ORIGINAL

Inhibition of GHRH aggravated acetaminophen-induced acute mice liver injury through GH/IGF-I axis

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Abstract. The aim of the current study is to investigate the effects of growth hormone releasing hormone (GHRH) antagonist on acetaminophen (APAP)-induced acute liver injury in mice. Healthy C57/B6L mice were orally treated with 200 mg/kg APAP with or without a 30-min pre-treatment with 300 µg/kg GHRH antagonist MZ-5-156. After 12 hours, serum, plasma, and liver samples from each mouse were collected for analyses. Our results showed that twelve-hour treatment with APAP caused obvious liver injury, elevated serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST) levels, increased oxidative stress, reduced expressions of antioxidant enzymes, accumulated expression of pro-inflammatory cytokines, and increased circulating levels of growth hormone (GH) and insulin-like growth factor-I (IGF-I). Pre-treatment with MZ-5-156 aggravated liver injury, further increased serum ALT and AST levels, exacerbated oxidative stress and inflammation induced by APAP. Treatment of MZ-5-156 also blocked the phosphorylation form and total form of Janus kinase 2 (JAK2) and signal transducer and activator of transcription 5 (STAT5). Treatment of GHRH super-agonist JI-38 immediately after MZ-5-156 treatment partly reversed the liver injury caused by APAP and MZ-5-156. In conclusion, GHRH plays essential protective role in APAP-induced acute liver injury *in vivo*. The protective properties of GHRH are partially through GH/IGF-I axis and JAK/STAT pathway.

Key words: GHRH antagonist, Acetaminophen, Liver injury, GH/IGF-I axis

DRUG-induced acute liver injury is an important cause of acute liver failure. In the United States, about 2,000 cases of acute liver failure occur annually. Half of the cases are from abuse or overuse of drugs [1]. Acetaminophen (also known as paracetamol, N-acetyl-p-aminophenol, APAP) is a well-documented over-the-counter antipyretic and analgesic drug mainly used for the relief of headache and other kinds of aches. It is also a well-characterized hepatotoxin with dose-dependent manner. When ingested in excessive dose, APAP can induce life-threatening acute hepatic injury [2]. After ingestion, APAP is metabolized by the cytochrome P450 system to form a highly reactive intermediate metabolite N-acetyl-pbenzoquinone-imine (NAPQI) [3]. Under normal circumstances, NAPQI will be eliminated by conjugation with glutathione (GSH) to form mercapturic acid which will be excreted into the urine [4]. Excessive administration of APAP may saturate the glucuronidation pathway, leading to structural and

functional dysfunctions of the cell induced by accumulated NAPQI [5]. Moreover, depletion of intracellular GSH after overdose APAP ingestion renders liver cells highly vulnerable to oxidative stress, inflammation, and necrosis [6]. The oxidative stress formed by APAP overdose is initiated by cellular superoxide formation through the uncoupling of cytochrome P450 enzymes to other enzymes and mitochondria or the activation of NADPH oxidase [7, 8]. Since GSH is the cofactor for glutathione peroxidase (GPx) detoxification of peroxides, depletion of GSH also leads to increased intracellular levels of peroxides [5].

Growth hormone releasing hormone (GHRH) is the releasing hormone of growth hormone (GH) through the binding of GHRH receptor in the anterior pituitary. Dysregulation of GHRH secretion and action induces abnormalities in body growth, protein regulation, bone growth, and metabolism [9]. GHRH is involved in the pathogenesis of a large number of cancers, including ovarian, breast, lung, prostatic, colorectal, and gastroenteropancreatic cancers, as these tumors are the most important non-hypothalamic source of GHRH [10]. *In vivo* studies showed that antagonist of GHRH (e.g. MZ-5-156) inhibited the growth of human prostate cancer [11,

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12], breast cancer [11], ovarian cancer [13], and pancreatic cancer [14]. Therefore, applying GHRH antagonist in the treatment of cancer has become a promising direction for cancer therapy. However, the role of GHRH in acute liver injury largely remains unclear. In the current study, we investigated the effects of GHRH antagonist MZ-5-156 and GHRH agonist JI-38 on APAP-induced acute liver injury in a mice model. Inhibition of GHRH exacerbated the oxidative stress and necro-inflammation induced by APAP partly though the GH/IGF-I axis and Janus kinase 2 (JAK2)/signal transducer and activator of transcription 5 (STAT5) pathway.

Materials and Methods

Animals and treatments

Fifty healthy male C57/B6L mice (~25 g, 8-9 weeks old) were purchased from animal center of Xinxiang Medical University. Then mice were housed in a well temperature- and humidity-controlled environment for 1 week before experiment. Mice were divided into five groups (n=10). Vehicle mice were freely accessed with regular chow and tap water. APAP treated mice were given oral APAP (200 mg/kg) with or without a 30-min pre-treatment of 300 µg/kg GHRH antagonist MZ-5-156 (i.v. injection). Vehicle-treated MZ-5-156 mice were also received the pre-treatment of MZ-5-156 as the control. To further test the effect of GHRH during liver injury, we treat the fifth group of mice with MZ-5-156 and GHRH agonist JI-38 (50 µg/kg, i.v. injection) before the APAP treatment. After 12 hours, mice were anesthetized and then sacrificed. The anesthesia agent was the mixture of ketamine (100 mg/kg) and xylazine (10 mg/kg). The pre-treatment period and dosage of MZ-5-156 and JI-38 were optimized by previous studies [15, 16]. Serum and plasma samples were obtained from whole blood by centrifugation at 3,000 rpm for 15 min. Liver sample from each mouse was collected for histological examination and molecular measurement. The entire animal experiment has been approved by the animal center of Xinxiang Medical University.

Biochemical assays

The serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST) levels of mice were detected by corresponding detection kits (Liaoning Taikang Medical Science Company, China) according to the manufacturer's protocols respectively.

Histological assessment

Liver tissue sample from each mouse were fixed in 10% phosphate-buffered formalin, and then embedded in paraffin blocks. Five-micrometer tissue sections were cut and stained with hematoxylin and eosin (H&E) for histological analysis under light microscope (Nikon, Japan).

Determining lipid peroxidation in liver tissue

Liver tissue was homogenized in Tris-HCl buffer (20 mM [pH 7.4]) and then centrifuged at 2500 × g at 4 °C for 10 min. Two hundred microliters of supernatant was analyzed for malondialdehyde (MDA) levels using a kit (Bioxytech MDA-586; Oxis Research, Foster City, CA) and the spectrophotometer was read at 586 nm.

Measurement of CAT and SOD activity

Twenty microliters of supernatant of homogenized liver tissue was analyzed for super oxide dismutase (SOD) and catalase (CAT) activity by using appropriate kits (CAT kit: Cayman Chemical; SOD kit: Sigma-Aldrich, Buchs, Switzerland).

RNA extraction and quantitative PCR measurement

Total RNA of each mouse was extracted from the liver sample using RNeasy Mini Kit (Qiagen, Valencia, CA) and then quantified with the spectrophotometer (Beckman Coulter, Fullerton, CA). The reverse-transcription of total RNA was performed by SuperScript™ First-Strand Synthesis System (Invitrogen, Calsbad, CA). The mRNA expression levels of tumor necrosis factor- α (TNF- α), interleukin 1 β (IL-1 β), and GPx1 were measured by quantitative real-time PCR conducted in ABI Prism 7300 machine with ABI SYBR green system (Applied Biosystems, Forster City, CA). Primer sequences are: (1) TNF- α : forward: 5'-CAG-GCGGTGCCTATGTCTC-3'; reverse: 5'-CGAT-CACCCCGAAGTTCAGTAG-3'; (2) IL-1 β : forward: 5'-GAAATGCCACCTTTTGACAGTG-3'; reverse: 5'-CTGGATGCTCTCATCAGGACA-3'; (3) GPx1: forward: 5'-CCACCGTGTATGCCTTCTCC-3'; reverse: 5'-AGAGAGACGCGACATTCTCAAT-3'. Parallel amplification of β -actin was used as the internal control. Fold change of each gene was calculated by using the comparative Ct method. All quantitative PCR procedures, including the design of primers, validation of PCR environment, and quantification methods were performed according the MIQE guideline [17].

Western blot analysis

To determine the role of JAK2 and STAT5 in GHRH inhibition, specific antibodies against the phosphorylated and normal forms of JAK2 and STAT5 (Cell Signaling, Danvers, MA) were used in Western blotting of mice liver samples. Antibody of CYP2E1 (Millipore, Billerica, MA) was also applied to characterize the expressional change of CYP2E1. Liver samples were homogenized in lysis buffer (150 mM NaCl, 10 mM HEPES, pH 7.9, 1 mM EDTA, 0.6% NP-40, 0.5 mM PMSF, 1 µg/mL leupeptin, 1 µg/mL aprotinin, and 10 µg/mL trypsin inhibitor). Samples were then sonicated and incubated for 30 minutes on ice. Debris was removed by centrifugation at 10,000 rpm. Protein concentrations of each sample were determined in order to calculate the loading amount. Samples were separated in a denaturing 10% polyacrylimide gel and transferred to a 0.1 µm pore nitrocellulose membrane. Nonspecific binding sites were blocked with tris-buffered saline (TBS; 40 mM Tris, pH 7.6, 300 mM NaCl) containing 5% nonfat dry milk for 1 hour at room temperature. Membranes were then incubated with appropriate primary antibodies in TBS with 0.1% Tween 20 (TBST). Membranes were washed and incubated with secondary antibodies conjugated to horseradish peroxidase to show the result bands with ECL buffer (GE Healthcare, UK). Parallel blotting of β-actin was used as internal control.

Measurement of circulating GH and IGF-I

To analyze the circulating levels of GH and IGF-I, commercial kits (GH: Millipore; IGH-I: Immunodiagnostic Systems, Fountain Hills, AZ) were used according to manufacturer's instructions in the plasma samples.

Statistical analysis

All values were expressed in the form of mean ± S.D. Statistical comparison between groups was done using

variance (ANOVA) and post hoc LSD tests. A $p < 0.05$ was considered to be statistically significant (SPSS version 17.0, IBM Corporation, Armonk, New York).

Results

Pre-treatment with MZ-5-156 aggravated the liver injury induced by APAP

In control mice, there was no abnormal morphological change could be observed under light microscope (Fig. 1A). Twelve-hour treatment of APAP induced classical damage in the mice liver, as demonstrated by necrosis and phagocyte filtration. The histological changes were more obvious around liver centrilobular veins (Fig. 1B). Pre-treatment with MZ-5-156 caused slight liver damage in vehicle-MZ-5-156 mice (Fig. 1C). Animals treated with MZ-5-156 followed by APAP showed worst degree of histological changes (Fig. 1D). Co-treatment of JI-38, the super-agonist of GHRH, significantly improved the histology of the liver (Fig. 1E). Serum levels of ALT and AST were consistent with the histological results. When compared with the control level, elevated serum ALT and AST levels by APAP treatment were further increased by the pre-treatment of MZ-5-156. Vehicle-treated MZ-5-156 mice also showed increases of both ALT and AST levels in the serum compared to those in control group, although the increases were not significant. Co-treatment with JI-38 partly reduced the levels of ALT and AST (Table 1).

MZ-5-156 pre-treatment exacerbated oxidative stress induced by APAP

After APAP treatment, protein expression of CYP2E1 was significantly elevated by approximately 2.7-fold. Pre-treatment with MZ-5-156 further increased such elevation (Fig. 2A). The level of serum MDA exhib-

Table 1 Effects of MZ-5-156 and APAP on serum ALT and AST levels

Group	ALT (IU/L) ^a	AST (IU/L) ^a
Control	59.6 ± 7.7	198.1 ± 20.5
APAP	1230.5 ± 111.4*	3470.9 ± 355.8*
MZ-5-156	77.5 ± 9.1	237.4 ± 24.4
MZ-5-156+APAP	2502 ± 211.8 [#]	4982.0 ± 515.7 [#]
MZ-5-156+JI-38+APAP	1058 ± 156.6*	2137 ± 246.0*

^a Values are means ± S.D., n=10; *Statistical significance: $p < 0.05$, compared with control group; [#] Statistical significance: $p < 0.05$, compared with control and APAP groups

Fig. 1 Representative results of hematoxylin and eosin (H&E) staining of mice liver tissues from each group. Mice were treated with 200 mg/kg acetaminophen (APAP) with or without a pre-treatment of 300 μ g/kg growth hormone releasing hormone (GHRH) antagonist MZ-5-156 and/or agonist JI-38. Magnification: 200x.

ited similar expressional change with that of CYP2E1 (Fig. 2B). As expected, treatment of APAP significantly down-regulated the mRNA expression level of SOD, CAT, and GPx1. Mice pre-treated with MZ-5-156 followed by APAP treatment showed lower expression of CAT and GPx1 than those of the APAP-treated mice (Fig. 2C-2E). Co-treatment of MZ-5-156 and JI-38 prior to APAP administration counteracted the effects of APAP and MZ-5-156 (Fig. 2).

APAP-induced hepatic inflammation was aggravated by MZ-5-156 pre-treatment

TNF- α and IL-1 β are important mediators of inflammatory response in the liver. After APAP treatment, mRNA expressions of TNF- α and IL-1 β were remark-

ably up-regulated by 5.5 folds and 2.1 folds, respectively. Pre-treatment with MZ-5-156 further elevated the expressions of these two genes to approximately 7.3 folds and 3.2 fold. Vehicle-treated MZ-5-156 also showed slightly elevated expression of TNF- α but no significant change of IL-1 β (Fig. 3). Pre-treatment with JI-38 partly reversed the effects of APAP and MZ-5-156 on the cytokines.

Effects of MZ-5-156 on APAP-treated mice were through inhibition of JAK2 and STAT5

The JAK/STAT pathway is an essential signal transduction component in GH-mediated physiological events [19]. To investigate the role of JAK2 and STAT5 on MZ-5-156 and APAP induced hepatotoxic-

Fig. 2 Levels of (A) CYP2E1 protein, (B) MDA formation, (C) SOD activity, (D) CAT activity, and (E) GPx1 mRNA expression from each group of mice. Mice were treated with 200 mg/kg acetaminophen (APAP) with or without a pre-treatment of 300 µg/kg growth hormone releasing hormone (GHRH) antagonist MZ-5-156 and/or agonist JI-38. Mark “*” stands for a statistically significant change compared to control group ($p < 0.05$). Mark “#” stands for a statistically significant change compared to APAP group ($p < 0.05$). Data presented are expressed as Mean $\pm$ S.D. (n=10). MZ, MZ-5-156; MZ+APAP, MZ-5-156 and acetaminophen co-treatment; M+I+A, MZ-5-156, JI-38 and acetaminophen co-treatment.

Fig. 3 Hepatic expressional changes of (A) TNF-α and (B) IL-1β from each group of mice. Mice were treated with 200 mg/kg acetaminophen (APAP) with or without a pre-treatment of 300 µg/kg growth hormone releasing hormone (GHRH) antagonist MZ-5-156. Mark “*” stands for a statistically significant change compared to control group ($p < 0.05$). Mark “#” stands for a statistically significant change compared to APAP group ($p < 0.05$). Data presented are expressed as Mean $\pm$ S.D. (n=10). MZ, MZ-5-156; MZ+APAP, MZ-5-156 and acetaminophen co-treatment; M+I+A, MZ-5-156, JI-38 and acetaminophen co-treatment.

ity, both phosphorylation form and total protein expression of these proteins were measured by Western blot. After APAP intoxication, phosphorylation of JAK2 and STAT5 was more obvious than that in control group. However, total protein expressions of JAK2 and STAT5 did not show significant change. Treatment of MZ-5-156, both in APAP-treated and vehicle mice, dramatically inhibited the phosphorylation and basal expressions of JAK2 and STAT5 (Fig. 4). JI-38 restored both phosphorylation and total protein levels of JAK2 and STAT5 in the liver.

Pre-treatment with MZ-5-156 reduced circulating levels of GH and IGF-I

To investigate the possible involvements of GH and IGF-I, circulating levels of these hormones were measured in the plasma. Treatment of APAP slightly increased the circulating levels of both GH and IGF-I. However, pre-treatments with MZ-5-156 dramatically reduced the circulating GH level and moderately decreased the IGF-I level in both vehicle- and APAP-treated mice (Table 2). As a super-agonist of GHRH, treatment of JI-38 strongly restored the level of GH and IGF-I.

Discussion

Antagonists of GHRH have been found to suppress the *in vivo* growth of a large number of experimental cancers, such as prostatic, mammary, ovarian, and lung carcinomas. The inhibitory effects of GHRH antagonists are partly from their inhibition of GHRH induced GH release from the pituitary, which in turn reduces the levels of hepatic and circulating IGF-I [10]. Another mechanism that contributes to the antitumor property of GHRH antagonism is based on the suppression of secreted IGF-I and IGF-II from the tumors [19]. Antagonists GHRH also exert antioxidative effect in tumors through activating p53, leading to the antioxidant defense of the cell [20, 21]. However, in this study, pre-treatment with GHRH antagonist MZ-5-156 aggravated the acute hepatotoxicity, including histological change, oxidative stress, and inflammation which were induced by APAP intoxication, suggesting a protective role of GHRH in toxin-induced acute liver injury. The protection of GHRH is partly through the GH/IGF-I axis and the JAK2/STAT5 pathway. Application of the super-agonist of GHRH, JI-38, further confirmed the essential roles of GHRH in acute liver injury. However, since serum levels of GH

Fig. 4 Representative results of phosphorylation form and total protein expression of (A) JAK2 and (B) STAT5 from each group of mice. Mice were treated with 200 mg/kg acetaminophen (APAP) with or without a pre-treatment of 300 µg/kg growth hormone releasing hormone (GHRH) antagonist MZ-5-156. β-actin was blotted as parallel control. Quantitative data showed the ratio between each form of gene against control. Mark “*” stands for a statistically significant change compared to control group ($p < 0.05$). Mark “#” stands for a statistically significant change compared to APAP group ($p < 0.05$). Data presented are expressed as Mean $\pm$ S.D. ($n=6$). MZ, MZ-5-156; MZ+APAP, MZ-5-156 and acetaminophen co-treatment; M+I+A, MZ-5-156, JI-38 and acetaminophen co-treatment.

Table 2 Effects of MZ-5-156 and APAP on circulating levels of GH and IGF-I

Group	GH (ng/mL) ^a	IGF-I (ng/mL) ^a
Control	73.5 ± 6.9	211.4 ± 18.6
APAP	94.5 ± 6.7*	224.1 ± 20.1*
MZ-5-156	12.8 ± 3.0 [#]	68.4 ± 7.5 [#]
MZ-5-156+APAP	15.4 ± 1.7 [#]	75.5 ± 8.2 [#]
MZ-5-156+JI-38+APAP	127.7 ± 11.1*	202 ± 19.8*

^a Values are means ± S.D., n=10; *Statistical significance: $p < 0.05$, compared with control group; [#]Statistical significance: $p < 0.05$, compared with control and APAP groups

and IGF-1 were completely reversed by GHRH agonist, whereas up-regulated phosphorylated JAK2 and total STAT5 induced by APAP were partially reversed by GHRH agonist, it is possible that other mechanisms than GH-IGF-1 axis may take part in APAP-induced hepatotoxicity. The limitation of this study is whether MZ-5-156 has direct actions on the liver or not is not clear. As a potent antagonist of GHRH, it probably aggravated liver injury through the pituitary-liver axis by reducing circulating and hepatic expression levels of GH and IGF-I. Further studies are needed to characterize its effect on the liver and the direct relationship between MZ-5-156 and GH.

A recent report demonstrated that GHRH agonist had a protective property after an acute myocardial infarction *in vivo* [22]. Since there was an expression of GHRH receptor in the cardiomyocytes, the protective effects of GHRH agonist after myocardial infarction was probably not through the GH/IGF-I axis. In addition, circulating levels of GH and IGF-I were not elevated by GHRH agonist treatment, further suggested the independence of GHRH effect from GH/IGF-I actions. Our study also found that GHRH was important for the protection against APAP induced hepatotoxicity by showing that inhibition of GHRH in the liver aggravated the acute injury caused by APAP. However, since the phosphorylation form and total protein expressions of JAK2 and STAT5 were significantly reduced by GHRH antagonist, it is reasonable to postulate that GH participated in this process. The change of circulating levels of GH and IGF-I further proved the involvement of GH/IGF-I axis in the protective effect of GHRH against APAP induced toxicity (Table 2). It is interesting that administration of GHRH agonist JI-38 dramatically restored the circulating levels of GH and IGF-I, solidly proved the involvement of GH/IGF-I axis. GH has been shown to enhance the

immune system and to reduce the acute inflammatory responses after sepsis, trauma, major operation, and thermal injury [23-27]. The essential protective roles of GH against acute liver injury have also been demonstrated in animal models. For example, cardiopulmonary bypass induced acute hepatic inflammation, oxidative stress, and apoptosis were reversed by co-treatment of recombinant human GH [28]. In the current study, the aggravated acute liver injury induced by MZ-5-156 and APAP co-treatments was primarily originated from the inhibition of GH release from the pituitary. However, we cannot exclude the possible autocrine and paracrine actions of GHRH in the liver, which is independent of the GH/IGF-I axis. In addition, previous studies have demonstrated that inhibition of GH (by using its antagonist G120K-PEG) inhibited the phosphorylation of JAK2 and following signaling pathways in mice. Administration of exogenous GH could partly reverse such phenomena [29, 30]. Another report showed that administrations of exogenous GHRH and GHRH antagonist (JMR-132) activated and suppressed the JAK2/STAT3 pathway (both phosphorylated and total forms of JAK2 and STAT3) *in vitro*, respectively [31]. So we believe that the effects of MZ-5-156 could be explained by the reduced plasma GH levels. Further investigations are needed to address this possibility. It should be noted that, according to a previous report, usage of ketamine and xylazine in anesthesia increased the plasma level of GH in rat [32]. However, in the current study, MZ-5-156 significantly reduced the level of GH, which exhibited more potent influence to the GH level than ketamine and xylazine could.

CYP2E1 is the key mediator of APAP-induced hepatotoxicity. Knockout of CYP2E1 prevented APAP-induced oxidative stress and injury through diminishing the production of the toxic electrophile, NAPQI [33]. Moreover, overexpression of CYP2E1 increased

the sensitivity of liver cells to APAP-induced toxicity *in vitro* [34]. In this study, up-regulated protein expression of CYP2E1 was further increased by the pre-treatment with MZ-5-156 but decreased through the addition of JI-38, suggesting the essential role of GHRH in the control of oxidative stress. Considering that inhibition of GHRH, GH, and IGF-I in the current study reduced the antioxidant system in the liver, it is reasonable to postulate the antioxidative role of GHRH/GH/IGF-I axis in acute liver injury. Two previous studies showed that application of GHRH antagonist attenuated oxidative stress in human cancer cells and aging cognitive deficiency mice, respectively [35, 36]. The inconsistency between the current study and these reports is probably from the differences between normal animal and cancer cell line, as well as the differences between the healthy and APAP-induced liver

injury animals. Application of GHRH antagonist has been proven to be therapeutic in many cancer cell lines in which it attenuates inflammation, oxidative stress, and apoptosis. While in APAP-induced acute liver injury mice, pre-treatment with MZ-5-156 blocked the production of GHRH from the pituitary to reduce the circulating and hepatic GH levels, which could protect liver against acute liver injury [28].

In conclusion, inhibition of GHRH by using GHRH antagonist MZ-5-156 aggravated acute liver injury, oxidative stress, and inflammation induced by APAP intoxication. This effect was associated with reduced circulating levels of GH and IGF-I, as well as impaired JAK2/STAT5 signaling pathway. Thus, GHRH was important for the basal hepatic defense against drug-induced acute liver injury.

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