Original Article

Γ-Aminobutyric acid promotes methionine-choline deficient diet-induced nonalcoholic steatohepatitis

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Abstract

Nonalcoholic steatohepatitis (NASH) is one of the most common liver diseases and a major cause of liver fibrosis worldwide. Γ-Aminobutyric acid (GABA) is one of the most abundant inhibitory neurotransmitters in the central nervous system. Recently, it has been reported that GABAergic signaling pathways are found in various non-neuronal tissues including the immune system and play a functional role. In the present study, we investigated whether administration of GABA has effects on NASH through its immunomodulatory effects. To test this hypothesis, C57BL/6 mice were fed a methionine–choline-deficient (MCD) diet for 8 weeks. After four weeks into MCD feeding, mice were provided with plain water (control) or water containing 2 mg/mL of GABA for the subsequent 4 weeks. Using this MCD diet-induced NASH model, we found that mice receiving GABA showed more severe steatohepatitis and liver fibrosis than control mice. This increased liver damage was confirmed by higher levels of serum alanine transaminase (ALT) and aspartate aminotransferase (AST) compared to the control group. In accordance with increased liver steatohepatitis, NASH-related and inflammatory gene expression (collagen α1, tissue inhibitor of metalloproteinase-1, TNF-α) in the liver was markedly increased in GABA-treated mice. Furthermore, GABA directly enhanced production of inflammatory cytokines including IL-6 and TNF-α in LPS activated RAW macrophage cells and increased TIB–73 hepatocyte death. Such effects were abolished when GABA was treated with bicuculline, a competitive antagonist of GABA receptors. These results suggest that oral administration of GABA may be involved in changes of the liver immune milieu and conferred detrimental effects on NASH progression.

Keywords: Γ-Aminobutyric acid, nonalcoholic steatohepatitis, methionine–choline-deficient diet, mice, GABA

Introduction

Nonalcoholic fatty liver disease (NAFLD) and its advanced stage, nonalcoholic steatohepatitis (NASH), are the most common causes of chronic liver disease in the world[1]. NAFLD and NASH are increasingly

prevalent, and major risk factors for their development including overnutrition, obesity, insulin resistance, glucose intolerance, and dyslipidemia[2]. NASH is characterized by steatosis with severe inflammation and fibrosis and can eventually lead to end-stage liver disease[3]; however, the complexity and pathology of physiologic events leading to development of NASH are not fully understood. Among related mechanisms, tumor necrosis factor α (TNF-α) appears to play a critical role in hepatic inflammatory cell recruitment in NAFLD and NASH[4–6]. Furthermore, endotoxin and lipopolysaccharide (LPS) contribute to progressive liver injury. Increased plasma endotoxin levels have been detected in mice with methionine-choline-deficient (MCD) diet-induced steatohepatitis and in humans with NAFLD[7].

Γ-Aminobutyric acid (GABA) is a non-protein amino acid that is produced by animals, plants, and micro-organisms and functions as a neurotransmitter in the central nervous system[8]. In mammals, GABA is produced by some peripheral tissue cells. GABA$_A$ receptors are expressed in adipose tissues and immunocompetent cells, such as macrophages and T cells, and exist due to the combinatorial assembly of their various subunits[9–11]. The presence of several GABA$_A$ receptor subunits has been described in mammals such as guinea pigs, humans, rats, and mice[12–15]. Especially in the liver, GABA$_A$ receptor consisting of the β3- and ε-subunit types is expressed in the human hepatocyte, whereas only the β3-subunit type is expressed in the rat hepatocyte[16]. Recent studies have shown that activation of GABA$_A$ receptors inhibits inflammatory diseases, such as type 1 diabetes, experimental autoimmune encephalomyelitis, and collagen-induced rheumatoid arthritis, in experimental animals[17–20]. Furthermore, GABA administration can regulate metabolic conditions by stimulating secretion of insulin or protein synthesis in the nervous system and improving the concentration of growth hormone[8,16,19]. Due to its physiologic functions, GABA is widely used in pharmaceuticals and also has the possibility of providing new enriched health products[21].

The potential activities of GABA have only focused on its beneficial anti-inflammatory effects in animal models. Further biologic activities of GABA including possible detrimental effects on the immune system have not yet been clearly investigated.

Therefore, in the present study, we investigated whether GABA administration has any effects on the progression of NASH in an MCD diet-fed mouse model.

Materials and methods

Animals and experimental protocol

Male C57BL/6 mice (5 weeks of age, 25-30 g body weight) obtained from Taconic Farms, Inc. (Samtako Bio Korea, O-San, South Korea) were used for experiments. Mice were maintained in a standard condition (24±2°C, 50±5% humidity), pathogen-free environment and were fed sterile normal diet (ND) or MCD diet and water *ad libitum*. Experimental procedures and animal management procedures were undertaken in accordance with the requirements of the Animal Care and Ethics Committees of Chonbuk National University.

Male C57BL/6 mice ($n = 6$ mice per group) were fed an MCD diet (Dyets Inc., PA, USA) for 8 weeks, which is an established model of NASH[22]. After four weeks into the MCD diet, mice were provided with plain water (control) or water containing 2 mg/mL of GABA (Sigma, St. Louis, USA) for the subsequent 4 weeks.

Histopathology

For histopathological examination of liver sections using light microscopy (BX-51, Olympus Corp., Tokyo, Japan), the liver tissues were collected, fixed in 10% neutral buffered formalin solution, routinely processed, and then embedded in paraffin. Tissue sections (6 μm) were prepared using a microtome (HM-340E, Thermo Fisher Scientific Inc., MA, USA) and placed on glass slides. Hematoxylin and eosin (H&E) staining was performed according to standard protocols. For lipid staining, 6 μm frozen liver sections were air-dried for 30 min, followed by fixation in 4% formaldehyde. Oil red O staining was performed using the previously described method[23].

Determination of liver fibrosis by Sirius red staining

Direct red 80 and Fast-green FCF (color index 42053) were obtained from Sigma-Aldrich Diagnostics. Liver sections were stained, and red-stained collagen fibers were quantified by the percentage of positive area per total liver section. All liver section images for each animal were analyzed using light microscopy and digital imaging software (analySIS TS, Olympus Corp., Tokyo, Japan). Data are expressed as the percentage of Sirius red-positive area per field.

Detection of in situ apoptosis by TUNEL staining

For detection of apoptotic cells in the liver, TdT-mediated dUTP nick-end labeling (TUNEL) staining was performed on cryosections using an ApopTaq

Peroxidase in situ apoptosis detection kit (Chemicon, Temecula, CA, USA) according to the manufacturer's instructions. The positive reaction was visualized with 3,3′-diaminobenzidine (DAB) substrate, and then nuclear counterstaining was performed using methyl green dye. TUNEL-labeled cells were quantified as the percentage of positive area per high-power field. A total of 10 high-power fields of liver tissue were analyzed from each animal using light microscopy and digital imaging software. Data are expressed as the percentage of TUNEL-positive area.

Biochemical assays

Serum levels of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) were determined using AM101-K spectrophotometric assay kits (ASAN Pharmaceutical, Hwasung, Korea). Triglyceride (TG) and total cholesterol contents in liver were determined using an AM202-K spectrophotometric assay kit (ASAN Pharmaceutical, Hwasung, Korea).

Quantitative real-time polymerase chain reaction (qPCR)

Total RNA was isolated from tissue using the Easy-Spin Total RNA extraction kit (*iNtRon* Biotech, Seoul, *Korea*). Following incubation with RNase-free DNase I (Promega, Madison, WI), reverse transcription was performed using a random primer and MultiScribe™ MuLV reverse transcriptase (Applied Biosystems, Foster City, CA) according to the manufacturer's instructions. cDNA was subjected to real-time PCR on a CFX96™ Real-Time PCR Detection System (Bio-Rad Laboratories, CA, USA) using SYBR Green I as a double-strand DNA-specific binding dye. After the reaction was complete, specificity was verified by melting curve analysis. Quantification was performed by comparing Ct values of each sample after normalization to GAPDH.

Immunohistochemical evaluation

For immunohistochemical staining, mouse livers were frozen in optimum cutting temperature (OCT) compound (Sakura Finetechnical Co., Tokyo, Japan). Six-μm-thick sections were cut, air-dried, and fixed in cold solution (1:1 mixture of acetone and methanol) for 15 minutes at −20°C. Non-specific binding was blocked with 3% peroxidase solution, followed by 10% normal goat serum. The Pollink-2 HRP Plus Rat-NM DAB detection kit (Golden Bridge International Inc., Mukilteo, WA, USA) was used for immunostaining. Briefly, the sections were incubated with anti-mouse CD11c antibody (eBioscience, San Diego, CA, USA) in a moist chamber for 30 minutes. They were then incubated with rat antibody enhancer (10 minutes), polymer-horseradish peroxidase (10 minutes), and DAB substrate (5 min). Finally, the sections were counterstained with hematoxylin and mounted with mounting medium. The sections were analyzed using light microscopy and digital imaging software. Data are expressed as the percentage of positive area per field.

Isolation of hepatic non-parenchymal cells (NPCs)

For isolation of hepatic NPCs, mouse livers were pressed through a 200-gauge stainless steel mesh. The liver cell suspension was suspended in RPMI-1640 medium and centrifuged at 50 *g* for 4 minutes. Supernatant containing NPCs was collected, washed in PBS, and resuspended in 40% Percoll (Sigma) in RPMI-1640 medium. The cell suspension was gently overlaid onto 70% Percoll and centrifuged for 20 minutes at 750 *g*. NPCs were collected from the interface, washed twice in PBS, and resuspended in RPMI-1640 medium.

Cell culture and treatment

The murine normal hepatocyte cell line TIB-73 and murine monocyte/macrophage cell line RAW264.7 were obtained from ATCC (Rockville, MD, USA). The cells were cultured at 37°C under 5% CO_2 in Dulbecco's modified Eagle's medium (DMEM, PAA Laboratories) supplemented with 10% fetal calf serum (Hyclone, Utah, USA), 4 mmol/L L-glutamine (PAA Laboratories), 100 IU/mL penicillin, and 100 μg/mL streptomycin (PAA Laboratories). GABA was dissolved in DMEM containing 1% FBS to make 20 mmol/L stock solution. Then, 100 and 200 nmol/L working solution was prepared. Bicuculline was dissolved in chloroform, and its solutions were made as follows: Bicuculline powder was added to chloroform by shaking intensively to yield a 100 mmol/L stock solution. Then, various concentrations of bicuculline working solution (25, 50, 100, and 200 μmol/L) were prepared. One μg/mL of LPS was used for macrophage cell line stimulation.

Measurement of ALT and cytokines

Cell culture supernatant concentrations of mouse TNF-α and IL-6 were measured with ELISA using a Quantikine kit from R&D Systems (Minneapolis, MN, USA). Cell culture supernatant and serum were used to measure ALT and AST levels. All experiments were performed according to the manufacturer's instructions.

Statistical analysis

All data are expressed as mean±standard error.
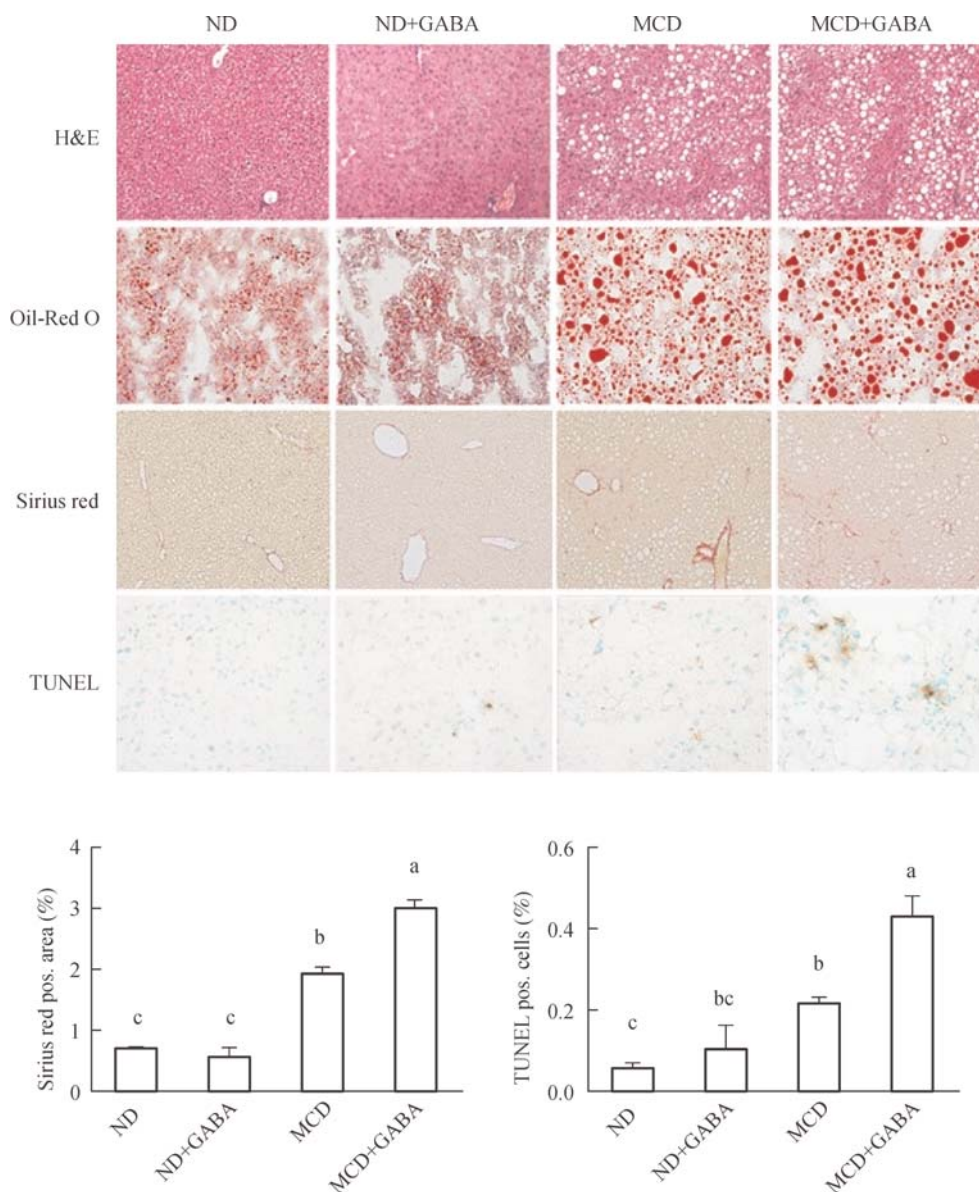
Fig. 1 GABA-treated mice show increased histopathologic changes on liver sections. Male mice were divided into 4 groups and fed a normal diet (ND) or methionine-choline deficient (MCD) diet for 8 weeks. After 4 weeks of MCD diet, the mice were treated with GABA (2 mg/ mL) or vehicle (N = 6-7/group) for another 4 weeks. Liver sections were stained with H&E, oil-red O, Sirius red, and TUNEL. Values are presented as mean±standard error per group. Samples were analyzed using a one-way ANOVA. Different letters indicate significant differences at $P < 0.05$, and bars sharing the same letter are not significantly different.

Differences between multiple groups were compared using one-way analysis of variance (ANOVA) in SAS version 9.1 (SAS Institute Inc., Cary, NC, USA), while individual comparisons were obtained using Duncan's Multiple Range Test (DMRT). A value of $P < 0.05$ was considered statistically significant.

Results

GABA enhances hepatocyte death and liver fibrosis

To evaluate the effect of GABA on liver metabolic function, C57BL/6 mice were fed an MCD diet for 8 weeks and provided with plain water containing 2 mg/ mL of GABA for the last 4 weeks of MCD feeding. There was no significant difference between the water- and GABA-treated groups in the mean amount of food intake or body weight of individual mice (data not shown).

As shown in *Fig. 1*, the MCD diet induced marked lipid accumulation with inflammatory cell infiltration in the liver, hepatocyte death, and liver fibrosis. Although no significant differences in lipid accumulation were observed between the GABA- and water-treated groups, GABA-treated, MCD diet-fed mice experienced a significant increase in liver fibrosis and hepatocyte death compared with mice fed MCD but not GABA.
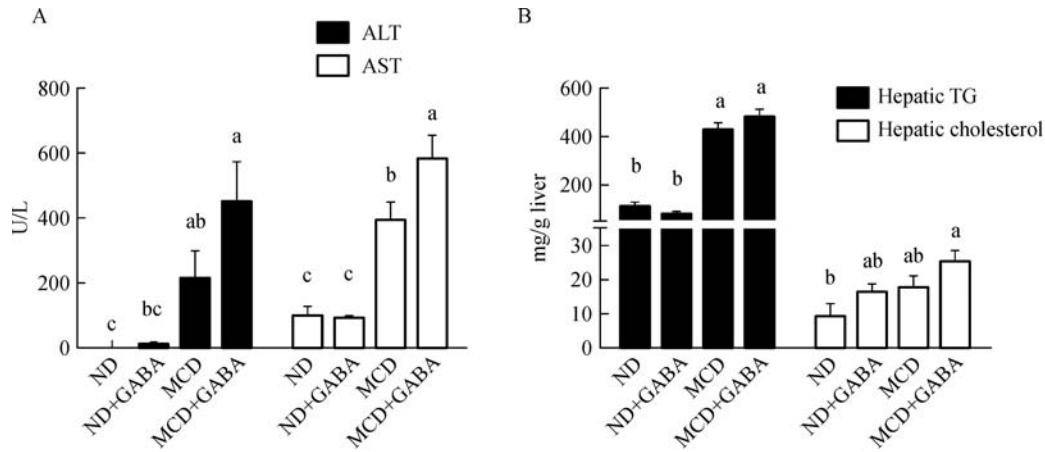
Fig. 2 GABA administration accelerated liver injury and steatosis. (A) Serum ALT and AST levels were measured (N = 6-7/group). (B) Hepatic triglyceride (TG) and total cholesterol contents were measured from normal and MCD-fed mice (N = 6-7/group) treated with vehicles or GABA (2 mg/mL) for 4 weeks. Values are presented as mean±standard error per group. Samples were analyzed using a one-way ANOVA. Different letters indicate significant differences at $P < 0.05$, and bars sharing the same letter are not significantly different.

Increased liver injury by GABA in MCD-fed mice was confirmed by higher levels of serum ALT and AST (**Fig. 2A**). In accordance with steatosis examined by oil red O staining, no significant differences were observed in hepatic TG or total cholesterol after GABA administration (**Fig. 2B**). These results suggest that GABA does not affect steatosis but does induce liver injury.

NASH-related gene expression is increased in GABA-treated, MCD diet-fed mice

To evaluate NASH-related gene expression, qPCR was performed on mouse livers. The mRNA levels of tissue *Timp1* and *Col1* were markedly increased in GABA-treated, MCD diet-fed mice compared to MCD

diet-fed mice not receiving GABA (**Fig. 3A**). Additionally, GABA-treated, MCD diet-fed mice showed slightly higher *TNF-α* mRNA expression compared to MCD diet-fed mice not receiving GABA (**Fig. 3B**). However, in NPCs isolated from liver using the Percoll density gradient method, *TNF-α* mRNA expression in GABA-treated, MCD diet-fed mice was significantly increased, indicating that severe inflammatory immune response occurs in the livers of these mice.

GABA regulates the immune response in MCD diet-induced NASH

To investigate the mechanism of NASH progression caused by GABA, changes in immune cell populations in the liver were examined. As shown in **Fig. 4**, MCD
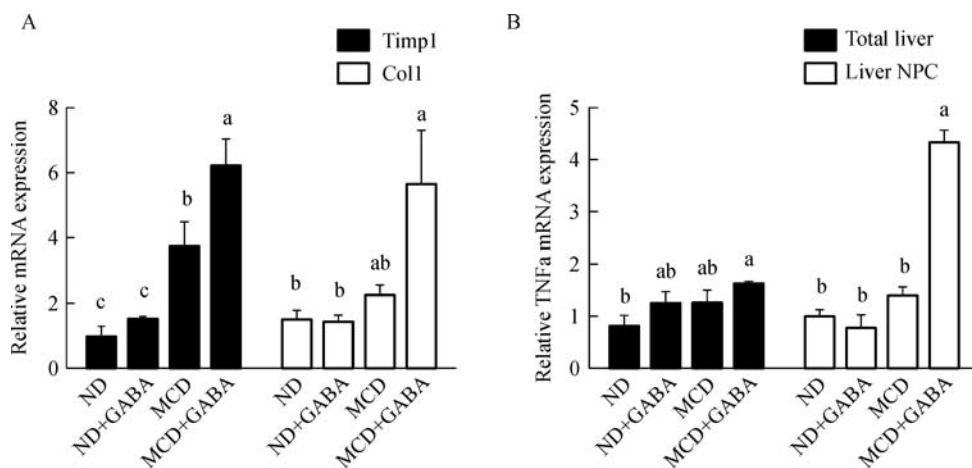
Fig. 3 Effects of GABA administration on the expression of liver fibrogenic genes. (A) Hepatic mRNA levels of *Timp1* and *col1* (fibrogenic markers) were determined by quantitative real-time PCR (N = 6-7/group). (B) Hepatic *TNF-α* mRNA levels in whole liver and NPCs from mice fed normal and MCD diets with vehicle or GABA (2 mg/mL) were determined by quantitative real-time PCR (N = 6-7/group). NPCs were isolated from the liver using the Percoll density gradient method. Values are presented as mean±standard error per group. Samples were analyzed using a one-way ANOVA. Different letters indicate significant differences at $P < 0.05$, and bars sharing the same letter are not significantly different.
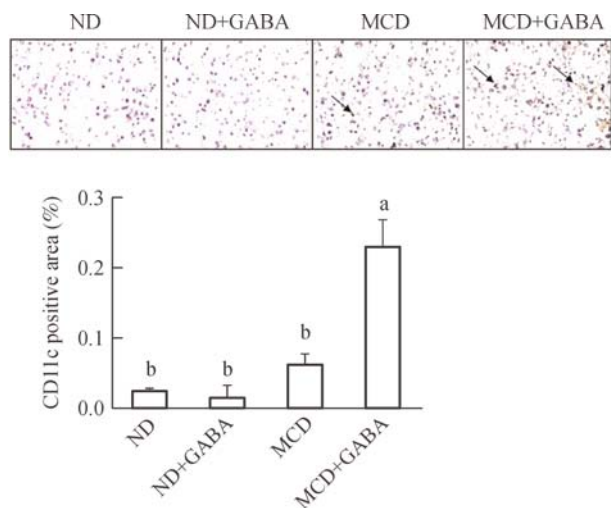
Fig. 4 **Immunohistochemistry confirmed increased dendritic cells in liver tissue from GABA-treated mice compared to MCD diet-fed mice (*N* = 6-7/group).** (A) Immunohistochemical staining of the liver (arrows) using antibodies against CD11c. (B) Quantification of CD11c-positive area (%). Values are presented as mean±standard error per group. Samples were analyzed using a one-way ANOVA. Different letters indicate significant differences at $P < 0.05$, and bars sharing the same letter are not significantly different.

Fig. 5 **GABA-regulated Kupffer cell subpopulation (M2) in MCD diet-induced NASH.** Hepatic mRNA levels of Arg1 and Mrc1, indicating an M2 response, were determined by qPCR. Data are presented as mean±SEM per group. ANOVA, **P < 0.05*. Samples were analyzed using a one-way ANOVA. Different letters indicate significant differences at $P < 0.05$, and bars sharing the same letter are not significantly different.
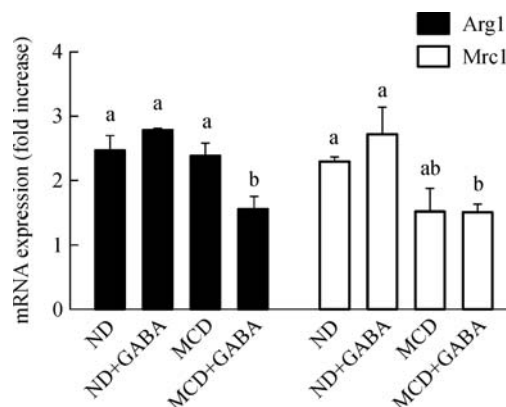
diet-fed mice with GABA administration displayed increased numbers of CD11c-expressing cells in the liver.

Macrophages are a heterogeneous population of myeloid-derived mononuclear cells that are a critical component of the innate immune response (classically activated proinflammatory M1 or alternatively activated anti-inflammatory M2 cells)[24]. Liver Kupffer cells (KC) have an important role in the development of hepatitis, and recent studies have shown that blocking the M2 activation program of KC exacerbates obesity-induced insulin resistance and decreases hepatocyte fatty acid oxidation[25].

In MCD diet-fed mice, treatment with GABA inhibited induction of the M2-related gene, arginase 1 (*Arg1*), compared to the levels in the control mice (***Fig. 5***). Altogether, these data indicate that GABA administration induces innate immune activation in the liver.

GABA directly enhances the inflammatory response in RAW 264.7 macrophages andincreases TIB–73 hepatocyte death

To determine the effects of GABA on innate immune cells, cultured macrophages were incubated with LPS and different concentrations of GABA for 24 hours. As indicated in ***Fig. 6A***, GABA treatment produced slightly increased TNF-α production, although there were no significant differences among the GABA

concentrations. In addition, IL-6 production was significantly increased by GABA treatment. To confirm the inflammation stimulatory effects of GABA, bicuculline, a competitive antagonist of GABA receptors, was treated with GABA in cultured macrophages. Production of TNF-α and IL-6 in GABA plus LPS treated macrophages was significantly regulated by bicuculline.

Normal hepatocyte cells, TIB-73, were incubated with different concentrations of GABA for 24 hours (***Fig. 6B***), and liver enzymes were measured in the cell supernatants. At a 100 nmol/L GABA concentration, AST and ALT values were notably increased. Such hepatotoxic effects of GABA were completely abrogated after bicuculline co-treatment. These results suggest that GABA directly induces an immune response and hepatocyte damage.

Discussion

The pathogenesis of steatosis and inflammation in NASH has been linked to multiple mechanisms including oxidative stress, mitochondrial damage in hepatocytes, and cytokine release[2,4]. Our present study shows that GABA exacerbates steatohepatitis in MCD diet-fed mice and shows similar results in different cell lines.

Recently, many studies have demonstrated that activation of GABA signaling inhibits inflammatory responses in type 1 diabetes, experimental autoimmune
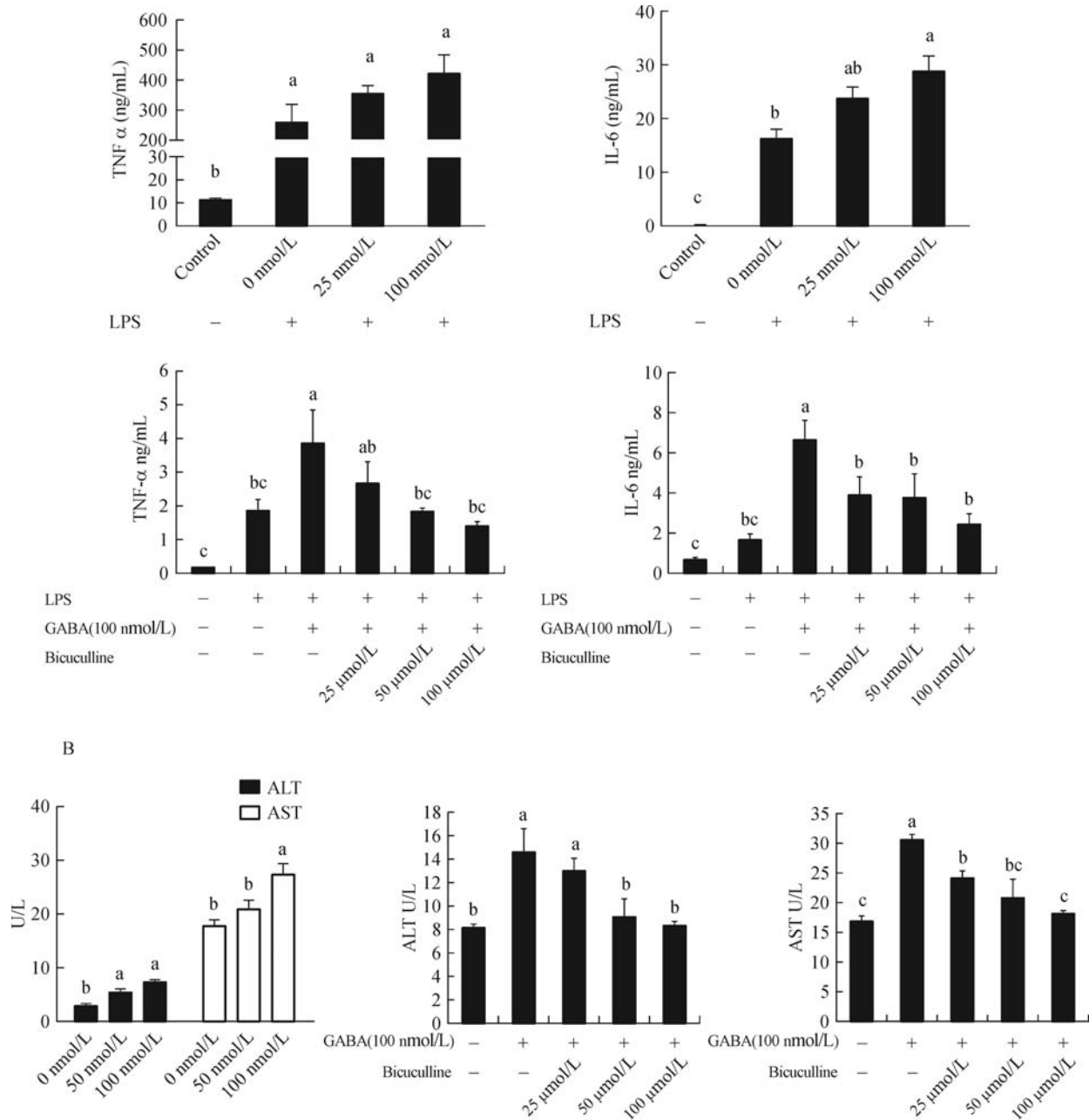
Fig. 6 GABA directly enhanced the inflammatory response in RAW 264.7 macrophages and increased TIB–73 hepatocyte death. (A) Cultured macrophages (5×10^6/mL) were incubated for 24 hours with GABA (25, 100 nmol/L), and TNF-α and IL-6 cytokines were measured in the medium ($N = 4$). Cultured macrophages (1×10^5/mL) were incubated for 24 hours with GABA (100 nmol/L) with different concentrations (25, 50, and 100 nmol/L) of bicuculline, and TNF-α and IL-6 cytokines were measured in the medium ($N = 4$). (B) TIB-73 hepatocytes (5×10^6/mL) were treated with GABA (0, 50, and 100 nmol/L) for 24 hours ($N = 4$), and AST and ALT values were measured. In addition, TIB-73 hepatocytes (1×10^5/mL) were incubated for 24 hours with GABA (100 nmol/L) with different concentrations (25, 50, and 100 nmol/L) of bicuculline, and AST and ALT values were measured. Data are presented as mean±SEM per group. Samples were analyzed using a one-way ANOVA. Different letters indicate significant differences at $P < 0.05$, and bars sharing the same letter are not significantly different.

encephalomyelitis, and rheumatoid arthritis in experimental animals[11,17,20]. We originally hypothesized that similar anti-inflammatory effects of GABA would be observed in an MCD diet-induced steatohepatitis model. In contrast to our expectations, GABA increased the severity of steatohepatitis and induced hepatic injury and inflammatory responsein in vivo and in vitro. From

the results of our additional experiments, both normal mice and those with high fat diet-induced obesity receiving GABA administration had no significant pathological changes in the liver compared to the control group (data not shown). These results indicate that GABA administration does not affect lipid metabolism in the liver (steatosis) but does affect

steatohepatitis. Previous studies have suggested that GABA has inhibitory effects on hepatocyte proliferation[26-27]. Indeed, ethanol consumption potentiates hepatic GABAergic activity, which in turn suppresses hepatocyte proliferation after partial hepatectomy[28]. Of note, direct treatment of GABA increased hepatocyte death as demonstrated by ALT and AST elevation (***Fig. 6B***). Collectively, our findings potentially support that GABA administration enhances MCD diet-induced liver injury through inhibition of hepatocyte proliferation or direct induction of hepatocyte stress, and subsequently increases liver inflammation. From this speculation, we need to question why MCD diet mice are more influenced by GABA than normal mice. There are several clues if we understand the MCD diet induced steatohepatitis model. First, severe steatosis can induce oxidative stress in hepatocytes, and damage associated molecular patterns from injured hepatocytes subsequently stimulate KCs and make severe inflammatory environment. Based on our preliminary study (data not shown), GABA treatment without LPS did not induce severe inflammation in macrophages, indicating that GABA's immune-stimulatory effects occurred in activated immune cells. Therefore, more detailed mechanisms of GABA's differential effects need to be more revealed. Second, stressed hepatocytes, especially via lipid accumulation might be more susceptible to hepatotoxicity induced by GABA. This notion is generally accepted that all hepatotoxins as well as GABA can induce more hepatotoxicity if hepatocytes are already stressed.

Although many researchers have shown the anti-inflammatory effects of GABA, little is known about the toxicity of GABA in human patients with liver disease. Moreover, the investigations of GABA have only focused on beneficial anti-inflammatory effects in animal models. Further biologic activities of GABA including possible detrimental effects on the immune system and various organs have not yet been clearly investigated.

To the best of our knowledge, this is the first report on the detrimental effects of GABA on NASH. Based on our findings, future studies are needed to elucidate the possible mechanisms of GABA's influence on NASH and GABAergic signaling.

Acknowledgments

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