Gastrodin Ameliorates Oxidative Stress and Proinflammatory Response in Nonalcoholic Fatty Liver Disease through the AMPK/Nrf2 Pathway

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This study was designed to investigate the antioxidative, antiinflammatory and metabolism-regulating effects of gastrodin (GSTD) in the treatment of nonalcoholic fatty liver disease (NAFLD). Oleic acid (OA) was used to induce steatosis in HL-7702 cells; a high-fat or high-fat and high-cholesterol diet was used to induce NAFLD in mice and rats. Our results showed that GSTD significantly increased hepatic superoxide dismutase (SOD) but decreased reactive oxygen species (ROS)/malondialdehyde (MDA) and reduced the mRNA levels of proinflammatory cytokines both in vitro and in vivo. GSTD promoted the phosphorylation of nuclear factor erythroid-2-related factor-2 (Nrf2) at serine (Ser) 40, stimulated its nuclear translocation and increased hepatic expression of heme oxygenase-1 (HO-1). GSTD activated AMP-activated protein kinase (AMPK), suppressed hepatic steatosis, lowered serum triglyceride (TG)/glucose and decreased body weight gain in animals with NAFLD. The stimulating effects of GSTD on the Nrf2 pathway as well as its antioxidative/antiinflammatory activities were abolished by compound C in OA-treated HL-7702 cells. In summary, our results demonstrate that GSTD activates the AMPK/Nrf2 pathway, ameliorates oxidative stress/proinflammatory response and improves lipid metabolism in NAFLD. Our findings may support the future clinical application of GSTD for the treatment of NAFLD to reduce hepatic steatosis, oxidative stress and proinflammatory response. Copyright © 2015 John Wiley & Sons, Ltd.

Keywords: gastrodin; hepatic steatosis; Nrf2; proinflammatory cytokines; AMP-activated protein kinase.

INTRODUCTION

Nonalcoholic fatty liver disease (NAFLD) is characterized by extra lipid accumulation in the liver. The progression of NAFLD could be divided into different stages, which include steatosis, nonalcoholic steatohepatitis (NASH), fibrosis/cirrhosis and eventually tumorigenesis (Henao-Mejia et al., 2012). NAFLD is usually accompanied with obesity, insulin resistance, diabetes or cardiovascular diseases, and the prevalence of NAFLD is about 14%–30% worldwide (Abd El-Kader and El-Den Ashmawy, 2015).

The pathophysiology of NAFLD is extremely complex, and the molecular mechanisms for its progression are not fully elucidated. A number of studies revealed that lipid-induced oxidative stress and proinflammatory response played critical roles in the development of NAFLD while chemicals with antioxidative or antiinflammatory activities were able to ameliorate the progression of NAFLD effectively in animal models (Henao-Mejia et al., 2012; Serviddio et al., 2008).

Gastrodin (GSTD, molecular weight 295.3, Fig. 5) is a water-soluble natural compound extracted from the root of Gastrodia elata BI., an ancient Chinese herbal medicine with a very long history of clinical application. Now GSTD is used in clinic in China to treat nervous system diseases such as vertigo, epilepsy and vertebral-basilar artery insufficiency (Hu et al., 2014; Wang et al., 2013). In animal models, GSTD also has beneficial effects against Parkinson’s disease and Alzheimer disease (Hu et al., 2014; Wang et al., 2013).

GSTD was reported to have antioxidative and antiinflammatory activities in neurons, cardiomyocytes and human bone marrow mesenchymal stem cells (Dai et al., 2011; Yang et al., 2013; Huang et al., 2015; Jiang et al., 2014a; Wang et al., 2014; Zhao et al., 2012; Peng et al., 2015). One study found that GSTD could increase the activity of superoxide dismutase (SOD), decrease reactive oxygen species (ROS) production and reduce the levels of proinflammatory cytokines in neurons and brain tissues upon pathological stimulation (Dai et al., 2011; Jiang et al., 2014a; Wang et al., 2014; Zhao et al., 2012; Peng et al., 2015). The antioxidative activity of GSTD was closely related to the induction of the nuclear factor erythroid-2-related factor-2 (Nrf2) pathway (Jiang et al., 2014a; Wang et al., 2014; Zhao et al., 2012; Peng et al., 2015). Upon activation, cytoplasmic Nrf2 is able to translocate into the nucleus, where it binds to the antioxidant response element (ARE) and stimulates the transcription of antioxidative enzymes such as heme oxygenase-1 (HO-1),
NADPH quinone oxidoreductase-1 (NQO-1) and SOD (Vomhof-Dekrey and Picklo, 2012; Cuadrado, 2015).

Our previous studies proved that powdered G. elata BI. lowered blood lipids, reduced liver fat accumulation and improved liver function in fat emulsion-fed rats (Geng et al., 2013). In mechanism studies (Geng et al., 2015), we used oleic acid (OA) to induce steatosis in cultured HL-7702 liver cells, and found that GSTD could decrease intracellular fat accumulation greatly. Importantly, we found that GSTD could activate the AMP-activated protein kinase (AMPK) pathway in HL-7702 cells, and its inhibitory effect on OA-induced fat accumulation was AMPK dependent.

Currently, it is not clear whether GSTD could suppress oxidative stress and inflammation in NAFLD; related cellular signaling pathways are not fully elucidated either. In addition, the detailed mechanisms of GSTD in modulating lipid metabolism need to be investigated. So, in the present study, we used in vitro and in vivo NAFLD models to clarify the above issues.

MATERIALS AND METHODS

**Chemicals and kits.** GSTD (lot number: 20120509, purity≥98%) was obtained from Zhejiang Cheng Yi Pharmaceutical Co., Ltd. (Zhejiang, China); OA, bovine serum albumin (BSA), compound C and dimethyl sulfoxide (DMSO) were purchased from Sigma-Aldrich Co. (St. Louis, MO, U.S.A.). Cell culture reagents including fetal bovine serum (FBS), RPMI-1640 medium and nonessential amino acids were purchased from Gibco-Invitrogen (Grand Island, NY, U.S.A.). Superoxide Dismutase Activity Colorimetric Assay Kit was from Abcam (Cambridge, MA, U.S.A.). Lipid Peroxidation Malondialdehyde (MDA) Assay Kit and 2′,7′-dichlorofluorescin diacetate (DCFH-DA) were from Sigma-Aldrich Co. (St. Louis, MO, U.S.A.). Protein Extraction Reagent, Pierce™ Bicinchoninic Acid (BCA) Protein Assay Kit, Protease Inhibitor Cocktail and NE-PER™ Nuclear and Cytoplasmic Extraction Reagents were from Thermo Fisher Scientific Inc. (Waltham, MA, U.S.A.). Cell culture reagents including fetal bovine serum, RPMI-1640 medium, and appropriate antibiotics in an atmosphere of 5% CO2 were purchased from Invitrogen (Waltham, MA, U.S.A.).

**RNA extraction and real-time reverse transcriptase-polymerase chain reaction (RT-PCR).** After treatment, total RNA was isolated from cells or tissues and reversely transcribed into cDNA using a commercially available kit according to the supplier’s protocols. Real-time PCR was performed with gene specific primers (Supplementary Table 1, Thermo Fisher Scientific Inc.) with glyceraldehyde-3-phosphate dehydrogenase (GAPDH) or ACTB as internal control. The reactions were performed in an ABI Prism® 7900 High-Throughput Real-Time PCR System (Applied Biosystems, Foster City, CA, U.S.A.) under conditions identical to those previously reported (Li et al., 2014). The comparative threshold cycle is used to quantitate the relative levels of target genes compared to an endogenous control. After normalization, the fold change in gene expression was calculated using the formula: 2-ΔΔCt, where ΔCt is the difference in cycle number between the target gene and the reference gene.

**In vitro induction of steatosis and drug administration.** OA was dissolved in sterile phosphate buffered saline (PBS) plus 5% BSA and stored at −20 °C. 2 × 10⁵ of HL-7702 cells were seeded onto 6-well plates and cultured overnight. After serum starvation, cells were treated with 0.6 mM of OA for 24 h to induce steatosis. Fat accumulation was confirmed by measurement of intracellular TG content as described in our previous report (Geng et al., 2015). GSTD was dissolved in sterile saline and administered to cells at indicated concentrations. We added GSTD to cells at the same time of OA administration and treated the cells for 24 h. In some cases, cells were treated with GSTD without OA after serum starvation; 24 h later, cell total proteins were harvested for western blot analysis.
Livers of the mice were also harvested and weighed; epididymal adipose tissues were collected and weighed. Blood samples were collected by retro-orbital puncture, and serum was isolated for the measurement of serum glucose, insulin, and homoeostasis model assessment-insulin resistance (HOMA-IR) was calculated as serum glucose (mM) × serum insulin (U/mL)/22.5 (Sarafidis et al., 2007). After washing and incubation with appropriate secondary antibodies, signals were developed with an ECL kit (EMD Millipore Corporation). In some cases, membranes were stripped for antibody binding, p-Nrf2 (Ser40), p-AMPKα (Thr172) and p-LKB1 (Ser428) levels were examined by specific antibodies. Blots were scanned and quantified as described previously (Li et al., 2014). Expression levels of target proteins were normalized to those of ACTB (for total or cytoplasmic protein) or histone H3 (for nuclear protein) and plotted as fold of control. Western blot. Cell and tissue total proteins, cell nuclear and cytoplasmic proteins were extracted or isolated according to the supplier’s protocols. After quantification, samples containing about 25 μg of protein were subjected to 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto Immobilon®-P polyvinylidene difluoride (PVDF) membranes (EMD Millipore Corporation, Billerica, MA, U.S.A.) by a BIO-RAD 170-3930 Mini Trans-Blot® Electrophoretic Transfer Cell (Bio-Rad, Hercules, CA, U.S.A.). After blocking, membranes were probed with antibodies against HO-1, Nrf2, AMPKα, LKB1, ACTB (for total or cytoplasmic protein) or histone H3 (for nuclear protein). After washing and incubation with appropriate secondary antibodies, signals were developed with an ECL kit (EMD Millipore Corporation). In some cases, membranes were stripped for antibody binding, p-Nrf2 (Ser40), p-AMPKα (Thr172) and p-LKB1 (Ser428) levels were examined by specific antibodies. Blots were scanned and quantified as described previously (Li et al., 2014); the expression levels of target proteins were normalized to those of ACTB (for total or cytoplasmic protein) or histone H3 (for nuclear protein) and plotted as indicated. p-Nrf2 (Ser40), p-AMPKα (Thr172) and p-LKB1 (Ser428) levels were normalized to those of Nrf2, AMPKα and LKB1, respectively.

Animal experiments. The protocols of the animal experiments were reviewed and approved by the Research Committee of the Institute of Materia Medica, Chinese Academy of Medical Sciences (CAMS); animals were cared for according to the institutional guidelines of CAMS. Six-week old male C57BL/6J mice (20.0 ± 2.00 g) were purchased from Vital River Laboratories (Beijing, China). All mice were housed in a room at 20–24 °C with 50–60% humidity and 12 h light/dark cycle. After 5 days of accommodation period, some mice were fed with regular rodent diet as normal diet (ND) control group (n = 8), while other rats were fed with a high-fat and high-cholesterol (HFHC) diet to induce NAFLD. The HFHC diet (Vital River Laboratories) was composed of 10% lard, 0.5% cholesterol, 0.1% cholate and 89.4% regular rodent diet. Eight weeks later, these rats were continuously fed with the HFHC diet and were left untreated (HFHC control group, n = 10) or treated with GSTD at 20 mg/kg (n = 10) or 50 mg/kg (n = 10) for 10 weeks, respectively.

Body weights and food intake of the rats were recorded twice weekly. At the beginning of the experiment, at the end of week 8 and then at 18, blood samples were collected by retro-orbital puncture for the measurement of serum CHO, TG and glucose; serum ALT and AST levels were assayed on week 18. At the end of the experiment, rats were sacrificed; their livers were harvested and weighed for calculation of liver index. A portion of every liver sample was fixed in 10% formaldehyde for hematoxylin and eosin (H&E) staining; the remaining samples were immediately frozen in liquid nitrogen.

Liver total RNA was extracted for real-time RT-PCR analysis of the expression levels of key genes involved in lipid synthesis and fatty acid oxidation (Supplementary Table 1). The mRNA levels of hepatic proinflammatory cytokines and SOD1 were also determined. In addition, liver total proteins were extracted for western blot analysis of p-Nrf2 (Ser40), Nrf2, p-AMPKα (Thr172), AMPKα and HO-1 levels. A part of each liver sample was homogenized for determination of TG content and CPT1 activity following the instructions of commercially available kits. Hepatic SOD activities and MDA contents were measured as described above.

In another set of animal experiment, male Sprague–Dawley (S.D.) rats (150 ± 10.0 g, Vital River Laboratories, Beijing, China) were used. After 5 days of accommodation period, some rats were fed with regular rodent diet as normal diet (ND) control group (n = 8), while other rats were fed with a high-fat and high-cholesterol (HFHC) diet to induce NAFLD. The HFHC diet (Vital River Laboratories) was composed of 10% lard, 0.5% cholesterol, 0.1% cholate and 89.4% regular rodent diet. Eight weeks later, these rats were continuously fed with the HFHC diet and were left untreated (HFHC control group, n = 10) or treated with GSTD at 20 mg/kg (n = 10) or 50 mg/kg (n = 10) for 10 weeks, respectively.

Body weights and food intake of the rats were recorded twice weekly. At the beginning of the experiment, at the end of week 8 and then at 18, blood samples were collected by retro-orbital puncture for the measurement of serum CHO, TG and glucose; serum ALT and AST levels were assayed on week 18. At the end of the experiment, rats were sacrificed; their livers were harvested and weighed for calculation of liver index. A portion of every liver sample was fixed in 10% formaldehyde for H&E staining; the remaining portions were immediately frozen in liquid nitrogen for real-time RT-PCR analysis of proinflammatory cytokines (Supplementary Table 1), western blot, as well as determination of hepatic TG, SOD and MDA.

Blocking experiment. After serum starvation, cells were left untreated or pretreated with 10 μM of compound C (dissolved in DMSO) for 1 h, followed by OA and GSTD treatment as indicated. Twenty four later, cells were harvested for determination of intracellular SOD/ROS/MDA, mRNA levels of proinflammatory cytokines, as well as p-AMPKα (Thr172), AMPKα, p-Nrf2 (Ser40), Nrf2 and HO-1 protein levels. Nuclear translocation of Nrf2 was also determined in this experiment.

Statistical analysis. Values are mean ± S.D. of at least three separate experiments for the in vitro studies. In animal experiments, values are mean ± S.D. of 6–10 animals in each group. After validation of the test for homogeneity of variance, differences among study groups were examined by one-way ANOVA followed by the Newman–Keuls test for multiple comparisons. p < 0.05 was considered to be statistically significant.
RESULTS

GSTD suppresses oxidative stress and proinflammatory response in OA-treated HL-7702 cells

OA was used to induce steatosis in cultured HL-7702 cells. In agreement with our previous report (Geng et al., 2015), the cells developed obvious TG accumulation after 24 h of OA incubation, which could be greatly ameliorated by GSTD (data not shown).

After treatment with 0.6 mM OA, cellular SOD1 mRNA level (Fig. 1B) and SOD activity (Fig. 1A) dropped by about 50% as compared to untreated control cells (p < 0.01). In parallel with the decline of SOD, ROS and MDA levels increased about two-fold in OA-treated cells (Fig. 1A). Co-administration of GSTD increased cellular antioxidation defense and scavenged excessive free radicals and lipid peroxidation products dose-dependently. As shown in Fig. 1A and 1B, 25 and 50 μg/mL of GSTD could partially but significantly increase SOD mRNA/activity and decrease ROS/MDA levels (p < 0.05 vs. OA alone). When the concentration of GSTD reached 100 μg/mL, SOD/ROS/MDA in OA-treated cells were completely restored to near baseline levels (p < 0.01 vs. OA alone).

The mRNA expression levels of proinflammatory cytokines (Fig. 1B) like TNF-α, IL-6 and cyclooxygenase-2 (COX2) increased dramatically after OA treatment (p < 0.01 or p < 0.001 vs. untreated control cells). GSTD could prevent the OA-induced upregulation of proinflammatory cytokines in a manner similar to those of oxidative stress-related parameters.

To confirm the results of proinflammatory cytokines obtained by real-time RT-PCR, we assayed the concentrations of TNF-α and IL-6 in cell culture medium by ELISA. As shown in Fig. 1C, HL-7702 cells released significantly more amounts of TNF-α and IL-6 into culture medium after OA treatment (p < 0.01 or p < 0.001 vs. untreated control cells). GSTD dose-dependently reduced the release of TNF-α and IL-6 from cells (p < 0.05 or p < 0.01 vs. OA alone).

Taken together, these results indicate that GSTD effectively ameliorates oxidative stress and proinflammatory response in OA-induced steatosis in cultured liver cells.

GSTD activates the AMPK and Nrf2 pathway, improves lipid/glucose homeostasis and ameliorates oxidative stress/proinflammatory response in animal models of NAFLD

Next, the in vivo activities of GSTD were studied in a mouse model of NAFLD. After 10 weeks of HFD-feeding, H&E staining of liver tissue sections (Fig. 2A) showed that the mice developed severe macro-vesicular hepatic steatosis with a large amount of inflammatory cells infiltrating the portal area. In accordance, liver TG content (Table 1) increased about 3.19-fold in mice with NAFLD (p < 0.001 vs. ND). Co-administration of GSTD at 10, 20 or 50 mg/kg greatly prevented liver steatosis (Fig. 2A), reduced liver TG accumulation (p < 0.01 vs. HFD) (Table 1) and inhibited inflammatory cell infiltration (Fig. 2A). The elevated liver weight and liver index caused by HFD-feeding were also significantly decreased by GSTD treatment (p < 0.05 vs. HFD) (Table 1).

In addition to the development of NAFLD, HFD-feeding resulted in hyperlipidemia, hyperglycemia as well as hyperinsulinemia in the mice (p < 0.05 or p < 0.01 vs. ND). The NAFLD mice developed significant insulin resistance as indicated by the increase of HOMA-IR (p < 0.01 vs. ND). GSTD lowered the elevated serum CHO only at the highest dose (Table 1). In contrast, all of the three doses of GSTD reduced serum TG, glucose, insulin and HOMA-IR greatly (p < 0.05 or p < 0.01 vs. HFD).

The daily food intake was nearly identical in the studied groups (Table 1). However, at the end of the experiment, the average body weight of the mice fed with HFD was significantly heavier than that of the ND control mice (p < 0.05). Compared to those fed with ND (p < 0.01), the
mice fed with HFD were an average of 6.2 g heavier after 10 weeks. GSTD administered at 10, 20 and 50 mg/kg inhibited weight gain in the mice significantly (p < 0.01 vs. HFD). The weight of the epididymal fat pad was also measured in our experiment. Results showed that it increased dramatically after HFD-feeding (p < 0.001 vs. ND), which was designated as 1. Liver total proteins were extracted for western blot analysis of p-Nrf2 (Ser40), Nrf2, p-AMPKα (Thr172), AMPKα and HO-1 (D). Representative blots are presented. Blots were scanned and quantified; the levels of p-Nrf2 (Ser40), p-AMPKα (Thr172) and HO-1 were normalized to those of Nrf2, AMPKα and ACTB, respectively, and plotted as fold of ND, which was designated as 1. Values are mean ± S.D. of six to eight animals in each group; ** p < 0.01, *** p < 0.001 vs. that of ND group; ## p < 0.01, ### p < 0.001 vs. that of HFD group.

Figure 2. Effects of GSTD in a mouse model of NAFLD. Male C57BL/6J mice were fed with a HFD to induce NAFLD; meanwhile, GSTD at 10, 20 or 50 mg/kg was orally administered to the animals for 10 weeks. At the end of the experiment, mice were sacrificed; their livers were dissected for H&E staining (A, × 400, scale bar = 50 μm). Liver total RNAs were extracted for real-time RT-PCR determination of the expression levels of key genes involved in lipid metabolism (B), proinflammatory cytokines and SOD1 (C). The expression levels of target genes were normalized to that of GAPDH and plotted as fold of ND, which was designated as 1. Liver total proteins were extracted for western blot analysis of p-Nrf2 (Ser40), Nrf2, p-AMPKα (Thr172), AMPKα and HO-1 (D). Representative blots are presented. Blots were scanned and quantified; the levels of p-Nrf2 (Ser40), p-AMPKα (Thr172) and HO-1 were normalized to those of Nrf2, AMPKα and ACTB, respectively, and plotted as fold of ND, which was designated as 1. Values are mean ± S.D. of six to eight animals in each group; ** p < 0.01, *** p < 0.001 vs. that of ND group; ## p < 0.01, ### p < 0.001 vs. that of HFD group.

As shown in Fig. 2B, after 10 weeks of HFD-feeding, the hepatic expression of lipogenic genes such as sterol regulatory element-binding protein 1c (SREBP1c), acetyl-CoA carboxylase 1 (ACC1) and fatty acid synthase (FAS) increased dramatically, while those of the lipolytic genes like CPT1A and acyl-coenzyme A oxidase 1 (ACOX1) declined significantly (p < 0.001 vs. ND). Co-administration of GSTD greatly reduced the mRNA levels of genes involved in lipid synthesis but upregulated those involved in lipolysis and fatty acid oxidation (p < 0.001 vs. HFD). Meanwhile, the compromised liver CPT1 activity (Table 1) in HFD-fed mice could be significantly restored by GSTD treatment (p < 0.01 vs. HFD). These results suggest that GSTD inhibits hepatic lipogenesis and promotes lipolysis in mice with NAFLD.

In agreement with in vitro results, HFD-feeding led to the downregulation of SOD mRNA and activity, but significantly increased the content of MDA and TNF-α/IL-6/COX2 mRNA levels in the liver (p < 0.001 vs. ND) (Fig. 2C and Table 1). GSTD greatly restored
Table 1. Effects of GSTD on metabolism and oxidative stress related parameters in C57BL/6J mice fed with a HFD

<table>
<thead>
<tr>
<th>Measurements</th>
<th>ND (n = 6)</th>
<th>HFD (n = 8)</th>
<th>HFD + GSTD 0 mg/kg (n = 8)</th>
<th>HFD + GSTD 20 mg/kg (n = 8)</th>
<th>HFD + GSTD 50 mg/kg (n = 8)</th>
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</thead>
<tbody>
<tr>
<td>Serum CHO (mM)</td>
<td>3.43 ± 0.47</td>
<td>5.20 ± 1.11*</td>
<td>4.44 ± 0.32</td>
<td>4.20 ± 0.56</td>
<td>3.51 ± 0.57***</td>
</tr>
<tr>
<td>Serum TG (mM)</td>
<td>0.60 ± 0.05</td>
<td>0.97 ± 0.12*</td>
<td>0.64 ± 0.13*</td>
<td>0.63 ± 0.11*</td>
<td>0.59 ± 0.09**</td>
</tr>
<tr>
<td>Serum glucose (mM)</td>
<td>3.65 ± 0.33</td>
<td>7.50 ± 1.20**</td>
<td>4.12 ± 0.62**</td>
<td>3.98 ± 0.42**</td>
<td>3.77 ± 0.35**</td>
</tr>
<tr>
<td>Serum insulin (µU/mL)</td>
<td>9.54 ± 1.33</td>
<td>16.5 ± 3.24**</td>
<td>9.90 ± 0.85**</td>
<td>9.80 ± 1.48**</td>
<td>9.48 ± 0.64**</td>
</tr>
<tr>
<td>HOMA-IR</td>
<td>1.55 ± 0.25</td>
<td>5.50 ± 0.98**</td>
<td>1.81 ± 0.21**</td>
<td>1.73 ± 0.25**</td>
<td>1.59 ± 0.13**</td>
</tr>
<tr>
<td>Food intake (g/mouse/d)</td>
<td>3.25 ± 0.21</td>
<td>3.19 ± 0.41</td>
<td>3.31 ± 0.38</td>
<td>3.11 ± 0.42</td>
<td>3.18 ± 0.45</td>
</tr>
<tr>
<td>Body weight, week 0 (g)</td>
<td>20.7 ± 1.80</td>
<td>20.1 ± 1.65</td>
<td>20.9 ± 2.10</td>
<td>20.2 ± 1.95</td>
<td>20.2 ± 2.00</td>
</tr>
<tr>
<td>Body weight, week 10 (g)</td>
<td>27.8 ± 2.56</td>
<td>33.3 ± 5.76*</td>
<td>28.9 ± 4.10**</td>
<td>27.9 ± 3.78**</td>
<td>27.3 ± 3.56**</td>
</tr>
<tr>
<td>Body weight gain (g)</td>
<td>7.10 ± 1.08</td>
<td>13.3 ± 1.98**</td>
<td>8.00 ± 1.21**</td>
<td>7.70 ± 0.98**</td>
<td>7.10 ± 0.89**</td>
</tr>
<tr>
<td>Epidymal fat weight (g)</td>
<td>0.04 ± 0.02</td>
<td>1.14 ± 0.22***</td>
<td>0.39 ± 0.14**</td>
<td>0.25 ± 0.07**</td>
<td>0.22 ± 0.08**</td>
</tr>
<tr>
<td>Liver weight (g)</td>
<td>0.60 ± 0.04</td>
<td>1.13 ± 0.12**</td>
<td>0.83 ± 0.10**</td>
<td>0.80 ± 0.08**</td>
<td>0.79 ± 0.06**</td>
</tr>
<tr>
<td>Liver index (%)</td>
<td>2.16 ± 0.38</td>
<td>3.39 ± 0.51**</td>
<td>2.87 ± 0.43</td>
<td>2.86 ± 0.37**</td>
<td>2.89 ± 0.37**</td>
</tr>
<tr>
<td>Liver TG (µmol/g)</td>
<td>7.34 ± 1.24</td>
<td>23.4 ± 3.98***</td>
<td>10.8 ± 1.78**</td>
<td>10.5 ± 2.27**</td>
<td>9.89 ± 1.25**</td>
</tr>
<tr>
<td>Liver CPT1 activity (µmol/min/mg)</td>
<td>6.21 ± 1.29</td>
<td>2.67 ± 0.42**</td>
<td>5.53 ± 1.11**</td>
<td>5.78 ± 1.25**</td>
<td>6.00 ± 1.30**</td>
</tr>
<tr>
<td>Liver SOD activity (µU/mg)</td>
<td>25.0 ± 2.89</td>
<td>9.61 ± 1.68***</td>
<td>18.0 ± 2.34**</td>
<td>24.9 ± 3.05**</td>
<td>26.4 ± 3.59**</td>
</tr>
<tr>
<td>Liver MDA (nmol/mg)</td>
<td>8.18 ± 1.34</td>
<td>24.1 ± 4.17***</td>
<td>12.6 ± 2.30</td>
<td>9.50 ± 1.75**</td>
<td>9.40 ± 1.69**</td>
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The daily food intake was not statistically different among the tested groups of rats (Supplementary Table 2). GSTD greatly reduced serum CHO (at 50 mg/kg), TG, glucose levels and inhibited body weight gain induced by HFHC diet-feeding. GSTD also upregulated hepatic p-AMPKα (Thr172), p-Nrf2 (Ser40), HO-1 protein levels and manifested antioxidative and antiinflammatory activities as in the mouse model of NAFLD (Supplementary Table 2 and Supplementary Fig. 1).

In the above experiments, GSTD was administered to the mice at the same time of HFHC-feeding. In order to confirm the efficacy of GSTD, we used a HFHC diet to induce NAFLD in S.D. rats and then treated the animals with GSTD. As shown in Supplementary Table 2, the rats became obviously obese after 8 weeks of HFHC diet-feeding; their serum CHO, TG and glucose levels were significantly elevated as well (data not shown). Continuous feeding of the HFHC diet for another 10 weeks led to severe steatosis, fibrosis and inflammatory cell infiltration in the rat liver (Supplementary Fig. 1A). However, 10 weeks of GSTD administration significantly reduced liver index and fat storage, restored liver morphology and function, and inhibited inflammatory cell infiltration in the liver (Supplementary Table 2 and Supplementary Fig. 1A).

The signaling pathways involved in Nrf2 activation by GSTD were not fully clarified. Previous reports showed that cellular AMPK is an upstream molecule for Nrf2 activation and a key mediator for the antioxidant and antiinflammatory response in rodent with NAFLD.

GSTD activates the LKB1/AMPK and Nrf2 pathways in HL-7702 cells

To confirm the stimulating effect of GSTD on the Nrf2 pathway, HL-7702 cells were treated with this compound for 24 h, and western blot analyses were performed. As shown in Fig. 3A, GSTD enhanced the phosphorylation of Nrf2 at Ser40 in a dose-dependent manner. As a result, the nuclear translocation of Nrf2 (Fig. 3B) and the protein expression level of HO-1 (Fig. 3A) increased greatly after GSTD administration (p < 0.05 or p < 0.01 vs. control). GSTD activated AMPK in HL-7702 cells (Fig. 3A), which was in agreement with our previous report (Geng et al., 2015). How the AMPK pathway is activated by GSTD is not clear. Here, our new result proved that GSTD significantly enhanced the phosphorylation of LKB1 at Ser428 (Fig. 3A).

GSTD activates Nrf2 and ameliorates oxidative stress/proinflammatory response through AMPK activation

The signaling pathways involved in Nrf2 activation by GSTD were not fully clarified. Previous reports showed that cellular AMPK is an upstream molecule for Nrf2 activation and a key mediator for the antioxidant and antiinflammatory response in rodent with NAFLD.

HFD-feeding resulted in a marked decrease of the AMPK activity (p < 0.01 vs. ND) (Fig. 2D), which was in accordance with the change of hepatic lipogenesis and lipolysis. Because of the crucial role of the Nrf2 pathway in maintaining cellular redox state and protecting cells against oxidative stress/proinflammatory response in rodents with NAFLD. As shown in Fig. 2D, hepatic Nrf2 and HO-1 protein levels were not affected by HFD. Interestingly, GSTD had no influence on liver total Nrf2 protein expression, but significantly increased p-Nrf2 (Ser40) level, which was proven to be crucial for Kelch-like ECH-associated protein 1 (Keap1) dissociation and nuclear distribution of Nrf2 (Vomhof-Dekrey and Picklo, 2012; Mo et al., 2014), we evaluated the influence of GSTD on Nrf2 in NAFLD. As shown in Fig. 2D, hepatic Nrf2 and HO-1 protein levels were significantly elevated as well (data not shown). Continuous feeding of the HFHC diet for another 10 weeks led to severe steatosis, fibrosis and inflammatory cell infiltration in the rat liver (Supplementary Fig. 1A).

antiinflammatory activities of some compounds (Mo et al., 2014; Endo et al., 2013; Jeong et al., 2009). It was possible that GSTD may stimulate the Nrf2 pathway through AMPK activation. We used compound C, a selective inhibitor of AMPK, to treat the cells in order to test our hypothesis.

As shown in Fig. 4A, OA alone had no influence on Nrf2 and HO-1 expression. And again, GSTD did not influence total Nrf2 protein level although it did increase its phosphorylation at Ser40 in OA-treated HL-7702 cells. As a result, the nuclear distribution of Nrf2 (Fig. 4B) and HO-1 expression (Fig. 4A) increased greatly because of the effects of GSTD administration (p < 0.01 vs. OA alone). These results demonstrated that GSTD could stimulate the Nrf2 pathway in HL-7702 cells in the background of OA challenge.

The cellular baseline AMPK activity was decreased by OA (in agreement with in vivo result) (Fig. 4A). The stimulating activities of GSTD on AMPKa (Thr172)/Nrf2 (Ser40) phosphorylation (Fig. 4A), Nrf2 nuclear translocation (Fig. 4B) and HO-1 expression (Fig. 4A) were completely blocked by compound C pretreatment (p < 0.01 vs. OA + GSTD). Accordingly, the activities of GSTD in reducing OA-induced oxidative stress (Fig. 4C) and proinflammatory response (Fig. 4D) were abolished by AMPK inhibition.

DISCUSSION

At the present time, an approved standard therapy for NAFLD is still not available clinically. In addition to diet control and exercise, agents such as lipid-lowering drugs and insulin sensitizers are often used to treat NAFLD (Henao-Mejia et al., 2012). However, some of those drugs are reported to increase the burden on the liver and disturb liver function. Here, we report that natural product GSTD, which is safe and low toxic in clinic, has integrated beneficial effects against oxidative stress/proinflammatory response and metabolic disorders in NAFLD.

Our results show that GSTD promoted Nrf2 nuclear translocation and HO-1 expression in liver cells. HO-1 works in close relationship with NAFLD. For example, it was reported to be upregulated in patients with NAFLD as an adaptive response (Malaguarnera et al., 2005), and its polymorphism was related to the risk of NAFLD in children (Chang et al., 2015). In the pathogenesis and progression of NAFLD, oxidative stress and proinflammatory response played important roles. The catalysates of HO-1, which include free iron, biliverdin/bilirubin and carbon monoxide, were reported to have antioxidative, antiinflammatory, cytoprotective and anti-fibrosis activities in the liver (Tsui et al., 2005). Now, HO-1 is considered a useful target for the treatment of metabolic diseases (Son et al., 2013). Our results indicate that GSTD suppressed liver fibrosis induced by HFHC diet-feeding. Our findings were in agreement with a recent report (Zhao et al., 2015), in which GSTD could ameliorate liver fibrosis induced by bile duct ligation. These results imply that the beneficial effects of GSTD on the liver may not be restricted to NAFLD.

Multiple cellular signaling pathways or kinases like AMPK (Mo et al., 2014; Endo et al., 2013), phosphatidylinositol 3-kinase (PI3K)/Akt (Rodriguez et al., 2013) as well as mitogen-activated protein kinases (MAPks) (Kensler et al., 2007) were reported to stimulate Nrf2 nuclear translocation. Among the three MAPks, extracellular signal-regulated kinase (ERK) and P38 were reported to be activated by GSTD to stimulate the Nrf2 pathway and inhibit oxidative stress in neurons (Jiang et al., 2014a; Wang et al., 2014; Zhao et al., 2012). However,
there were also reports indicating that GSTD suppressed the expression of proinflammatory cytokines in lipopolysaccharide (LPS)-challenged cardiomyocytes and neurons through inhibition of MAPK phosphorylation (Dai et al., 2011; Yang et al., 2013). In metabolic disorder models, OA and HFD-feeding were reported to activate the MAPK pathways (Soto-Guzman et al., 2008; Sankar et al., 2015). How the MAPK pathways are modulated by GSTD under overnutrition and metabolic stress is unknown and requires further investigation.

Our results strongly suggest that GSTD stimulates the Nrf2 pathway through AMPK activation in liver cells, as AMPK inhibition by compound C totally abolished the activities of GSTD on Nrf2 (Ser40) phosphorylation, nuclear translocation as well as HO-1 expression. Celluar AMPK could be activated by upstream kinases like liver kinase B1 (LKB1) and Ca²⁺/calmodulin-dependent protein kinase II (CaMKII), as well as inhibition of mitochondrial function (Yin et al., 2012). It was reported that GSTD could block the activation of CaMKII in neurons (Jiang et al., 2014b). Here, our results clearly demonstrate that GSTD activated LKB1 in liver cells. The phosphorylation of LKB1 at Ser428 was shown to be essential for its ability to bind and activate AMPK (Xie et al., 2006). So, it is obvious that LKB1 activation is at least one of the mechanisms used by GSTD to stimulate AMPK in the liver.

AMPK is a key molecule that controls energy balance in organisms, and now, it is considered an important molecular target for the treatment of metabolic disorders such as dyslipidemia, diabetes, fatty liver and obesity (Steinberg and Kemp, 2009). Our results revealed that GSTD activated hepatic AMPK significantly in animals with NAFLD, which resulted in overall improvement of metabolic disorders.

GSTD inhibited lipogenesis and promoted lipolysis in HFD-fed mice. The expression of lipogenic genes like ACC1 and FAS is stimulated by SREBP1c, which could be downregulated by AMPK activation (Liao et al., 2014). Upon activation, AMPK could phosphorylate ACC2 and inhibit its enzymatic activity, which in turn stimulates CPT1 activity and fatty acid β-oxidation (O’Neill et al., 2014; Park et al., 2002). We noticed that both the CPT1 activity and mRNA were upregulated by GSTD in the mouse liver. CPT1A and ACOX1 are rate-limiting enzymes for mitochondrial or peroxisomal fatty acid β-oxidation (Park et al., 2002; Oaxaca-Castillo et al., 2007) and are target genes of peroxisome proliferator activated receptor α (PPARα) (Shi et al., 2013). AMPK was reported to phosphorylate and stimulate PPARα upon activation (Li et al., 2007). Whether or not GSTD could activate hepatic PPARα, either directly or via the AMPK pathway, is a topic for further study.
Unlike its strong activities in modulation of TG and glucose metabolism, we noticed that the influence of GSTD on serum CHO was seemingly only mild. Similar results were obtained in hyperlipidemic rats treated with the powder of *G. elata* BI. (Geng et al., 2013). These results suggest that GSTD may regulate CHO metabolism through different mechanisms.

In addition to metabolic regulation, AMPK was also responsible for the antioxidative and antiinflammatory activities of GSTD in OA-treated liver cells, as compound C abolished the stimulating effect of GSTD on SOD and blocked its inhibitory effects on TNF-α/IL-6/COX2 expression. These results were in agreement with the AMPK-dependent Nrf2 activation by GSTD. Experimental data from our study support that the AMPK may be activated by GSTD as a pivotal pathway to modulate metabolism and oxidative stress/proinflammatory response simultaneously in NAFLD (Fig. 5).

GSTD reduced blood glucose and insulin resistance in NAFLD mice in our present study. And interestingly, GSTD was revealed to activate the PI3K/Akt pathway (Yang et al., 2013; Peng et al., 2015), which is critical for insulin stimulated glucose uptake. Furthermore, GSTD suppressed MAPK activation and the expression of proinflammatory cytokines in LPS-challenged cardiomyocytes in a PI3K/Akt-dependent manner (Yang et al., 2013).

PI3K/Akt activation is associated with the inhibition of glycogen synthase kinase-3 (GSK-3). Recent studies demonstrated that GSK-3 negatively regulated Nrf2 by promoting its phosphorylation at Ser335/338 and subsequent degradation (Cuadrado, 2015). So theoretically, the activation of PI3K/Akt pathway may lead to Nrf2 upregulation. Indeed, some studies report that GSTD increased the protein expression level of Nrf2 in neurons and tissues (Wang et al., 2014; Zhao et al., 2012; Peng et al., 2015). However, our results indicated that in liver cells, GSTD promoted Nrf2 phosphorylation at Ser40 rather than directly upregulating its expression. Whether or not GSTD could influence the phosphorylation of Nrf2 at Ser335/338 and modulate the expression of Nrf2 in a tissue specific manner needs to be further investigated. The influence of GSTD on the PI3K/Akt pathway in NAFLD and its crosstalk between AMPK as well as the Nrf2 pathway are now under investigation in our laboratory.

In conclusion, our results in the present study indicate that natural botanical compound GSTD activates the AMPK/Nrf2 pathway, ameliorates hepatic oxidative stress/proinflammatory response and significantly improves metabolic disorders in NAFLD. Considering the safety-profile and long history of this drug, our findings will provide scientific basis for the clinical application of GSTD to treat NAFLD in the future.

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Conflict of Interest

The authors declare no conflict of interest.

REFERENCES


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