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Original Article

Omega-3 PUFA ameliorates hyperhomocysteinemia-induced hepatic steatosis in mice by inhibiting hepatic ceramide synthesis

Yong-qiang DONG, Xing-zhong ZHANG, Lu-lu SUN, Song-yang ZHANG, Bo LIU, Hui-ying LIU, Xian WANG*, Chang-tao JIANG*

Department of Physiology and Pathophysiology, School of Basic Medical Sciences, Peking University, Key Laboratory of Molecular Cardiovascular Science, Ministry of Education and Beijing Key Laboratory of Cardiovascular Receptors Research, Beijing 100191, China

Abstract

Hyperhomocysteinemia (HHcy) is a key risk factor in hepatic steatosis. In this study, we applied a metabolomic approach to investigate the changes in the metabolite profile due to HHcy-induced hepatic steatosis and the effects of omega-3 PUFA (polyunsaturated fatty acid) supplementation in mice. HHcy was induced in mice by giving DL-Hcy (1.8 g/L) in drinking water for 6 weeks, then the mice were sacrificed, and the metabolic profiles of the liver and plasma were analyzed through UPLC-ESI-QTOFMS-based lipidomics. Hepatic triglycerides and cholesterol were further assayed. The expression of ceramide metabolism-related genes was measured by quantitative PCR. Compared with control mice, HHcy mice exhibited hepatic steatosis with a notable increase in ceramide-related metabolites and subsequent upregulation of ceramide synthesis genes such as *Sptlc3*, *Degs2*, *Cer4* and *Smpd4*. Omega-3 PUFA was simultaneously administered in HHcy mice through chow diet containing 3.3% omega-3 PUFA supplement for 6 weeks, which significantly ameliorated Hcy-induced hepatic steatosis. The decrease in hepatic lipid accumulation was mainly due to reduced hepatic levels of ceramides, which was partly the result of the lower expression of ceramide synthesis genes, *Sptlc3* and *Degs2*. Similar beneficial effects of DHA were observed in Hcy-stimulated primary hepatocytes *in vitro*. In summary, Hcy-induced ceramide elevation in hepatocytes might contribute to the development of hepatic steatosis. Furthermore, downregulation of ceramide levels through omega-3 PUFA supplementation ameliorates hepatic lipid accumulation. Thus, ceramide is a potential therapeutic target for the treatment of hepatic steatosis

Keywords: nonalcoholic fatty liver disease; hepatic steatosis; hyperhomocysteinemia; omega-3 PUFA; DHA; ceramide; metabolomics

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Introduction

Nonalcoholic fatty liver disease (NAFLD) is a chronic liver disease that may progress from steatosis to nonalcoholic steatohepatitis (NASH)[1]. NAFLD is one of the most common liver disorders, affecting 17%-33% of the general population in the USA[2]. A recently recognized risk factor of NAFLD is hyperhomocysteinemia (HHcy), whose hallmarks include a sulfur-containing amino acid derived from metabolism of methionine, and elevated plasma Hcy levels (>10 μmol/L) define HHcy. HHcy is an independent risk factor involved in several metabolic disorders[3-7]. Recently, plasma Hcy levels have been reported to be positively correlated with the development of hepatic steatosis[8]. In addition, a high-methionine diet (HMD) promotes hepatic steatosis in mice[9, 10]. Although several studies have suggested that HHcy perturbs lipid metabolism via enhanced CD36 expression[9, 11], the metabolite profile changes and precise targets for the treatment of HHcy-induced hepatic steatosis remain unknown.

Omega-3 fatty acids belong to the family of polyunsaturated fatty acids, which exert various benefits against metabolic syndromes such as obesity and insulin resistance[12, 13]. Omega-3-mediated improvements in lipogenesis and hepatic lipid metabolism have been reported in numerous studies[14, 15]. Other studies have demonstrated that supplementation with omega-3 impedes the development of NAFLD by suppressing of lipogenic genes such as *Srebp1c*[14, 16]. However, to date, the lipid metabolic profiles of the liver after supplementation with omega-3 remained unclear. In this study, we determined the mechanism underlying omega-3 PUFA's amelioration of

*To whom correspondence should be addressed.
E-mail jiangchangtao@bjmu.edu.cn (Chang-tao JIANG);
xwang@bjmu.edu.cn (Xian WANG)
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hepatic steatosis induced by HHcy.

We found that ceramides are more abundant in HHcy-induced fatty livers, specifically, hepatic levels of saturated ceramides were significantly increased in HHcy mice compared with control mice, possibly because of the upregulation of ceramide synthases in hepatocytes induced by Hcy. Omega-3 supplementation ameliorated HHcy-induced hepatic steatosis through the inhibition of ceramides in the liver, thus suggesting that ceramide may be a potential therapeutic target for the treatment of patients with hepatic steatosis.

Materials and methods

Reagents

DL-homocysteine (Hcy) and docosahexaenoic acid (DHA) were purchased from Sigma-Aldrich Chemical (St Louis, MO, USA). Ceramides were obtained from Avanti Polar Lipids (Alabaster, AL, USA).

Animals

All procedures involving mice were approved by the Peking University Animal Care and Use Committee. Mice (C57BL/6J background) were housed in an animal facility and given free access to water with or without DL-Hcy (1.8 g/L) for 6 weeks as previously described[3, 4, 6]. Each group of mice was fed a chow diet either containing 4% fat by weight or supplemented with approximately 3.3% omega-3 PUFAs comprising DHA/EPA (D/E, 3:1) (33 mg/g; a generous gift from Abbott, USA) as previously described in different mouse models[17, 18]. Body weight was measured every 2 weeks during the experiments.

Histological analysis

Oil red O staining was performed on frozen liver sections through a standard protocol, as previously described[9].

Triglyceride and cholesterol content quantification

Total triglycerides and total cholesterol in liver were measured with enzymatic kits from Wako Life Sciences (Richmond, VA, USA) and Thermo Scientific (Waltham, MA, USA) as described previously[9].

Cell culture

Murine primary hepatocytes were isolated as previously described[9, 19, 20]. Hepatocytes were cultured in growth medium, RPMI-1640 medium supplemented with 10% fetal bovine serum. After a 4-h attachment, cells were treated as indicated in the experiments.

Sample preparation for metabolomics analysis

For hepatic lipidomics analysis, approximately 10 mg of liver were homogenized with 200 μL of H_2O and then extracted with 1200 μL of a cold chloroform:methanol (2:1) solution containing 2 μmol/L ceramide (19:0) as an internal standard. The homogenate was vortexed at 4 °C for 20 min then centrifuged for 20 min at 13 000 revolutions per minute. The lower organic phase was transferred to a new tube and evaporated under a vacuum. The residue was suspended in 200 μL of a chloroform:methanol (1:1) solution and then diluted with an isopropanol:acetonitrile:H_2O (2:1:1) solution.

Lipidomics determination

Samples were analyzed with a Thermo Scientific™ Q Exactive™ hybrid quadrupole Orbitrap mass spectrometer equipped with a Thermo Scientific™ Dionex™ UltiMate™ 3000 Rapid Separation LC (RSLC) system to perform UPLC separations. The UPLC conditions were set as follows. The column was an HSS T3 column (2.1 mm×100 mm, 1.7 μm, Waters) operated at 45 °C. The two UPLC buffers were A-water and B- methanol, both of which contained 0.1% formic acid. The mobile phase gradient was set as: 95% A at 0.1 min; 80% A at 3 min; 25% A at 4.5 min; 0% A at 6.5 min; 0% A at 15 min; 95% A at 15.5 min; 95% A at 17 min. Ceramides and other lipid metabolites were identified by comparing the parent ion mass and MS/MS fragmentations to acknowledged database such as https://metlin.scripps.edu/ or http://www.hmdb.ca. Peak extraction and integration were operated with Xcalibur 2.2 SP1.48 software (Thermo Fisher Scientific, USA), the relative quantification of each metabolite was calculated by comparison to internal standard ceramide (19:0). The mass spectrometer parameters of the full mass scan were set as follows. The pos HESI-II spray voltages were 3.7 kV. The heated capillary temperature was 320 °C, and the heated vaporizer temperature was 300 °C. The sheath gas pressure was 30 psi, and the auxiliary gas pressure was 10 psi. The resolution was 70 000. The auto gain control target was under 1×10^6. The maximum isolation time was 50 ms, and the m/z range was 150–1500.

Data processing and multivariate data analysis

Xcalibur 2.2 SP1.48 software (Thermo Fisher Scientific, USA) was used for peak extraction and integration. Statistical models, including PLS-DA analysis and heat maps, were created with the MetaboAnalyst 3.0 web service (http://www.metaboanalyst.ca/). The relative level of each analyte was calculated after normalization of the peak area to that of the internal standard.

Statistical analysis

The data are expressed as the mean±SEM. Statistical analyses were performed with two-tailed Student's *t*-tests and one-way ANOVA with Tukey's confirmation. *P* values less than 0.05 were considered statistically significant.

Results

HHcy induces hepatic steatosis *in vivo*

To evaluate the role of HHcy in hepatic steatosis, wild-type C57BL/6J mice were administered drinking water with or without Hcy (1.8 g/L) for 6 weeks. The food consumption, body weight and fasting plasma glucose levels remained unchanged between the HHcy and control mice (Supplementary Figure S1A-S1C). However, Oil red O staining of the liver sections revealed a significant increase in the number of hepatic lipid droplets in the HHcy mice (Figure 1A), which

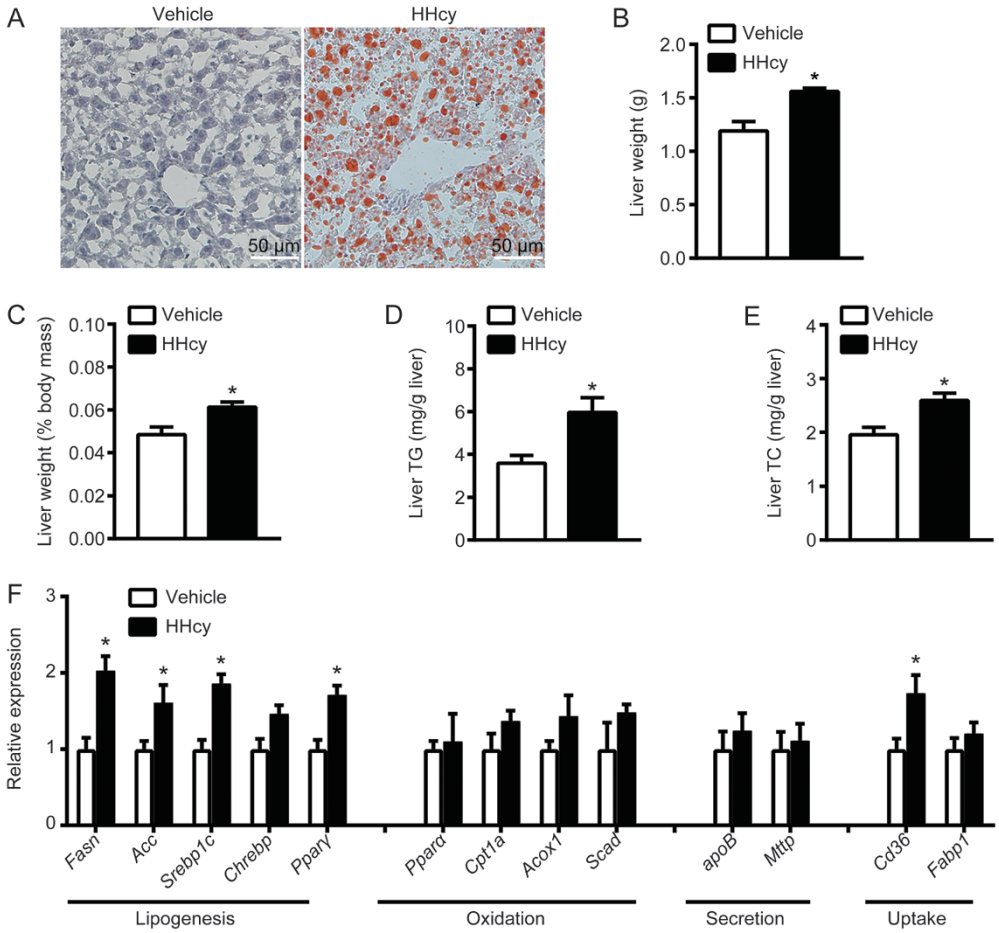

Figure 1. Effects of HHcy on lipid accumulation *in vivo*. (A) ORO staining of lipids in representative liver sections. (B) Liver weight. (C) Ratios of liver weight/body weight. (D) Hepatic TG content. (E) Hepatic TC content. (F) The mRNA levels of genes involved in hepatic lipogenesis, FFA oxidation, TG secretion and FFA uptake. (A-F) Male C57BL/6 mice (6 weeks old) were treated with vehicle or Hcy (1.8 g/L) for 6 weeks (n=6/group). All data are presented as the mean±SEM. Two-tailed Student's t-test: *P<0.05 compared with vehicle.

was concomitant with elevated liver weight (Figure 1B) and liver/body mass ratios (Figure 1C). Hepatic triglycerides (Figure 1D) and cholesterol (Figure 1E) were also increased in mice treated with HHcy compared with control. In addition, fatty acid synthesis-related genes, such as fatty acid synthase (*Fasn*), acetyl-CoA carboxylase (*Acc*), sterol response element–binding protein 1c (*Srebp1c*) and peroxisome proliferator-activated receptor gamma (*Pparγ*) in the liver as well as fatty acid uptake-related genes, including cluster of differentiation 36 (*Cd36*), were markedly upregulated in HHcy mice. In contrast, the expression of genes involved in fatty acid β-oxidation and TG secretion was not affected in the HHcy treatment group (Figure 1F). These results indicate that HHcy may induce lipid perturbation and hepatic steatosis.

The hepatic ceramide levels are significantly elevated after Hcy treatment

To further determine the metabolic profiles of HHcy-induced hepatic steatosis, UPLC-ESI-QTOFMS-based lipidomics analysis was performed to investigate the metabolites in the liver. Heat map analysis and the VIP scores of the biomarkers showed that ceramides were among the metabolites that exhibited differences between the groups (Figure 2A-2C). The hepatic saturated C18:0, C20:0, C22:0 and C24:0 ceramides were significantly upregulated in HHcy mice (Figure 2D), whereas the metabolic profiles of the plasma showed no differences (Supplementary Figure S2A, S2B). These results suggest that HHcy significantly increases liver ceramide levels.

Hcy promotes ceramide synthesis both *in vivo* and *in vitro*

To further investigate the mechanism by which Hcy mediated the increase in ceramide metabolites, ceramide metabolism-related genes in the liver were examined. qPCR analysis showed that the mRNAs encoded by ceramide synthesis-related genes such as serine palmitoyl transferase long chain base 3 (*Sptlc3*), ceramide synthase 4 (*Cers4*), degenerative spermatocyte homolog 2 (*Degs2*) and sphingomyelin phosphodiesterase 4 (*Smpd4*) were significantly increased *in vivo* (Figure 3A). Moreover, we found that Hcy also promoted the gene expression of *Sptlc3* and *Degs2* in primary hepatocytes stimulated with Hcy (100 μmol/L) for 24 h (Figure 3C). However, the mRNAs encoded by genes involved in ceramide

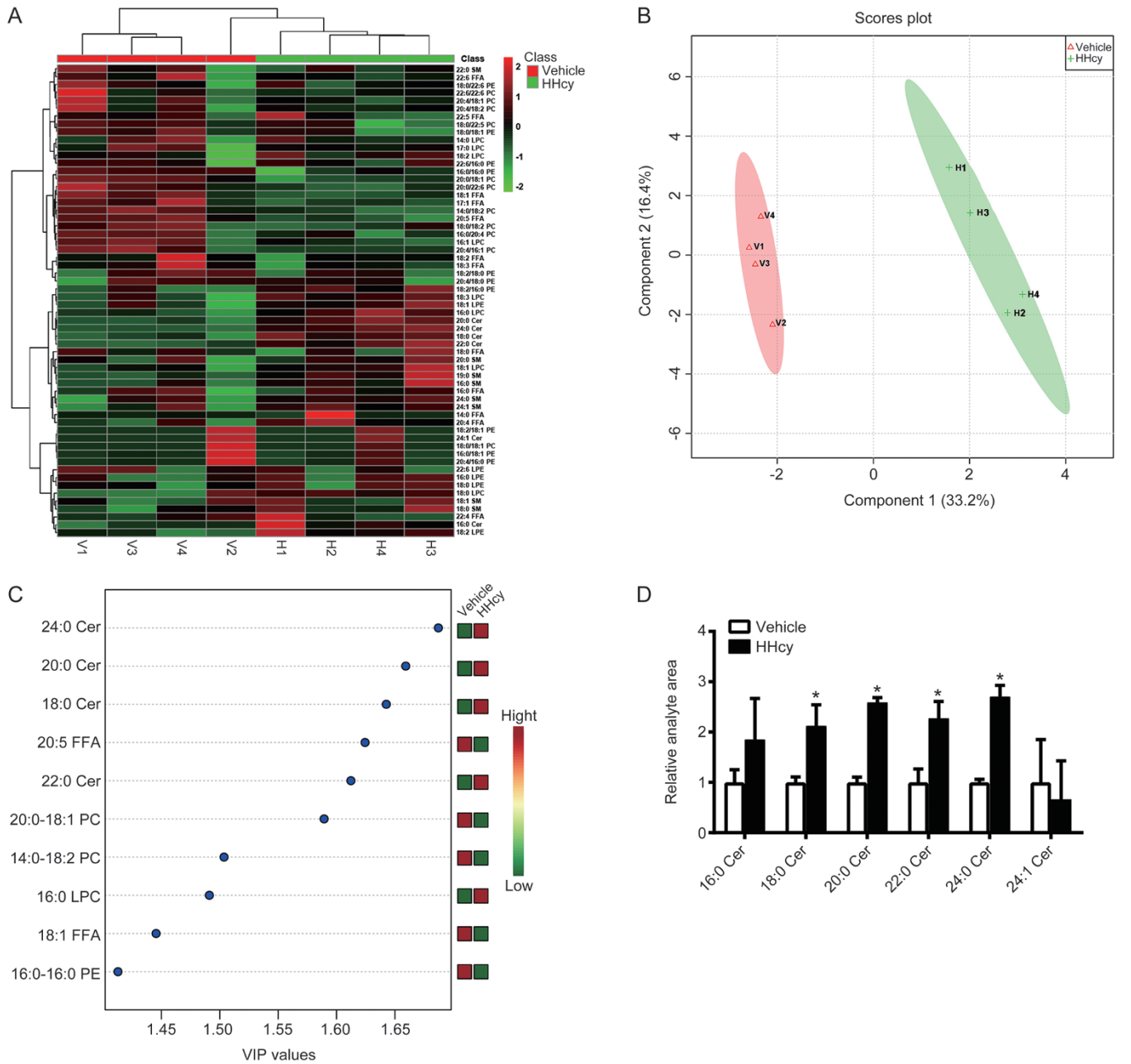

Figure 2. Metabolic profiling of HHcy mice and control mice. (A) Heat map of lipid metabolites in liver. (B) Score scatter plot of the partial least squares discriminant analysis (PLS-DA) model of lipid metabolites in the liver; the red plot represents the vehicle group, and the green plot represents the HHcy group. (C) Variable importance in projection (VIP) plot generated from the PLS-DA, displaying the top 10 most important metabolite features in the liver. (D) The levels of saturated ceramides in the liver. (A-D) Male C57BL/6 mice (6 weeks old) were treated with vehicle or Hcy (1.8 g/L) for 6 weeks (n=4/group). (D) The data are presented as the mean±SEM. Two-tailed Student's t-test: *P<0.05 compared to vehicle.

catabolism, including sphingomyelin synthase 1 and 2 (*Sgms1* and *Sgms2*), alkaline ceramidase 1 and 2 (*Acer1* and *Acer2*), ceramide kinase (*Cerk*) and sphingosine kinase1 and 2 (*Sphk1* and *Sphk2*) showed no significant changes compared with corresponding controls in both the livers from Hcy-treated mice and primary hepatocytes treated with Hcy (100 μmol/L) for 24 h (Figure 3B, 3D). Together, these results suggest that Hcy stimulates ceramide synthesis primarily by inducing the expression of genes involved in ceramide synthases in the hepatocytes of mice.

Omega-3 PUFA treatment ameliorates HHcy-induced hepatic steatosis

It has been demonstrated that dietary omega-3 PUFA ameliorates the development of liver dysfunction and steatosis[14, 21]. Therefore, we determined whether HHcy-induced hepatic steatosis might be alleviated by omega-3 PUFA supplementation. Omega-3 PUFA decreased HHcy-induced lipid accumulation

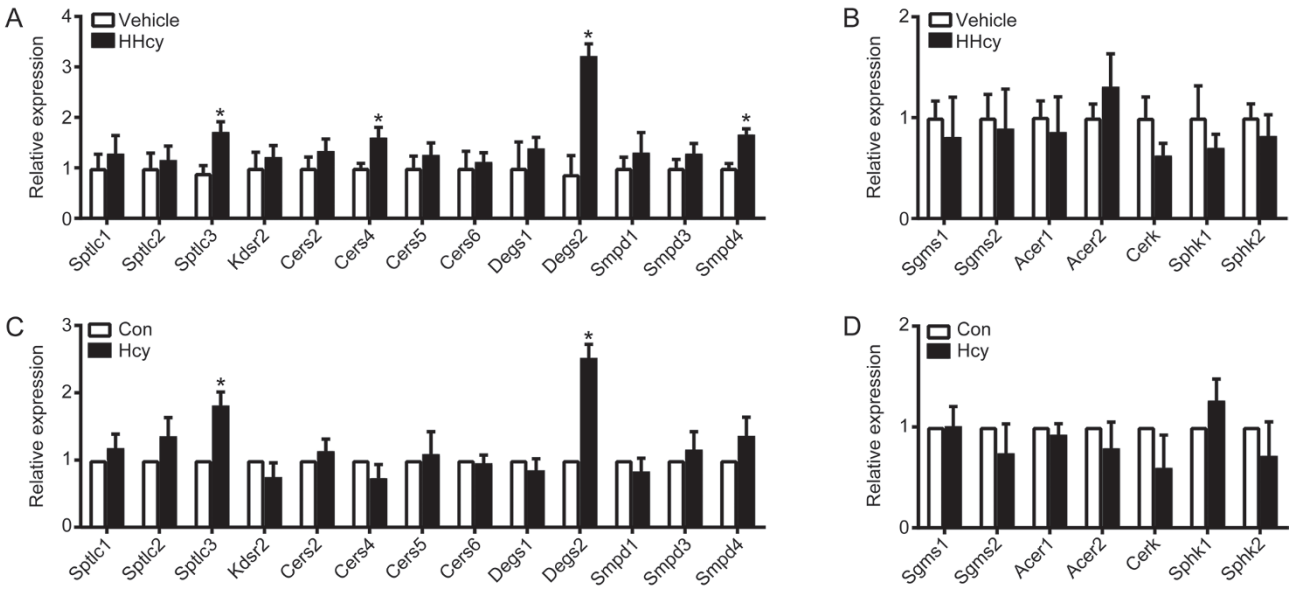

Figure 3. Hcy induces ceramide synthases production both *in vivo* and *in vitro*. (A) The mRNA levels of ceramide synthases in the liver. (B) The mRNA levels of ceramide hydrolases in the liver. (A, B) Male C57BL/6 mice (6 weeks old) were treated with vehicle or Hcy (1.8 g/L) for 6 weeks (n=6/group). (C) The mRNA levels of ceramide synthases in primary hepatocytes. (D) The mRNA levels of ceramide hydrolases in primary hepatocytes. (C, D) Primary hepatocytes were treated with Hcy (100 μmol/L) for 24 h (n=5). All data are presented as the mean±SEM. Two-tailed Student's t-test: *P<0.05 compared with vehicle or control as appropriate.

in the liver without affecting the food consumption, body weight and fasting plasma glucose levels (Figure 4A, Supplementary Figure S1D–S1F). In line with this, the liver weight and liver/body mass ratios were reduced in mice treated with omega-3 PUFA (Figure 4B, 4C). Moreover, omega-3 PUFA protected from HHcy-augmented hepatic TG and TC accumulation (Figure 4D, 4E) as well as mRNA expression of *Fasn*, *Srebp1c* and *Pparγ* (Figure 4F). These data suggest that omega-3 PUFA might ameliorate HHcy-induced hepatic steatosis.

Omega-3 PUFA modifies ceramide metabolism

To determine the changes in the metabolic profiles in the liver after treatment with omega-3 PUFA, UPLC-ESI-QTOFMS-based metabolomics analysis was used to detect the lipid composition. Heat map analysis and a PCA model of the UPLC-ESI-QTOFMS negative mode data from mouse livers showed distinct metabolic profiles among the vehicle-, HHcy- and HHcy+omega3-treated groups (Figure 5A, 5B). Further analysis indicated that omega-3 PUFA abrogated HHcy-induced increases in the saturated ceramide levels in the liver (Figure 5C). These results suggest that the inhibition of ceramides by omega-3 PUFA might lead to improvements in HHcy-induced hepatic steatosis.

Omega-3 PUFA decreases ceramide synthase both *in vivo* and *in vitro*

To further investigate the mechanism by which omega-3 PUFA decreased the levels of ceramide metabolites, ceramide metabolism-related genes were examined. qPCR analysis showed that the expression of genes corresponding to ceramide synthase, *Sptlc3* and *Degs2*, were increased by HHcy, and this effect was reversed by omega-3 PUFA supplementation (Figure 6A, 6B). Docosahexaenoic acid (22:6 omega-3, DHA) is a major polyunsaturated fatty acid (PUFA) in the omega-3 series. When pretreatment of 24 h Hcy-stimulated primary hepatocytes with DHA (20 μmol/L), the expression of ceramide synthases were significantly attenuated by DHA (Figure 6C, 6D). Together, these findings indicate that the beneficial effects of omega-3 PUFA on HHcy-induced hepatic steatosis are, at least in part, due to the suppression of ceramide synthases in HHcy mice.

Discussion

NAFLD is currently the most common hepatic disorder worldwide[22-24]. Increasing evidence indicates that HHcy is a new risk factor for hepatic steatosis[8-10]. Several studies have reported that HHcy induces hepatic steatosis by disrupting lipid metabolism[9, 25]. Here, we adopted a metabolomic approach to explore the metabolic profiles of hepatic steatosis induced by HHcy and to search for the potential therapeutic targets for treating hepatic steatosis. We found that ceramides (particularly saturated ceramides) were elevated with the development of HHcy-induced hepatic steatosis. Furthermore, the hepatic ceramide levels in HHcy mice supplemented with omega-3 PUFA were significantly decreased, and these mice showed improvements in hepatic lipid accumulation. Interestingly, analysis of the expression of genes involved in ceramide metabolism showed that the HHcy-induced increased ceramide metabolic enzymes in hepatocytes were markedly attenuated in the presence of omega-3 PUFA both *in vivo* and *in vitro*.

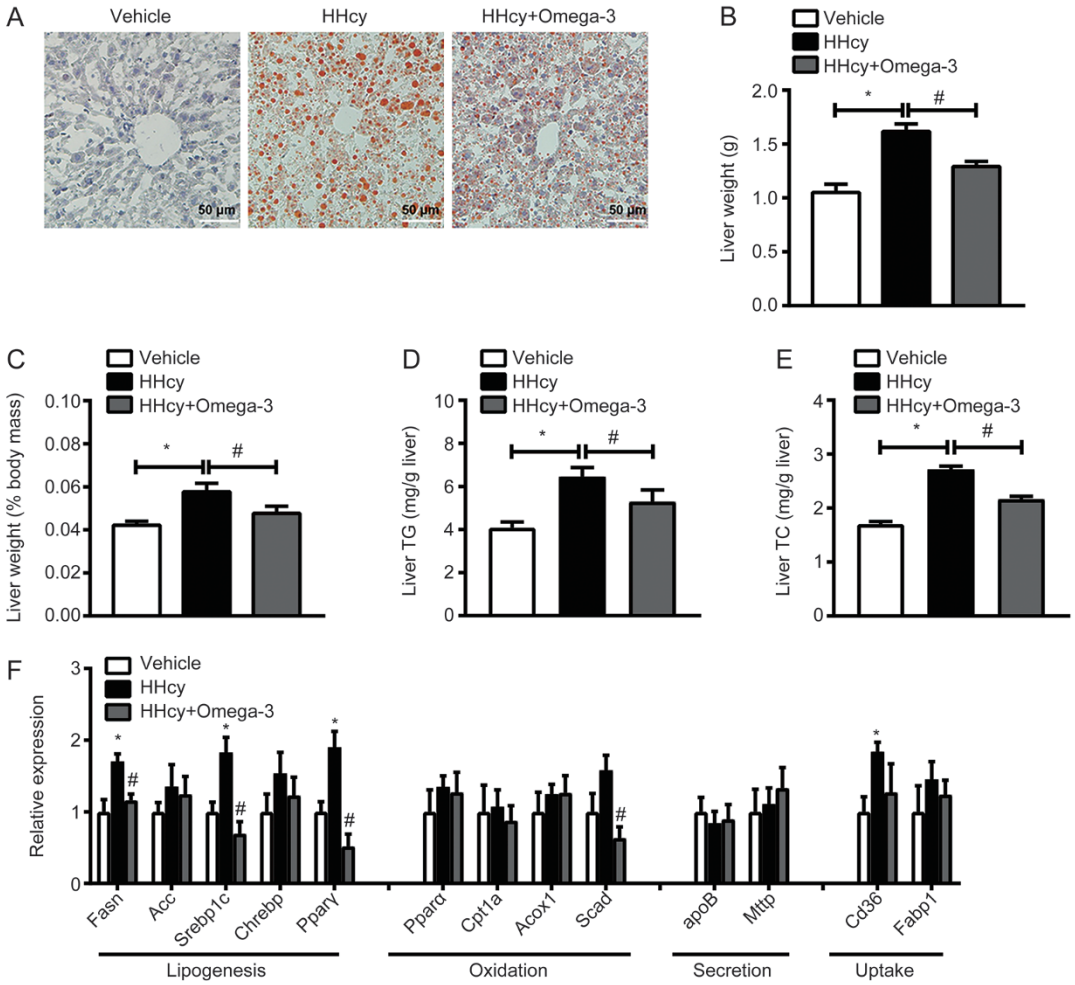

Figure 4. Administration of omega-3 PUFA attenuates the development of HHcy-induced hepatic steatosis. (A) ORO staining of lipids in representative liver sections. (B) Liver weight. (C) Ratios of liver weight/body weight. (D) Hepatic TG content. (E) Hepatic TC content. (F) The mRNA levels of genes involved in hepatic lipogenesis, FFA oxidation, TG secretion and FFA uptake. Hcy-treated mice were fed a standard chow diet or a chow diet supplemented with omega-3 PUFA (33 mg/g) for 6 weeks and were compared with mice administered vehicle for 6 weeks (n=6/group). All data are presented as the mean±SEM. (B-F) One-way ANOVA with Tukey's correction: *P<0.05 compared with vehicle; $^{\#}P$<0.05 compared to HHcy.

Ceramide, which serves as the backbone for sphingolipids, has been linked with insulin resistance and NAFLD and is considered as a biomarker for these diseases[19, 26]. A series of studies have revealed that increased ceramides in the liver and plasma are positively correlated with the development of hepatic insulin resistance and steatosis in rodents[27-29]. A clinical study has further confirmed that the concentration of hepatic ceramides is higher in patients with NAFLD[26]. Furthermore, it has been reported that saturated ceramides such as C16:0 ceramide induce lipid accumulation by upregulating the genes involved in hepatic lipogenesis in a mouse model[30]. Besides C16:0 Cer, numerous studies have demonstrated that the levels of C18:0, C20:0 and C24:0 saturated ceramides were also increased in HFD-induced hepatic steatosis[29, 31-34]. The inhibition of the *de novo* synthesis of saturated ceramides significantly decreased hepatic lipid accumulation and insulin resistance[30, 34]. In line with the results of these studies, we found that ceramides, especially saturated ceramides such as C18:0, C20:0, C22:0 and C24:0, were positively correlated with the progression of HHcy-induced hepatic steatosis. Furthermore, the metabolic profiles in the plasma were unchanged, thus suggesting that the accumulation of ceramides was primarily localized to the liver. Of note, the disruption of hepatic ceramides by omega-3 PUFA led to a decrease in HHcy-induced hepatic steatosis. These results suggested that ceramides play a crucial role in HHcy-induced hepatic steatosis.

In addition, other studies have reported that the inhibition of hepatic ceramide synthesis also ameliorates HFD-induced hepatic steatosis in a mouse model[30, 35]. Collectively, these reports have suggested that inhibiting hepatic ceramide generation and accumulation might be a common target for treating NAFLD and hepatic steatosis.

To explore the mechanism by which Hcy increased the hepatic ceramide levels, we measured the different enzymatic pathways involved in Hcy–regulated ceramide metabolism. Previous studies have revealed that ceramide synthases

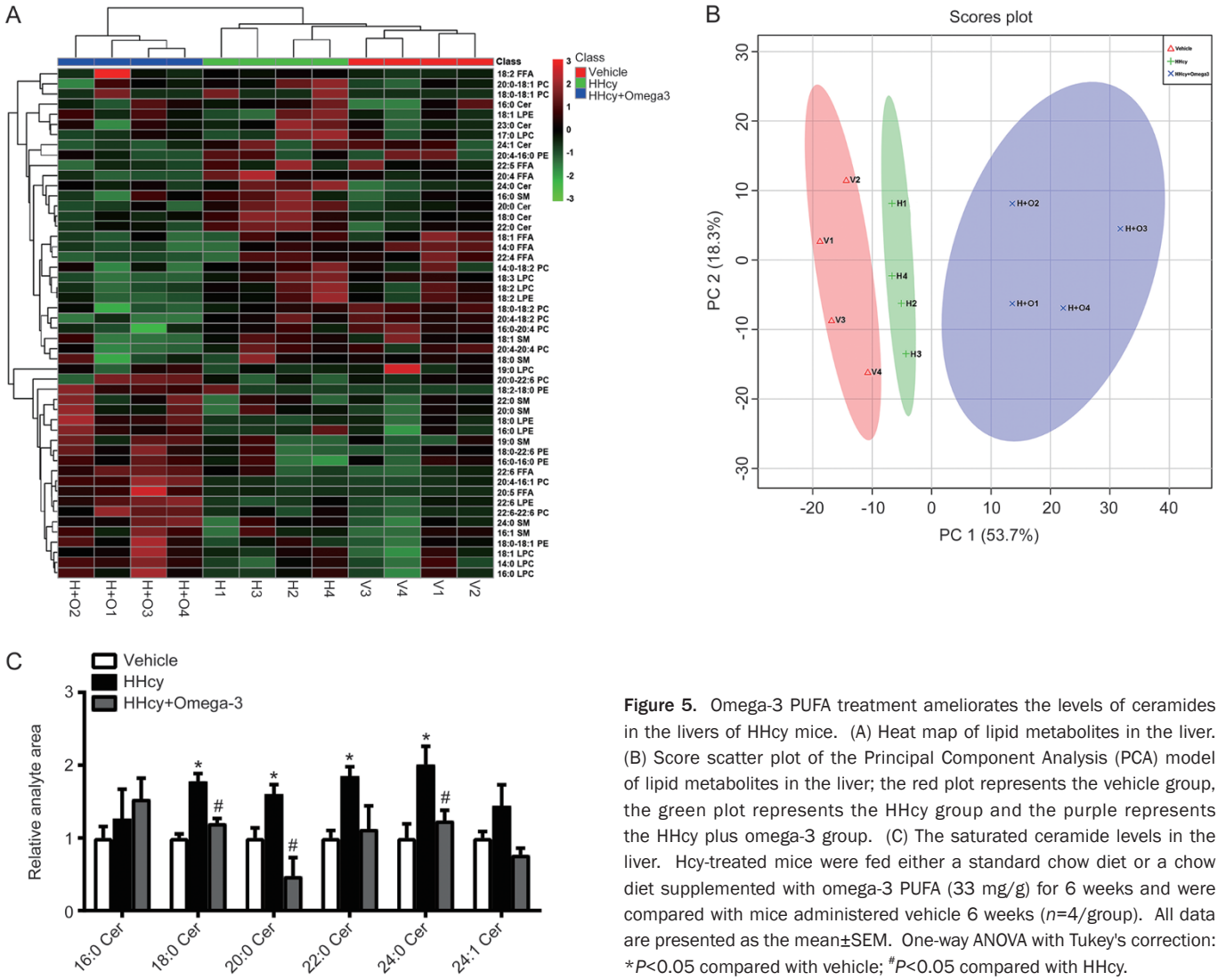

Figure 5. Omega-3 PUFA treatment ameliorates the levels of ceramides in the livers of HHcy mice. (A) Heat map of lipid metabolites in the liver. (B) Score scatter plot of the Principal Component Analysis (PCA) model of lipid metabolites in the liver; the red plot represents the vehicle group, the green plot represents the HHcy group and the purple represents the HHcy plus omega-3 group. (C) The saturated ceramide levels in the liver. Hcy-treated mice were fed either a standard chow diet or a chow diet supplemented with omega-3 PUFA (33 mg/g) for 6 weeks and were compared with mice administered vehicle 6 weeks (n=4/group). All data are presented as the mean±SEM. One-way ANOVA with Tukey's correction: $^{*}P<0.05$ compared with vehicle; $^{\#}P<0.05$ compared with HHcy.

are responsible for the increase in ceramides[36]. It has been reported that the genes involved in ceramide synthesis are stimulated by various endogenous factors such as thyroid hormones and angiotensin II[37, 38]. Therefore, ceramide synthase may mediate the actions of these agents or factors as an important second messenger. In the present study, we found that genes involved in ceramide synthesis, such as *Sptlc3* and *Degs2*, were dramatically increased after treatment with Hcy both *in vivo* and *in vitro*; additionally, these increases were accompanied by elevated levels of ceramides in HHcy mice. However, abolishing the expression of *Sptlc3* and *Degs2* by omega-3 significantly ameliorated HHcy-mediated increases of hepatic ceramide, thus suggesting that ceramide synthases were involved in Hcy-induced ceramide production.

Hcy treatment has been reported to significantly promote *de novo* ceramide synthases in the kidney, thereby potentially contributing to glomerulosclerosis[39, 40]. Further disruption of acid sphingomyelinase, a ceramide-producing enzyme, dramatically attenuates the production of ceramides and improves glomerular oxidative stress induced by HHcy[41]. In addition, ceramide hydrolytic enzymes (*Acer, Sphk, Sgms, Cerk*) in the kidney were unchanged after treatment with Hcy, thus further supporting our findings that increased ceramide levels in the livers of HHcy mice were mediated primarily through the ceramide synthases without affecting ceramide hydrolases.

However, it should be noted that this study determined only the levels of ceramide synthase (*Sptlc3* and *Degs2*) genes, and the roles of *Sptlc3* and *Degs2* in HHcy-induced hepatic steatosis require further elucidation in genetic model animals.

The mechanism of omega-3 PUFA has been demonstrated in different physiological systems, including the regulation of hepatic lipid metabolism[42]. Omega-3 PUFA supplementation has been reported to ameliorate HFD-induced hepatic steatosis by inhibiting genes involved in lipogenesis, including sterol regulatory element-binding protein 1c (*SREBP-1c*)[14]. In addition, dietary omega-3 fatty acids can rescue the fructose-provoked ER stress response, thereby decreasing FFA oxidation and the deposition of hepatic lipids[43]. In the present study, we showed that omega-3 PUFA ameliorates hepatic steatosis induced by HHcy, possibly as a result of decreases in

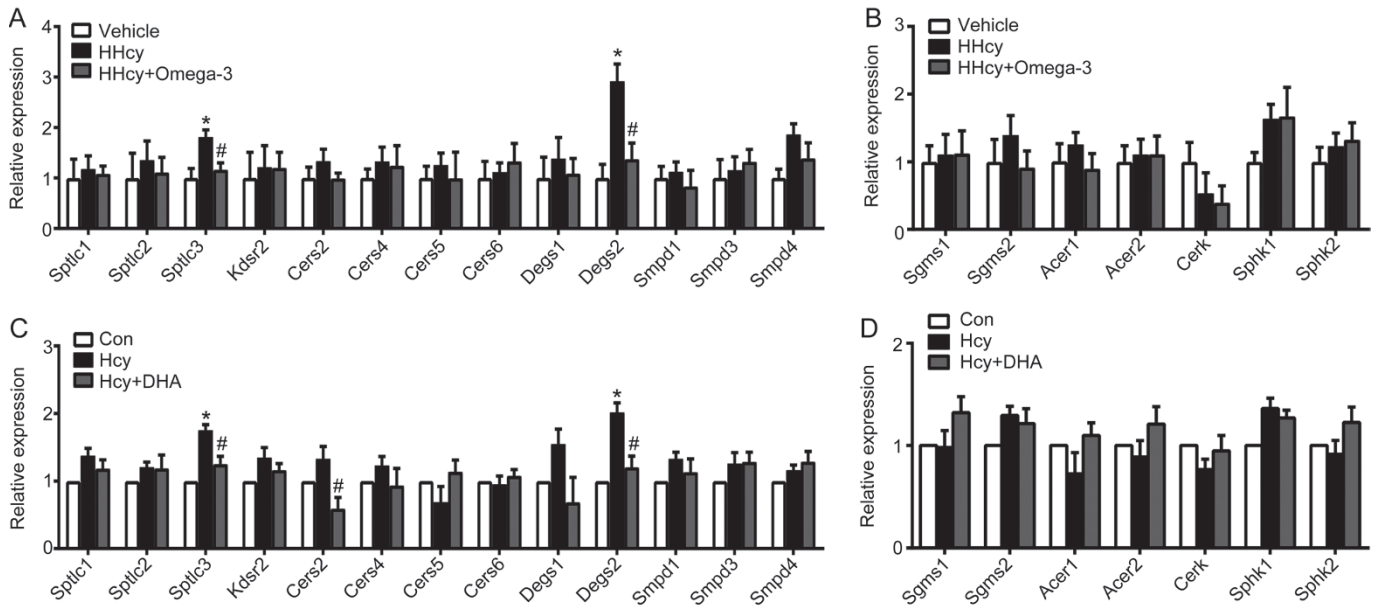

Figure 6. Omega-3 PUFA decreases the expression of ceramide synthases induced by Hcy in hepatocytes. (A) The mRNA levels of ceramide synthases in the liver. (B) The mRNA levels of ceramide hydrolases in the liver. Hcy-treated mice were fed either a standard chow diet or a chow diet supplemented with omega-3 PUFA (33 mg/g) for 6 weeks and were compared with mice administered vehicle for 6 weeks (n=6/group). (C) The mRNA levels of ceramide synthases in primary hepatocytes. (D) The mRNA levels of ceramide hydrolases in primary hepatocytes. Primary hepatocytes were pretreated with DHA (20 μmol/L) for 1 h before Hcy administration (100 μmol/L) for 24 h (n=5). (A-D) All data are presented as the mean±SEM. (A-D) One-way ANOVA with Tukey's correction: $^{*}P<0.05$ compared with vehicle or control as appropriate; $^{\#}P<0.05$ compared to HHcy or Hcy as appropriate.

hepatic ceramide levels and ceramide synthases production. Furthermore, the assembly of DHA into phospholipids in caveolae has been found to lead to decreased ceramide generation and to consequently inhibit cytokine signaling in human retinal endothelial cells[44].

In summary, our current findings demonstrate that HHcy-induced ceramide production is involved in the development of hepatic steatosis and that this activity is primarily due to the upregulation of ceramide synthases in hepatocytes. Dietary omega-3 PUFA might abolish HHcy-induced lipid accumulation by decreasing the generation of hepatic ceramides and may serve as a potential therapy for treating patients with hepatic steatosis.

Acknowledgements

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Author contribution

Yong-qiang DONG, Xing-zhong ZHANG, Song-yang ZHANG, Lu-lu SUN, and Hui-ying LIU designed and performed the experiments and analyzed the data; Bo LIU contributed to the histology experiments; Xian WANG and Chang-tao JIANG designed and supervised the research; Yong-qiang DONG, Song-yang ZHANG, Xian WANG and Chang-tao JIANG wrote and edited the manuscript. All the authors approved the final manuscript.

Supplementary information

Supplementary information is available on the website of Acta Pharmacologica Sinica.

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