Acetaminophen induced gender-dependent liver injury and the involvement of GCL and GPx

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ABSTRACT: Acetaminophen (AP) is widely used as the antipyretic and analgesic drug in clinic, and it can induce serious liver injury in the case of excessive abuse. The present study showed that AP (400 mg/kg) induced obvious liver injury, while in male mice the hepatotoxicity induced by AP was much more serious than in female mice as indicated by the results of alanine aminotransferase (ALT) activity and reduced glutathione (GSH) amount. Further, the enzymatic activity and protein expression of glutamate-cysteine ligase (GCL) and glutathione peroxidase (GPx) were all higher in female mice liver than in male after the administration of AP (200 mg/kg). Meanwhile, AP (10 mM) decreased GCL and GPx activity in isolated mouse hepatocytes in the time-dependent manner, while the inhibitors of GCL and GPx can augment AP induced-cytotoxicity. Taken together, our results demonstrate the gender-related liver injury induced by AP and the important role of GCL and GPx in regulating such hepatotoxicity.

Keywords: Acetaminophen, gender, hepatotoxicity, GCL, GPx

1. Introduction

Acetaminophen (AP) also known as paracetamol, is widely used in clinical as antipyretic and analgesic drug. AP can induce serious liver injury when it is used overdose, or even sometimes it is used under the recommended dose. As AP belongs to the type of over-the-counter (OTC) drug, so it can be easily purchased by people, and thus leads to the abuse of this drug. The abuse of AP makes the problem about it-induced acute liver failure more frequent and thus becomes a big problem for clinicians. Drug-induced liver injury (DILI) has been the serious medical problem and with significant mortality in clinical situations, among which AP has been the primary cause of drug-induced acute liver failure in American (1).

Reduced glutathione (GSH) is an important antioxidant reducing sulfhydryl (-SH) tripeptide, and plays important roles in counteracting with oxidative stress injury and maintains cellular redox balance (2,3). Intracellular GSH and its related enzymes such as glutamate-cysteine ligase (GCL), glutathione peroxidase (GPx), glutathione reductase (GR), and glutathione-s-transferase (GST) constitute the key antioxidant system in body to counteract with oxidative stress injury (4). AP will be converted into the metabolite N-acetyl-p-benzoquinoneimine (NAPQI) by liver cytochrome P450s 2E1 (CYP2E1), CYP1A2, CYP2D6, CYP3A, etc. (5). Generally, NAPQI will be detoxified by GSH, while when GSH is exhausted; it will form covalent binding with macromolecules such as protein or DNA, and thus leads to acute liver failure (6). N-Acetyl-L-cysteine (NAC), the precursor of GSH biosynthesis, is generally used as the antidote to AP-induced hepatotoxicity in clinic (7,8).

Experimental and clinical research demonstrates that gender-dependent difference will lead to the big difference of liver injury caused by exogenous toxins. There is already report that gender is a critical factor involved in AP-induced liver injury, and male animals are more susceptible than female (9). Generally, there is the difference in metabolic enzymes between male and female, thus gender is a critical factor affects drug metabolism and pharmacokinetics, which will contribute to the gender-dependent hepatotoxicity. But the previous study already demonstrated that metabolism did not contribute to the gender-dependent liver injury induced by AP (9). The present study is designed to observe AP-induced gender-dependent liver injury, and the potential regulating of GSH antioxidant system.

2. Materials and Methods

2.1. Chemicals and reagents

β-Actin polyclonal antibody was purchased from Cell
Signaling Technology (Danvers, MA, USA). Anti-GPX-1, GCLC/GCLM antibodies were the products of Santa Cruz Biotechnology Corporation (Santa Cruz, CA, USA). Peroxidase-conjugated goat anti-Rabbit IgG (H + L) and peroxidase-conjugated goat anti-mouse IgG (H + L) were obtained from Jackson ImmunoResearch (West Grove, PA, USA). Nitrocellulose membranes were obtained from Bio-Rad (Hercules, CA, USA) and enhanced chemiluminescence detection system was from Amersham Life Science (Buckinghamshire, UK). GSH, oxidized glutathione (GSSG), and NADPH were purchased from Roche Diagnostics GmbH (Mannheim, German). AP, 5, 5’-dithio-bis-(2-nitrobenzoic acid) (DTNB), glutathione reductase, d,L-buthionine-(S,R)-sulfoximine (BSO), mercaptosuccinic acid (MA), and all other reagents unless indicated were obtained from Sigma Chemical Co. (St. Louis, MO, USA).

2.2. Experimental animals

Specific pathogen free female and male ICR mice (18-22 g body weight) were purchased from Shanghai Laboratory Animal Center (Shanghai, China). The mice were fed with a standard laboratory diet and given free access to tap water. The animal room was maintained at a temperature of 22 ± 1°C with a 12 h light-dark cycle (6:00-18:00) and 65 ± 5% humidity. All mice were received humane care in compliance with the institutional animal care guidelines approved by the Experimental Animal Ethical Committee of Shanghai University of Traditional Chinese Medicine. Both sexes of mice were divided into 3 groups, vehicle group, the group treated with AP for 200 and 400 mg/kg body weight, respectively, and each group contained 8 mice.

2.3. Treatments of animals

AP was dissolved in warm saline. Female and male mice were given a single doses of AP (200 and 400 mg/kg, i.p.) and solvent control group were treated with warm saline. Animals were killed 4 h after administration.

All blood was collected for measurement of serum alanine and aspartate aminotransferases (ALT/AST) activities. Livers were also collected and washed with ice-cold saline and then quick-frozen in liquid nitrogen for further experiments.

2.4. Measurement of GSH levels

Liver reduced and oxidized glutathione levels were determined by the DTNB assay according to the previous reported method with a minor modification (10). Briefly, mice liver tissues were homogenized in cold metaphosphoric acid (5%) buffer, and then centrifuged at 10,000 g for 10 min and the supernatant was transferred to new tubes for detection of glutathione levels. The reaction mixture contained 1 mM EDTA, NADPH (0.24 mM), glutathione reductase (0.06 units), DTNB (86 μM) and samples. Yellow 5-thio-2-nitrobenzoic acid (TNB) formation was monitored at 412 nm. GSSG was measured by first masking the GSH in the metaphosphoric acid (5%) buffer with 10 mM 2-vinylpyridine in the presence of 6% triethanolamine and derivatized in 37°C overnight as previously described (11). Protein concentrations were determined according to the method of Bradford (12), using bovine serum albumin as standard. The levels of glutathione were calculated based on tissue protein concentration and expressed as nM per mg protein.

2.5. Enzymatic assays

ALT and AST were determined according to the methods reported previously (13). GCL activity was assayed according to the previous reported method (14). The activity of GCL was calculated based on tissue protein concentration and expressed as U/mg protein. One unit of GCL activity was equal to the quantity of the oxidation of 1 mM NADPH per min.

GR activity was assayed as described method (15). The activity of GR was calculated based on tissue protein concentration and expressed as mlU/mg protein. One unit of GR activity is defined as 1 mM GSSG catalyzed per min.

GPx activity was assayed according to the previous reported method (16). A unit of enzyme activity is defined as the amount of enzyme catalyzing the formation of 1 μM of GSH per min, and the activity of GPx was calculated based on tissue protein concentration.

GST activity was assayed according to previous reported method (17). A unit of GST activity is defined as the amount of enzyme catalyzing the formation of 1 μM of product per min, and GST activity is defined as the units of enzyme activity per mg protein.

2.6. Western-blot analysis

Liver tissues were homogenized in lysis buffer containing 50 mM Tris (pH 7.5), 1 mM EDTA, 150 mM NaCl, 20 mM NaF, 0.5% NP-40, 10% glycerol, 1 mM phenylmethylsulfonyl fluoride, 10 μg/mL aprotinin, 10 μg/mL leupeptin, 10 μg/mL pepstatin A and then centrifuged at 3,000 g for 3 min and the supernatant was transferred to new tubes. Protein concentrations were assayed and normalized to equal protein concentration. Proteins were separated by SDS-PAGE and blots were probed with appropriate combination of primary and HRP conjugated secondary antibodies.

2.7. Mice primary hepatocytes isolation

Mice primary hepatocytes were isolated by an improved two-step perfusion separation as previously described (18). Cell viability, as determined by trypan blue exclusion, was generally > 85%. Freshly isolated hepatocytes were seeded in 60 mm dishes (1 × 10⁶ cells/dish) or 96-well plates (2 × 10⁵ cells/well) in M199 containing 10% fetal
bovine serum, 100 U/mL penicillin/streptomycin, 2 mM glutamine, 10 ng/mL epidermal growth factor (EGF) and 100 nM dexamethasone and cultured at 37°C with 5% CO₂.

2.8. Determination of cellular GCL and GPx activity

Mice hepatocytes were incubated with AP (5 mM) for 12 and 24 h, and then the cells were harvested with PBS. As for GCL assay, the cells were lysed with ultrasonication in lysis buffer (50 mM Tris (pH 7.5), 0.25 M sucrose, 0.1 mM EDTA, 0.7 mM β-mercaptoethanol and 1 mM phenylmethylsulfonyl fluoride) on ice and then centrifuged at 10,000 g, 4°C for 60 min. The supernatant of the cells was transferred to new tubes for GCL assay. The method of cellular GCL activity assay was the same as described above in the liver tissue. As for GPx assay, the cells were also lysed with ultrasonication in PBS and then centrifuged at 3,000 g, 4°C for 3 min. The supernatant was transferred to new tubes for cellular GPx activity assay. The method for cellular GPx activity assay was the same as described above in the liver tissue.

2.9. Cell viability determination

Mouse hepatocytes were pretreated with BSO (200 μM, pretreated 12 h) or incubated with MA (7 mM) 24 h after AP treatment. After treated with AP for 48 h, cells were further incubated with 500 μg/mL 3-(4,5-dimethyl-thiazol-2-yl) 2,5-diphenyltetrazolium bromide (MTT) for 4 h. The functional mitochondrial dehydrogenases in survival cells can convert MTT to blue formazan, which was dissolved in 10% SDS-5% iso-butanol-0.01 M HCl (19). The optical density was measured at 570 nm with 630 nm as a reference and cell viability was normalized as a percentage of control. Each experiment was performed at least three times by using hepatocytes freshly isolated from different mice.

2.10. Statistical analysis

The results were expressed as means ± SEM. Differences between groups were assessed by one-way ANOVA with LSD post hoc test. The analyses were carried out using the SPSS 11.5 software package for Windows. p < 0.05 was considered as statistically significant.

3. Results

3.1. AP-induced liver injury in male and female mice

The elevation of serum ALT and AST activity is the major indicator for the liver injury (20). As shown in Figure 1, we can see that AP (400 mg/kg) increased serum ALT and AST activity. Meanwhile, the increase of serum ALT activity in male mice was higher than that in female (p < 0.01).

GSH is the liver important antioxidant. As shown in Figure 2, we found that AP (400 mg/kg) decreased liver GSH amount, while increased liver GSSG amounts, and thus led to the damage on the balance of liver GSH and GSSG. Further, we found that there was a difference in the basal GSH and GSSG amounts, and they were

Figure 1. AP-induced liver injury in male and female mice. (A) ALT activity, (B) AST activity. Data were expressed as means ± SEM (n = 8). # p < 0.05 compared to female mice under the same treatment.

Figure 2. Effects of AP on liver GSH and GSSG amounts in male and female mice. (A) GSH amounts, (B) GSSG amounts. Data were expressed as means ± SEM (n = 8). * p < 0.05, *** p < 0.001 compared to female mice under the same treatment.
both higher in female than in male ($p < 0.01$, $p < 0.05$). With the administration of AP (200 mg/kg), liver GSH amount was higher in female than in male ($p < 0.001$), while there was no difference in male and female after the administration of AP (400 mg/kg).

3.3. Effects of AP on GCL, GR, GPx, and GST enzymatic activity in male and female mice

Next, we observed the effects of AP on the enzymatic activity of GSH-related antioxidant enzymes. The results in Figure 3 showed that AP (200 mg/kg) decreased liver GCL, GR, GPx, and GST activity in male mice, and decreased liver GCL, GPx, and GST activity in female mice. In female mice, GCL and GPx activity were higher than that in male ($p < 0.05$, $p < 0.001$). Further, after the administration of AP (200 mg/kg), GCL and GPx activity were higher in female than that in male ($p < 0.05$, $p < 0.01$).

3.4. Effects of AP on GCLC and GPx1 protein expression in male and female mice

Further, we observed the expression of catalytic subunit of GCL (GCLC) and modified subunit of GCL (GCLM), and GPx1 protein. The results of Western blot (Figure 4) showed that GCLC and GPx1 protein expression in female were higher than in male after treated with AP (200 mg/kg).

3.5. GCL and GPx was involved in AP-induced cytotoxicity in mice hepatocytes

As shown in Figure 5, we found that AP (5 mM) obviously decreased cellular GCL and GPx activities in cultured mice hepatocytes ($p < 0.05$, $p < 0.001$). Further, BSO and MA, which are the specific inhibitor of GCL and GPx, both augmented AP-induced cytotoxicity ($p < 0.01$, $p < 0.001$).
4. Discussion

DILI is the liver damage caused by the parent drug or its metabolites, and now it is a serious problem during drug development and also for the use of many established drugs. It is reported that about 1,000 kinds of drugs can cause liver damage, and the severe cases can cause liver failure and require liver transplantation or even death (21). Prevention and treatment of DILI has become the challenging and persistent research topic of international medical community, pharmaceutical industry, and medical institution. Among all the drugs, AP overdose is currently the most frequent cause of acute liver failure in the United States and Great Britain (22).

In the present study, we found that the elevation of serum ALT activity induced by AP was higher in male than in female mice (Figure 1). ALT is the sensitive indicator of liver injury, and our results confirmed the previous study that male mice was more sensitive to AP-induced liver injury (9).

There are already reports that cellular GSH is important for the detoxification of AP-induced liver injury (6,7). In the present study, our results showed that AP (200 mg/kg) had no much effect on GSH amount in female mice, but in male mice GSH amount was obviously decreased, and thus led to the significant difference in GSH amount between male and female mice. Our results indicate that GSH may be a more sensitive indicator than ALT/AST for AP-induced liver injury. Further results showed that AP (200 mg/kg)-induced the decrease of GCL and GPx was higher in male mice than in female. Also, the results of Western-blot demonstrated that the expression of catalytic subunit of GCL (GCLC) and GPx-1 were higher in female than in male after given AP (200 mg/kg), which may contribute to its high activity in female mice. GCL and GPx are both antioxidant enzymes, of which GCL is critical for the biosynthesis of GSH, while GPx counteracts with cellular excessive reactive oxygen species (ROS) via utilizing GSH (23,24). There is already report that GCL is critical for AP-induced gender-related liver injury (25,26), and our present results further suggest that GPx may also be involved in regulating AP-induced gender-related liver injury.

In our results we can see that AP (200 mg/kg) obviously decreased GCL, GPx, GR, and GST enzymatic activity, and the dose is lower than the dose of 400 mg/kg, which induced the elevation of ALT/AST. The result suggests that GCL, GPx, GR, and GST may be the more sensitive indicators than ALT/AST for AP-induced liver injury. Further results showed that AP (200 mg/kg)-induced the decrease of GCL and GPx was higher in male mice than in female. Also, the results of Western-blot demonstrated that the expression of catalytic subunit of GCL (GCLC) and GPx-1 were higher in female than in male after given AP (200 mg/kg), which may contribute to its high activity in female mice. GCL and GPx are both antioxidant enzymes, of which GCL is critical for the biosynthesis of GSH, while GPx counteracts with cellular excessive reactive oxygen species (ROS) via utilizing GSH (23,24). There is already report that GCL is critical for AP-induced gender-related liver injury (25,26), and our present results further suggest that GPx may also be involved in regulating AP-induced gender-related liver injury.

Next, the results demonstrated the decrease of GCL and GPx activity after the administration of AP for the indicated time. Further, BSO and MA, which are the specific inhibitors of GCL and GPx (26-29), markedly augmented the cytotoxicity of AP on cultured mouse hepatocytes. These results indicate the important roles of GCL and GPx in regulating AP-induced hepatotoxicity, which may contribute to AP-induced gender-dependent liver injury.
Taken together, our results demonstrated the gender-dependent liver injury induced by AP, further we found that GCL and GPx was critical for regulating gender-related difference in the hepatotoxicity induced by AP. The present study reminds clinic doctors to pay attention to the gender-dependent liver injury induced by AP, and also shall consider the individual differences of liver GSH antioxidant system.

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