcoholism. In this study, the adopted model was highly close to the human's drinking pattern and could mimic the acute episode of alcoholic hepatitis after a single drunkenness of chronic alcohol dependent patients. Therefore, this model is beneficial for investigating the effect and potential mechanism of BT against alcohol induced lipid accumulation and metaflammation. Targeting lipin1/2-meidated P2X7r, the development of BT would provide scientific values against adipose metabolic disease and inflammation.

MATERIALS AND METHODS

Chemical reagents

Betulin (BT) (BET201102212) was purchased from Skyherb Technologies (Hangzhou, China). Primary antibodies of SREBP1 (ab3259), lipin1 (ab181389), lipin2 (ab176347), P2X7r (ab48871), NLRP3 (ab4207), FANS (ab22759), PPAR γ (ab19481) and GAPDH (ab8245) were purchased from Abcam (Cambridge, MA, USA). Primary antibodies of PPAR α (sc-9000), PGC-1 α (sc-518025), IL-6 (sc-28343), IL-18 (sc-133127), IL-1 α (sc-393998) and caspase-1 (sc-514) were obtained from Santa Cruz Biotechnology Inc (Santa Cruz, CA, USA). Primary antibody of IL-1 β (AF-401-NA) was purchased from R&D. Horseradish peroxidase (HRP)-conjugated goat anti-rabbit (ab97051) and rabbit anti-mouse (ab6728) were purchased from Abcam. Horseradish peroxidase (HRP)-conjugated rabbit anti-goat (HAF017) was purchased from R&D. The BCA Protein Assay Kit was obtained from Beyotime (Jiangsu, China). The Mouse IL-1 β Uncoated ELISA Kit was purchased from Invitrogen (Carlsbad, CA, USA) and mouse IL-6 ELISA Kit was obtained from NeoBioscience (EMC004; Shenzhen, China). DMSO was purchased from Sigma Chemical Co (St. Louis, MO, USA). Fetal bovine serum (FBS) and Dulbecco's modified Eagle medium (DMEM) basic (1X) were purchased from Gibco (MA, USA). All other chemical reagents were analytical grade.

Cell culture and treatment

AML-12 cells and macrophage-like murine cell line Raw 264.7 were cultured in Dulbecco's Modified Eagle's Medium (DMEM), contained with 10% fetal bovine serum (FBS), 100 U/mL penicillin and 100 mg/mL streptomycin at 37°C under 5% CO₂. AML-12 cells and Raw 264.7 cells were cultured in 6-well plates at a density of 1×10⁶ per well, and grown to full adherence for 24 h. The cells were treated with EtOH (50 mM), LPS (1 μg/mL) or different concentrations of BT for 24 h. Then, these cells were detected by Western blot, immunofluorescence staining or cell staining experiments, etc.

MTT

AML-12 cells were cultured in 96-well plates at a density of 1×10⁴ per well. Until fully adherent, the cells were treated with BT (0-100 μM) or EtOH (0-400 mM) for 24 h. Subsequently, each pore was added with 3-(4, 5 dimethylthiazol-2-yl)-2,5-diphenylterazolium bromide (MTT) for 3 h, then added DMSO to dissolve blue crystals. The absorbance was measured at 492 nm.

Oil red O staining

AML-12 cells were washed with 1×PBS, fixed with 4% paraformaldehyde solution and permeabilized with 0.1% TritonX-100 at room temperature. After immersed in 60% isopropanol, the cells were added Oil Red O working fluid, and counterstained with hematoxylin and sealed with glycerin gelatin. All the stained cells were examined with a light microscope (Olympus, Tokyo, Japan).

Cell immunofluorescence staining

AML-12 cells were stimulated by EtOH (50 mM) and Raw 264.7 cells were stimulated by LPS (1 μg/mL) or EtOH (50 mM), then cultured with or without BT. The cells were washed with 1×PBS, fixed with 10% paraformaldehyde solution and permeabilized with 0.1% TritonX-100 on ice, then blocked with 5% goat serum. The cells were incubated with primary antibodies overnight at 4°C, and incubated with corresponding secondary antibodies at room temperature. The nucleus was stained with DAPI and photographed with Olympus IX70 fluorescence microscope (Olympus).

Small-interference-RNA (siRNA) transfection

AML-12 cells were transfected with 50 nM control siRNA, lipin1-siRNA or lipin2-siRNA (RiboBio, Guangzhou, China) using the Lipofectamine RNAiMAX reagent (Thermo Fisher Scientific Inc., Waltham, MA, USA). The cells were then harvested 48 h post-transfection for followed-up experiments.

Animal experiments

Male C57BL/6 mice (body weight 20-22 g) were purchased from Changchun Yisi Laboratory Animal Technology Co., Ltd (Changchun, Jilin, China). Animals were acclimatized under 20 ± 2°C, relative humidity of 55 ± 5% and a 12 h light-dark cycle conditions, and allowed free access to standard diet and water ad libitum. The experiment procedures followed guidelines for the care and use of laboratory animals and were approved by Yanbian University (Yanji, China) (Permission number, 20171217).

Mice were randomly divided into five groups: Normal group, EtOH group, EtOH+BT-20 group, EtOH+BT-50 group and pair-fed group. Except normal and pair fed groups, the other groups were given a Lieber-DeCarli alcoholic liquid diet containing 5% EtOH (L-D, TP4030D, Trophic Animal Feed High-tech Co., Ltd, Nantong, China). In the first week, the concentration of EtOH in L-D diet was gradually added from 1% to 5%. On the last day, all mice except for normal and pair-fed groups were given a single gavage 5% EtOH (5 g/kg). Mice in normal group were fed with normal diet, and pair-fed group were fed with L-D control diet (TP4030C). BT groups were daily gavaged with BT (20 or 50 mg/kg) for 4 weeks. After the last alcohol administration 9 h, all mice were sacrificed under anesthesia. Blood samples and liver tissues were collected and stored at -80°C. The detailed process is described in Fig. 2.

Determination of serum biochemical parameters

Serum aspartate aminotransferase (AST), alanine aminotransferase (ALT) and triglycerides (TG) were determined according to the manufacturer's manufacturing instructions by Nanjing Jiancheng Bioengineering Institute (Nanjing, China).

Enzyme-linked immunosorbent assays for IL-1 β and IL-6

Mouse IL-1 β and IL-6 protein level was measured by Mouse

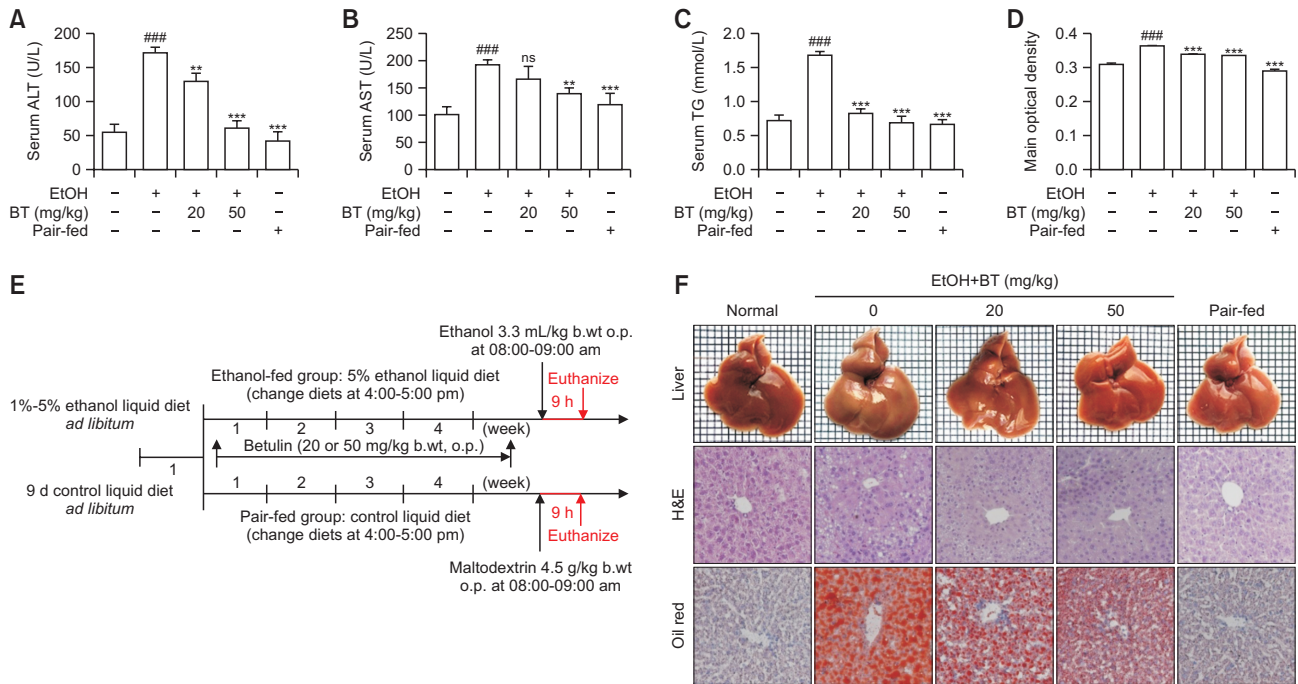


Fig. 2. BT alleviates hepatic steatosis and injury in chronic EtOH administration mice. (A) Serum ALT activities. (B) Serum AST activities. (C) Serum TG levels. (D) Positive area of Oil Red O staining was analyzed with Image Pro-Plus 6.0 (RRID:SCR_016879, Media Cybernetics, Inc., MD, USA). (E) Animal experimental design. (F) Liver appearance pictures, Hematoxylin and Eosin (H&E) stain and Oil red O stain (400×). ###*p*<0.001 vs normal group, ***p*<0.01 and ****p*<0.001 vs EtOH group, ns, not significant. All histograms represent the mean ± SD of at least three independent assays.

IL-1β Uncoated ELISA Kit (Invitrogen) and mouse IL-6 ELISA Kit (EMC004, NeoBioscience) according to the manufacturers' instructions.

Histopathological examination

Liver tissues were fixed with 10% formalin solution, dehydrated with EtOH and embedded in paraffin, then cut into 5 μm sections. For haematoxylin and eosin (H&E) staining, liver sections dewaxing hydration, and then stained with haematoxylin and eosin. For Oil Red O staining, liver cryosections were stained with Oil Red O working fluid and haematoxylin. For immunohistochemistry staining, liver tissue sections antigen restoration, treated with 10% H₂O₂ and blocking with 5% goat serum. The sections were incubated with primary and secondary antibodies, and then visualized with DAB (Maixin Biol, Fu Zhou, China). Finally, the cell nucleus was stained with hematoxylin and sealed with neutral gum. For immunofluorescence staining, liver cryosections were fixed with methanol and acetone, blocked with 5% goat serum, followed with primary antibodies, specific fluorescent antibody and DAPI. All the liver sections were performed by microscopy (TI-E, Nikon, Tokyo, Japan).

Western blotting

Protein samples were lysed by RIPA buffer. Equal quantities of protein samples were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and then transferred to polyvinylidene fluoride (PVDF) membranes (GE healthcare, Freiburg, Germany). The membranes were blocked with 5% skim milk, incubated with the primary anti-

bodies at 4°C overnight, followed with appropriate HRP-conjugated secondary antibodies, and visualized with ECL Detection Reagent (Bio-Rad, Hercules, CA, USA). The intensities of bands were quantified by Quantity One (Bio-Rad).

RT-PCR

Total RNA was extracted from liver tissue using RNA Extraction Kit according to manufacturer's protocols and its concentrations were measured by Nanodrop ND-1000 (Thermo Fisher Scientific, Wilmington, DE, USA). Samples of RNA were reverse-transcribed into cDNA. RT-PCR was performed using pre-set appropriate primers for the gen as described in Table 1. The PCR products were run on 2% agarose gel and stained with ethyl bromide. GAPDH was used as the internal to normalize transcript level of special genes.

Statistical analyses

All the data were expressed as mean ± SD and calculated via one-way analysis of variance and Tukey's multiple comparison tests. Calculations were performed using GraphPad Prism (GraphPad Software, San Diego, CA, USA) and a value of *p*<0.05 was considered as statistically significant.

RESULTS

BT regulates lipid deposition in EtOH-stimulated AML-12 cells

We examined the effects of EtOH or BT on the viability of AML-12 cells by MTT. AML-12 cells were treated with EtOH (0-

Table 1. The primer sequences for RT-PCR

Gene	Primer sequence	Product size
IL-18	5'-GATCAAAGTGCCAGTGAACC-3' 5'-AACTCCATCTTGTGTGCC-3'	866
GAPDH	5'-CTTGTGCAGTGCCAGCC-3' 5'-GCCCAATACGGCCAAATCC-3'	1254
SREBP1	5'-CTTAGCCTCTACACCAACTG-3' 5'-AGGAATACCCTCCTCATAGC-3'	4299
IL-1 β	5'-GTACATCAGCACCTCACAAG-3' 5'-CACAGGCTCTCTTTGAACAG-3'	1328
IL-1 α	5'-CTTGAGTCGGCAAAGAAATC-3' 5'-GAGATGGTCAATGGCAGAAC-3'	1974
P2X7r	5'-AGCGGAAAGAGCCTGTCATC-3' 5'-GCAGGATGTTTCTCGTGGTG-3'	3680
NLRP3	5'-GGTGTGGAATTAGACAACACTGC-3' 5'-TCAAAGACGACGGTCAGCTC-3'	4470
Caspase1	5'-ACATCCTTCATCCTCAGAAAC-3' 5'-GATAATGAGGGCAAGACGTG-3'	1533
TNF- α	5'-TCACACTCAGATCATCTTCTC-3' 5'-AGACTCCTCCCAGGTATATG-3'	1619
IL-6	5'-TCCTCTCTGCAAGAGACTTC-3' 5'-CCAGTTTGGTAGCATCCATC-3'	1087

400 mM) and BT (0-100 μ M) for 24 h. The results showed that EtOH (0-25 mM) and BT (0-25 μ M) had no effect on AML-12 cell viability (Fig. 1B, 1C). Oil Red O staining results showed that the red-stained lipid droplets stimulated with EtOH (50 mM) were obviously alleviated by BT treatment (Fig. 1D). In EtOH-stimulated AML-12 cells, the expression of SREBP1 and lipin1 was significantly increased, and the expression of lipin2 was decreased compared with that in the normal group. BT treatment effectively downregulated the expression of SREBP1 and lipin1 and upregulated the expression of lipin2 compared with EtOH treatment (Fig. 1E). The same results were obtained by immunofluorescence (Fig. 1F, 1G). Thus, BT could ameliorate lipid accumulation by mediating SREBP1 and lipin1/2 in EtOH-stimulated AML-12 cells.

BT ameliorates the inflammatory response by blocking P2X7r-NLRP3 activation in AML-12 or RAW 264.7 cells stimulated with EtOH or LPS

In EtOH-stimulated AML-12 cells, P2X7r and NLRP3 expression was significantly increased compared with that in the normal group, while BT treatment could significantly decrease P2X7r and NLRP3 expression (Fig. 3A). Immunofluorescence staining results also confirmed the above results (Fig. 3B, 3C). These results demonstrated that BT could block P2X7r-NLRP3 activation against EtOH stimulation in AML-12 cells. Moreover, the NLRP3 inflammasome is widely expressed in macrophages exposed to inflammatory stimuli, and we used RAW 264.7 cells to explore how BT regulates P2X7r/NLRP3 in macrophages. In LPS- or EtOH-stimulated RAW 264.7 cells, P2X7r and NLRP3 expression was significantly increased compared with that in the normal group, while BT treatment significantly decreased P2X7r and NLRP3 expression (Fig. 3D). Similarly, immunofluorescence staining also showed that BT treatment could significantly downregulate the expression of P2X7r and NLRP3 caused by LPS or EtOH (Fig. 3E, 3F). These results suggested that BT could improve the

EtOH-induced hepatocyte inflammatory response by inhibiting P2X7r-NLRP3 reduce the activation of P2X7r-NLRP3 in LPS- or EtOH-induced macrophages.

Lipin1/2 are indispensable for BT regulating lipid accumulation and metaflammation

To further confirm that BT alleviates lipid accumulation and metaflammation by lipin1/2-mediated P2X7r, AML-12 cells were transfected with siRNA against lipin1 or lipin2 and then treated with EtOH or BT, respectively. Expression of lipin1 or lipin2 was significantly decreased by treating with an siRNA lipin1 or siRNA lipin2. Lipin1 deficiency resulted in a down-regulation on SREBP1 and P2X7r expressions, while the EtOH-stimulated up-regulation of SREBP1 and P2X7r were strengthened by lipin2 deficiency. These results illustrated that EtOH could regulate SREBP1 and P2X7r expressions by targeting lipin1/2. In AML-12 cells with silencing lipin1, BT significantly decreased the expressions of SREBP1 and P2X7r compared with siRNA against lipin1 and EtOH group; lipin1-deficiency promoted the regulation of BT on SREBP1 expression (Fig. 4A). In AML-12 cells with silencing lipin2, BT significantly decreased the expressions of SREBP1 and P2X7r compared with siRNA against lipin2 and EtOH group; lipin2-deficiency blocked the regulation of BT on SREBP1 and P2X7r expressions (Fig. 4B). These results suggested that BT could target lipin1/2 to block the P2X7r-NLRP3, which might be potential therapeutic strategy for BT against lipid accumulation and metaflammation caused by EtOH.

BT alleviates hepatic steatosis and injury in chronic EtOH administration mice

Compared with the normal and pair-fed groups, serum ALT, AST and TG levels were significantly increased in the EtOH group, indicating that chronic EtOH administration could induce liver injury and lipid accumulation. BT treatment obviously downregulated these serum parameters compared with

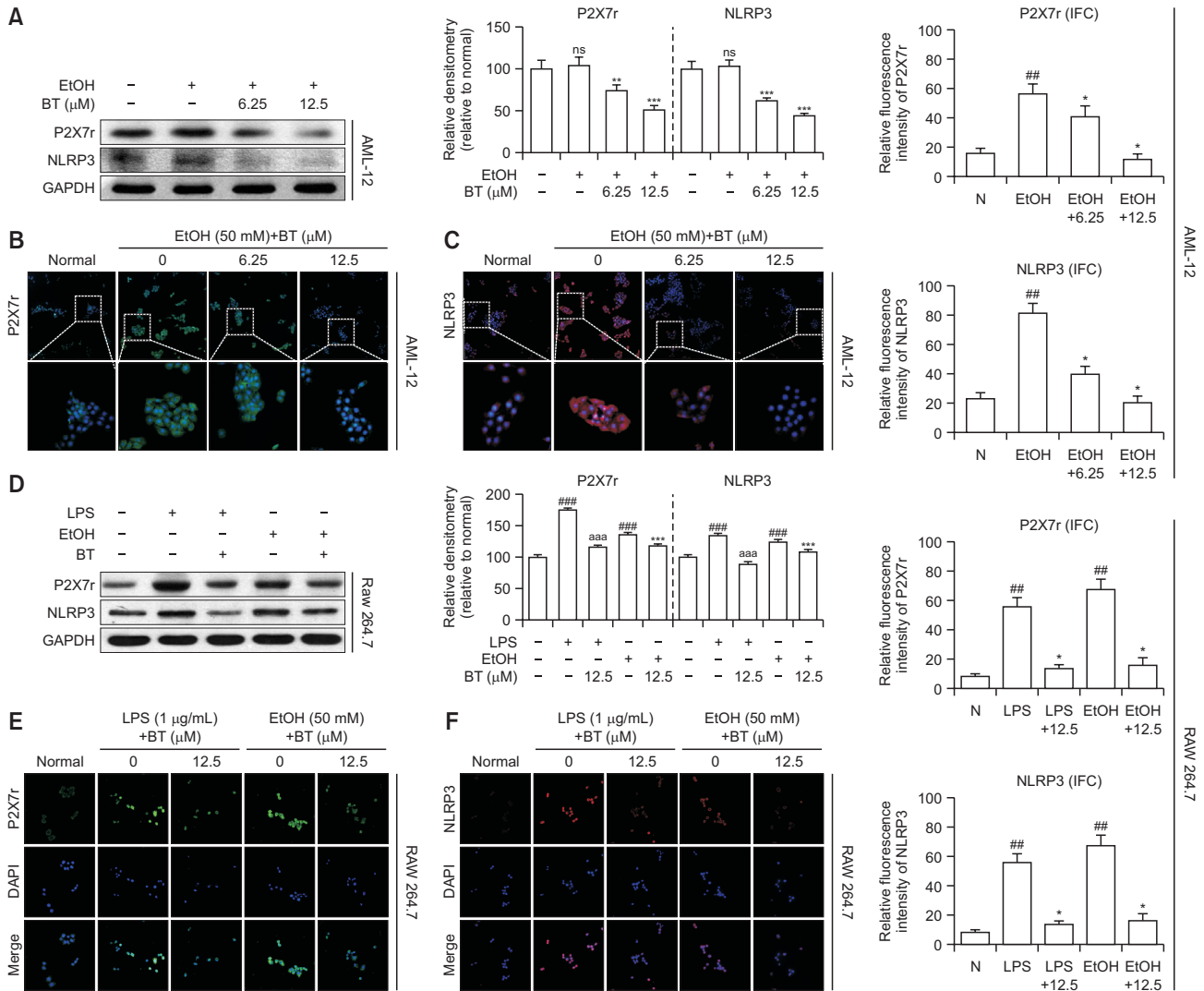


Fig. 3. BT ameliorates the inflammatory response by blocking P2X7r-NLRP3 activation in AML-12 or RAW 264.7 cells stimulated with EtOH or LPS. (A) Representative Western blotting analysis for expressions of P2X7r and NLRP3 in AML-12 cells. (B) Immunofluorescence staining of P2X7r expression in AML-12 cells (100× or 600×). (C) Immunofluorescence staining of NLRP3 expression in AML-12 cells (100× or 600×). (D) Representative Western blotting analysis for expressions of P2X7r and NLRP3 in RAW 264.7 cells. (E) Immunofluorescence staining of P2X7r expression in RAW 264.7 cells (600×). (F) Immunofluorescence staining of NLRP3 expression in RAW 264.7 cells (600×). The GAPDH was used as internal reference to normalize the data. For immunofluorescence staining, ^{##}*p*<0.01 vs normal group; ^{*}*p*<0.05 vs EtOH group or LPS group. ^{###}*p*<0.001 vs normal group, ^{**}*p*<0.01, ^{***}*p*<0.001 vs EtOH group, ^{aaa}*p*<0.001 vs LPS group, ns, not significant. All histograms represent the mean ± SD of at least three independent assays.

EtOH treatment (Fig. 2A-2C). Liver tissue in EtOH group appeared whitish in color and swollen compared with normal group, while these changes were reversed by BT treatment, and pair-fed group was no significant difference compared with normal group. In the EtOH group, H&E and Oil Red O staining showed massive red-stained lipid droplets, inflammatory cell infiltration and nuclear condensation compared with the normal group, while BT treatment significantly ameliorated these histological changes (Fig. 2D, 2F). These results suggested that BT treatment could obviously relieve hepatic steatosis and injury caused by chronic EtOH administration.

BT regulates SREBP1 and PPARα/γ expression in chronic EtOH-treated mice

SREBP1 is an important transcriptional regulator in fatty acid synthesis and results in the upregulation of triglyceride synthesis and further to fatty liver. In the EtOH group, the protein and mRNA expression levels of SREBP1 were significantly increased compared with those in the normal group and were decreased by BT treatment. In addition, chronic EtOH administration elevated the protein expression of FASN and PPARγ and decreased the protein expression of PPARα compared with the normal group. While BT treatment significantly upregulated the expression of PPARα and downregulated the expression of FASN and PPARγ compared with EtOH treatment (Fig. 5A). Immunohistochemical staining further

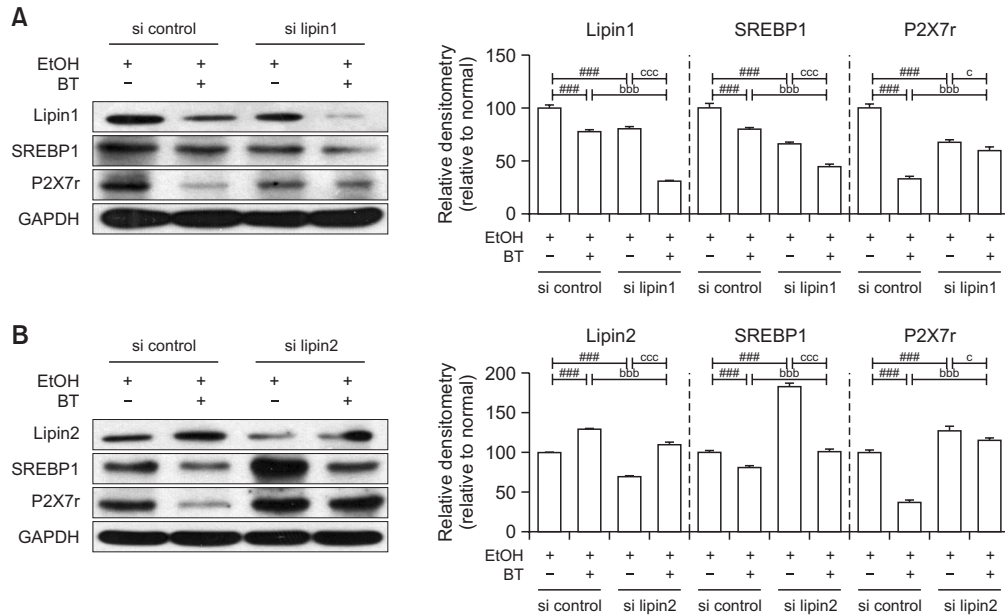


Fig. 4. BT regulates lipid accumulation and inflammatory response in AML-12 cells specifically silencing with siRNA lipin1/2. (A) Representative Western blotting analysis for expressions of lipin1, SREBP1 and P2X7r in AML-12 cells specifically silencing with siRNA lipin1. (B) Representative Western blotting analysis for expressions of lipin2, SREBP1 and P2X7r in AML-12 cells specifically silencing with siRNA lipin2. The GAPDH was used as internal reference to normalize the data. ###*p*<0.001 siRNA control group vs siRNA control-BT group, ^{bbb}*p*<0.001 siRNA control-BT group vs siRNA lipin1-BT group, ^c*p*<0.05, ^{ccc}*p*<0.001 siRNA lipin1 group vs siRNA lipin1-BT group. All histograms represent the mean ± SD of at least three independent assays.

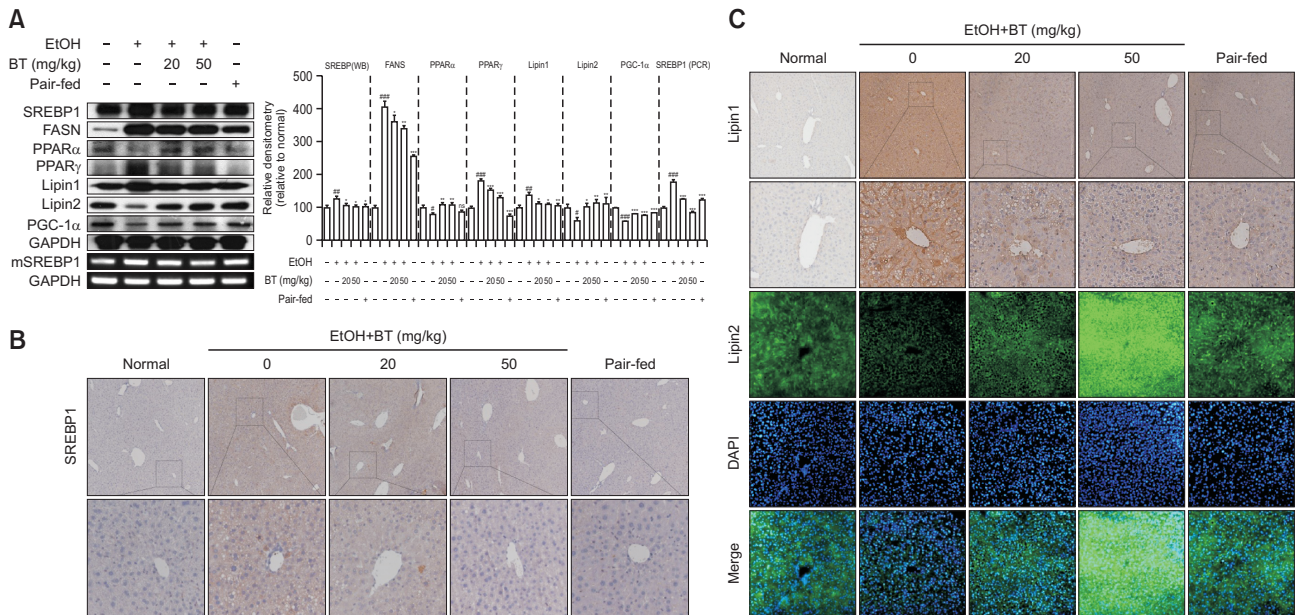


Fig. 5. BT regulates SREBP1 and PPAR α/γ expression in chronic EtOH-treated mice. (A) Representative Western blotting analysis for expressions of SREBP1, FASN, PPAR α , PPAR γ , lipin1, lipin2 and PGC-1 α ; RT-PCR analysis for expressions of SREBP1. (B) Immunohistochemical staining of SREBP1 present in 100 \times and 400 \times magnification. (C) Immunohistochemical staining of lipin2 present in 100 \times and 400 \times magnification. Densitometric values were normalized against GAPDH. ###*p*<0.001, #*p*<0.01, **p*<0.05 vs normal group, **p*<0.05, ***p*<0.01, ****p*<0.001 vs EtOH group, ns, not significant. All histograms represent the mean ± SD of at least three independent assays.

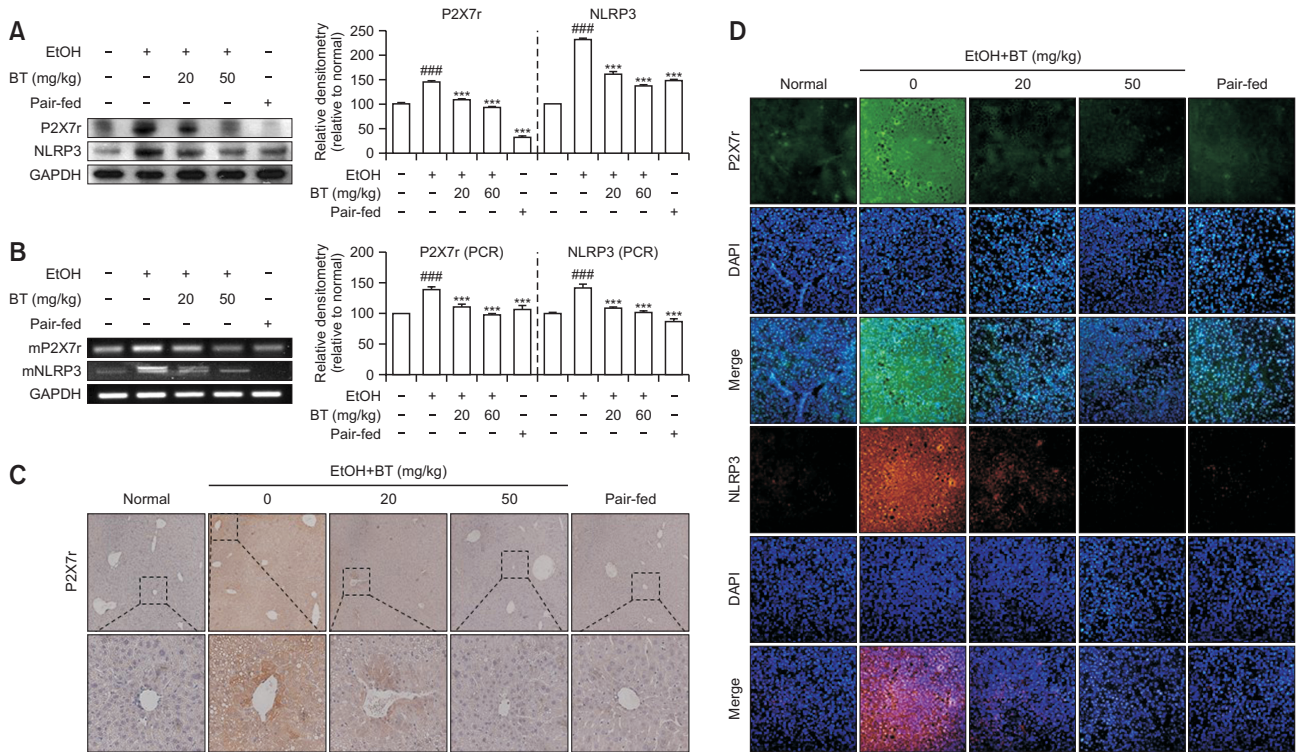


Fig. 6. BT regulates P2X7r and NLRP3 expressions in chronic EtOH administration mice. (A) Representative Western blotting analysis for expressions of P2X7r and NLRP3. (B) Representative RT-PCR analysis for expressions of P2X7r and NLRP3. (C) Immunohistochemical staining of P2X7r present in 100× and 400× magnification. (D) Immunofluorescence staining of P2X7r present in 100× magnification and immunofluorescence staining of NLRP3 present in 100× magnification. Densitometric values were normalized against GAPDH. ###*p*<0.001 vs normal group, ****p*<0.001 vs EtOH group. All histograms represent the mean ± SD of at least three independent assays.

confirmed that BT could obviously decrease the positive (in brown) expression of SREBP1 caused by chronic EtOH administration (Fig. 5B). In the pair-fed group, the expression levels of SREBP1, FASN, PPAR α and PPAR γ were not significantly different compared with those in the normal group (Fig. 5A). These results demonstrated that BT could inhibit hepatic lipogenesis in chronic EtOH-treated mice.

BT regulates lipin1 and lipin2 expressions in chronic EtOH administration mice

Chronic EtOH administration obviously increased the expression of lipin1, as well as decreased the expression of lipin2 compared with normal group. BT treatment could reverse the expressions of lipin1 and lipin2 (Fig. 5A). Immunohistochemistry and immunofluorescence staining results also confirmed that BT inhibited lipin1 expression and increased lipin2 expression caused by chronic EtOH administration (Fig. 5C). As a transcription cofactor of multiple metabolic pathways, the expression of PPAR gamma coactivator-1 α (PGC-1 α) was decreased caused by alcohol exposure, while BT treatment increased its expression (Fig. 5A). These results showed that the regulation of lipin1/2 might be necessary for BT to alleviate lipid deposition and hepatic steatosis in chronic EtOH administration mice.

BT inhibits the secretion of inflammatory cytokines by blocking P2X7r and NLRP3 expression in chronic EtOH-treated mice

In the EtOH group, the protein expression levels of P2X7r and NLRP3 were significantly increased compared with those in the normal group, while BT treatment reversed the P2X7r and NLRP3 levels that were increased by EtOH challenge (Fig. 6A). RT-PCR, immunohistochemistry and immunofluorescence staining also confirmed the same results (Fig. 6B-6D). To further assess the anti-inflammatory effects of BT, we used Western blot, real-time PCR and ELISA kits to examine the mRNA and protein expression levels of inflammatory cytokines, respectively. As expected, we observed high protein levels of IL-1 β and IL-6 in EtOH-induced mouse livers compared with normal mice, while BT treatment reduced IL-1 β and IL-6 levels (Fig. 7A, 7B). Similarly, the protein and mRNA expression levels of IL-6, IL-18, IL-1 α , IL-1 β , caspase-1 and TNF- α were markedly increased after chronic EtOH administration and were reduced by BT treatment (Fig. 7C, 7D). These results suggested that BT could inhibit the secretion of inflammatory factors by blocking P2X7r and NLRP3, thereby alleviating the inflammatory response caused by chronic EtOH administration.

DISCUSSION

The current study demonstrated that BT could obviously

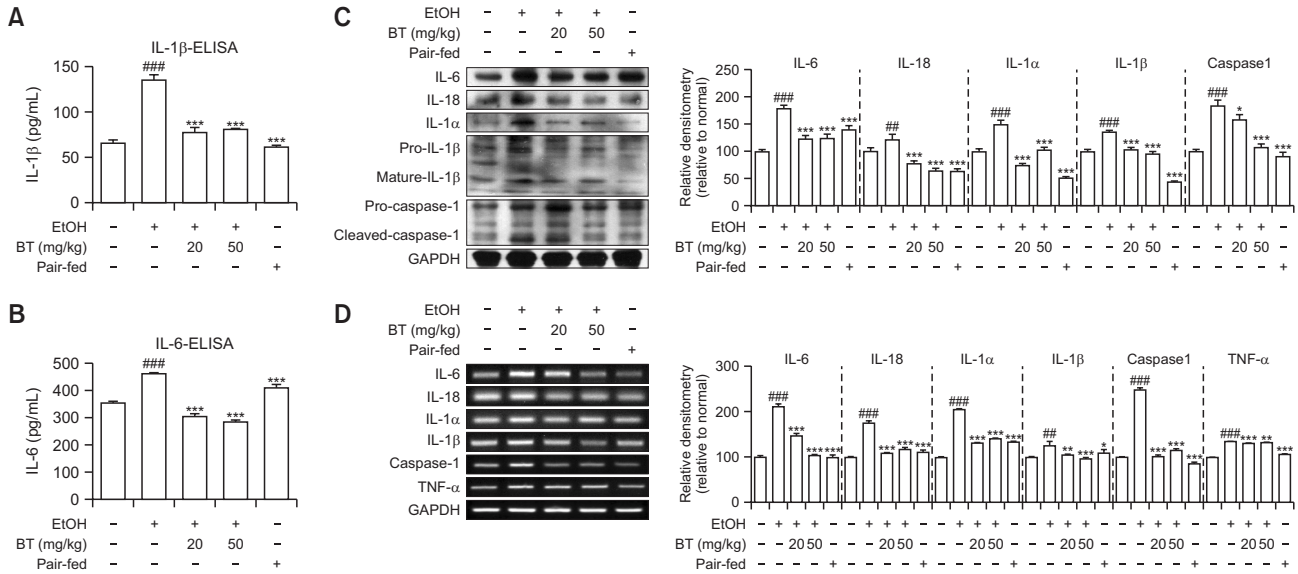


Fig. 7. BT inhibits the secretion of inflammatory cytokines by blocking P2X7r and NLRP3 expression in chronic EtOH-treated mice. (A) Protein expression of IL-1β was determined by ELISA kit. (B) Protein expression of IL-6 was determined by ELISA kit. (C) Representative Western blotting analysis for expressions of IL-6, IL-18, IL-1α, IL-1β and Caspase-1. (D) Representative RT-PCR analysis for expressions of IL-6, IL-18, IL-1α, IL-1β, Caspase-1 and TNF-α. Densitometric values were normalized against GAPDH. ###*p*<0.001, #*p*<0.01 vs normal group, **p*<0.05, ***p*<0.01, ****p*<0.001 vs EtOH group. All histograms represent the mean ± SD of at least three independent assays.

ameliorate liver lipid deposition and metaflammation by reducing serum ALT, AST and TG levels, regulating SREBP1, PPARα/γ, lipin1/2 and PGC-1α expressions and inhibiting inflammation factors production. Moreover, BT could inhibit P2X7r-NLRP3 signaling pathways to against lipid metaflammation, which were verified in chronic EtOH administration mice and EtOH/LPS-stimulated AML-12 cells and Raw 264.7 cells. Lipin1 deficiency suppressed SREBP1 and P2X7r expressions, while lipin2 deficiency showed the opposite results. Interestingly, the lipin1/2 deficiency significantly affected the regulation of BT on SREBP1 and P2X7r to further attenuate lipid accumulation and inflammation caused by EtOH. Therefore, BT targeted lipin1/2-mediated P2X7r to ameliorate steatosis and inflammation in chronic alcoholic liver disease, which suggested that BT would be a promising therapeutic candidate for chronic alcoholic liver disease.

In previous studies, we found that BT could alleviate acute alcohol-induced liver injury (Wan *et al.*, 2013; Bai *et al.*, 2016). Short-term acute drinking cannot completely mimic human alcoholic liver disease, whereas long-term chronic alcohol accumulation can induce metabolic diseases, such as alcoholic fatty liver development to hepatitis, cirrhosis, and even liver cancer (Liu, 2014; Wu *et al.*, 2016). To simulate chronic alcohol-induced lipid accumulation and metaflammation, the experimental animals were fed an L-D liquid diet containing 5% EtOH for 4 weeks, and followed by a single EtOH gavage on the last day (Bertola *et al.*, 2013). *In vitro*, AML-12 cells were directly incubated with medium containing 50 mM EtOH.

Growing evidence has demonstrated that lipogenesis plays a key role in the development of alcoholic liver disease. SREBP1 is a key transcription factor controlling adipogenesis. Mature SREBP1 enters the nucleus and activates the transcription of genes involved in cholesterol and fatty acid synthesis (Shimano, 2000). Our previous study and other scholars' achievements have shown the evidence that SREBP1

is the key point for excessive lipid accumulation in the liver (Ruiz *et al.*, 2014; Yao *et al.*, 2017a; Song *et al.*, 2018). Thus, the current study found that BT significantly inhibited the expression of SREBP1 to regulate lipid accumulation caused by EtOH. Lipin1 is a member of the lipin family, which can interact with the transcriptional coactivator PGC-1α to increase hepatic fatty acid oxidation (Bi *et al.*, 2015). In addition, PPARα/γ are also involved in fatty acid metabolism and transport (Galli *et al.*, 2001; Nanji *et al.*, 2004; Han *et al.*, 2021). In our previous studies, alcohol exposure upregulated SREBP1, FASN, PPARγ and lipin1 expression and inhibited PPARα activity, further limiting fatty acid degradation and increasing fat accumulation (Yao *et al.*, 2017b; Zhang *et al.*, 2020). As expected, our data showed that BT could reverse the changes in of SREBP1, FASN, PPARα/γ, lipin1/2 and PGC-1α caused by chronic alcohol administration, further relieving lipid deposition and the development of chronic alcoholic liver disease.

Except for lipid metabolism, the continuous inflammatory response could lead to inflammatory infiltration, hepatocyte necrosis and liver fibrosis, which cannot be reversed (Cui *et al.*, 2021). P2X7r is an ATP-activated ionotropic purinergic receptor, whereas extracellular ATP is sensed by immune cells as a danger signal (Huang *et al.*, 2014). P2X7r could mediate the maturation and secretion of interleukin-1β (IL-1β) and activate the NLRP3 inflammasome (Ferrari *et al.*, 2006). Immature pro-IL-1β requires cleavage into mature IL-1β under the cleavage of caspase-1, and the activation of caspase-1 depends on NLRP3 inflammasome complex formation (Ogura *et al.*, 2006). In the current study, BT inhibited the activation of P2X7r and the NLRP3 inflammasome, further inhibiting the cleavage of caspase-1. Lacking the cleavage of caspase-1, immature pro-IL-1β could also not be cleaved into mature IL-1β, thereby exerting its anti-inflammatory activity. Moreover, studies have shown that lipin2 can reduce the K⁺ efflux promoted by ATP and IL-1β production and regulate P2X7r

pore formation. Furthermore, the production of IL-1 β was significantly increased in lipin2 knockout human macrophages, while the primary BM-derived macrophages showed that IL-1 β production was abrogated when key elements of the NLRP3 inflammasome were knocked out. These results revealed the importance of lipin2 in the regulation of P2X7r and NLRP3 inflammasome activation (Lordén *et al.*, 2017). The current study found that lipin1 deficiency inhibited SREBP1 and P2X7r expressions, whereas lipin2 deficiency improved their expressions on the contrary. And BT treatment could further suppress the expressions of SREBP1 and P2X7r. Thus, BT may relieve the lipid accumulation and metaflammation caused by EtOH through targeting lipin1/2 to block the P2X7r-NLRP3 signaling pathway.

In summary, the study revealed the hepatoprotective effect of BT against chronic alcohol-induced lipid accumulation and metaflammation through targeting lipin1/2-mediated P2X7r signaling pathway, which supplied fundamental experimental support and potential clinical value. However, it's still needed to subsequent deeper study to explore the metabolism, bio-availability and drug delivery system of BT before applications.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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