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RESEARCH PAPER

Identification of matrine as a promising novel drug for hepatic steatosis and glucose intolerance with HSP72 as an upstream target

Xiao-Yi Zeng[1], Hao Wang[1], Fang Bai[2], Xiu Zhou[1], Song-Pei Li[1], Lu-Ping Ren[1], Ruo-Qiong Sun[1], Charlie C L Xue[1], Hua-Liang Jiang[2], Li-Hong Hu[2] and Ji-Ming Ye[1]

[1]*Lipid Biology and Metabolic Disease, Health Innovations Research Institute and School of Health Sciences, RMIT University, Melbourne, Vic, Australia, and* [2]*Shanghai Institute of Materia Medica, Chinese Academy of Sciences, Shanghai, China.*

Correspondence
Ji-Ming Ye, Health Innovations Research Institute and School of Health Sciences, RMIT University, PO Box 71, Bundoora, Melbourne, Vic. 61-3-99257745, Australia. E-mail: jiming.ye@rmit.edu.au

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BACKGROUND AND PURPOSE

Matrine is a small molecule drug used in humans for the treatment of chronic viral infections and tumours in the liver with little adverse effects. The present study investigated its therapeutic efficacy for insulin resistance and hepatic steatosis in high-fat-fed mice.

EXPERIMENTAL APPROACH

C57BL/J6 mice were fed a chow or high-fat diet for 10 weeks and then treated with matrine or metformin for 4 weeks. The effects on lipid metabolism and glucose tolerance were evaluated.

KEY RESULTS

Our results first showed that matrine reduced glucose intolerance and plasma insulin level, hepatic triglyceride content and adiposity in high-fat-fed mice without affecting caloric intake. This reduction in hepatosteatosis was attributed to suppressed lipid synthesis and increased fatty acid oxidation. In contrast to metformin, matrine neither suppressed mitochondrial respiration nor activated AMPK in the liver. A computational docking simulation revealed HSP90, a negative regulator of HSP72, as a potential binding target of matrine. Consistent with the simulation results, matrine, but not metformin, increased the hepatic protein level of HSP72 and this effect was inversely correlated with both liver triglyceride level and glucose intolerance.

CONCLUSIONS AND IMPLICATIONS

Taken together, these results indicate that matrine may be used for the treatment of type 2 diabetes and hepatic steatosis, and the molecular action of this hepatoprotective drug involves the activation of HSP72 in the liver.

Abbreviations

ACC, acetyl-CoA carboxylase; ACOX1, peroxisomal acyl-coenzyme A oxidase 1; AMPK, AMP-activated protein kinase; CH, chow diet; CS, citrate synthase; FAS, fatty acid synthase; GTT, glucose tolerance test; H&E, haematoxylin-eosin; HF, high-fat diet; HSF1, heat shock factor 1; HSP72, heat shock protein 72; HSP90, heat shock protein 90; iAUC, incremental AUC; IKK, IκB kinase; NAFLD, non-alcoholic fatty liver disease; RER, respiratory exchange ratio; SCD-1, stearoyl-CoA desaturase 1; SREBP-1, sterol regulatory element-binding proteins 1; T2D, type 2 diabetes; UCP2, uncoupling protein 2

Tables of Links

TARGETS	
Nuclear hormone receptors[a]	**Transporters**[b]
PPARα	UCP2
Enzymes[c]	
ACC	GSK3β
AMPK	IKKα
AST	IKKβ
ERK1	JNK
ERK2	PKCε
FAS	
Other protein targets	
α-tubulin	HSP72

LIGANDS	
Adiponectin	Leptin
IL-1β	Metformin
IL-6 (HSF1)	Palmitate
Insulin	TNFα

These Tables list key protein targets and ligands in this article which are hyperlinked to corresponding entries in http://www.guidetopharmacology.org, the common portal for data from the IUPHAR/BPS Guide to PHARMACOLOGY (Pawson *et al.*, 2014) and are permanently archived in the Concise Guide to PHARMACOLOGY 2013/14 ([a,b,c]Alexander *et al.*, 2013a,b,c).

Introduction

Type 2 diabetes (T2D) accounts for ~90% of all cases of diabetes, which was estimated to affect 382 million people worldwide in 2013 and has become a major threat to global development (International Diabetes Federation, 2013). Pharmacotherapy is an essential part in the treatment of T2D and management of associated complications. Among different anti-diabetic therapeutics, metformin is one of the most widely used drugs and is recommended as the first-line treatment for T2D (Inzucchi *et al.*, 2012). As T2D progresses, a second pharmaceutical agent with a different molecular mode of action from metformin is often required to achieve the desired therapeutic effect (Inzucchi *et al.*, 2012). However, the current therapeutic options for T2D are still limited due to the lack of chronic efficacy or undesirable side effects (Inzucchi *et al.*, 2012). Consequently, identification of new therapeutics with novel mechanisms and tolerable safety profiles is critical for the treatment of T2D.

Currently, the vast majority of lead compounds, identified by high-throughput screening, failed to translate into novel anti-diabetic drugs largely due to safety concerns in humans. We therefore used an alternative approach (drug repurposing) to identify new anti-diabetic compounds by searching existing therapeutics, which are used in humans chronically for diseases with reported features potentially linked to the pathogenesis of T2D. Drug repurposing is gaining increasing interest in recent years, as it can potentially avoid many common issues (e.g. poor bioavailability, side effects) encountered by new identified leads in conventional drug discovery and development (Strittmatter, 2014). In this study, we chose to target the liver because it is one of the major tissues involved in blood glucose regulation and can develop insulin resistance, which is a fundamental abnormality of T2D and closely linked with hepatosteatosis (Samuel *et al.*, 2010). Based on these criteria and our screening, we selected matrine to investigate its potential for the treatment of T2D and hepatic steatosis. Matrine is a small molecule (MW 238) (Figure 1A) used clinically for the treatment of viral hepatitis and hepatic tumours in the form of capsule or i.v. injection solution (e.g. China Food and Drug Administration approvals H20010242 or H20044669) (China Food and Drug Administration, 2014) with little adverse effects (Liu *et al.*, 2003). Interestingly, recent studies showed that both viral hepatitis and hepatocellular carcinoma are associated with increased lipogenesis and suppressed fatty acid oxidation, which together contribute to the development of hepatic steatosis (Syed *et al.*, 2010; Calvisi *et al.*, 2011). Furthermore, patients with hepatitis C also develop insulin resistance and associated metabolic syndrome (Bugianesi *et al.*, 2012), and treatments targeting disturbed lipid metabolism have been shown to inhibit the progression of hepatic viral infection (Sagan *et al.*, 2006). Recent work in our laboratory showed that oxymatrine, which is converted to matrine *in vivo* (Wang *et al.*, 2005), can reduce lipid content *in vitro* (Zeng *et al.*, 2012a).

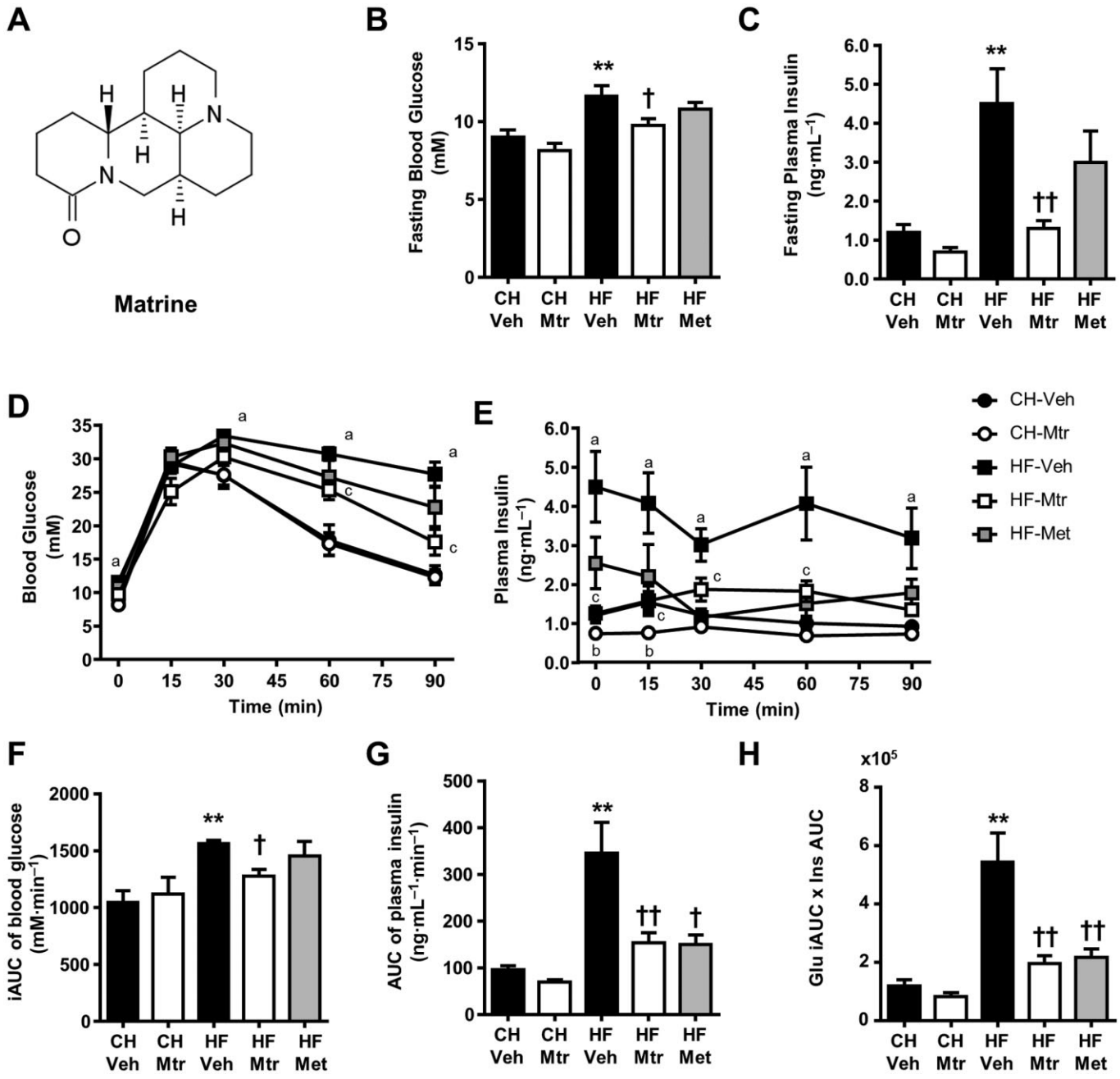

Figure 1

Matrine improved fasting blood glucose, plasma insulin and glucose tolerance in HF-fed mice. Molecular structure of matrine (A). Ten-week-old C57BL/6J mice were fed with a CH or a HF diet for 14 weeks. Matrine or metformin was administered for the last 4 weeks. After drug treatment, blood glucose (B) and plasma insulin (C) were measured following a 5–7 h fast. Blood glucose (D) and plasma insulin (E) were monitored in an i.p. GTT (glucose dose, 1 g·kg^{-1}) after the 2 week drug treatment. iAUC of blood glucose (F). AUC of plasma insulin (G). Whole-body insulin index (H) was expressed as blood glucose AUC × plasma insulin AUC. Data are mean ± SEM. *P < 0.05, **P < 0.01 versus CH-Veh; †P < 0.05, ††P < 0.01 versus HF-Veh. ^{a}P < 0.05, CH-Veh versus HF-Veh; ^{b}P < 0.05, CH-Veh versus CH-Mtr; ^{c}P < 0.05, HF-Veh versus HF-Mtr. n = 8 per group.

In this study, we investigated the therapeutic efficacy of this hepatoprotective drug for insulin resistance and hepatic steatosis in mice induced by a high-fat diet (HF) and the underlying mechanism. We also examined whether matrine may have a distinct therapeutic profile in the liver compared with metformin. Here we report that matrine significantly improved glucose tolerance and reduced hepatosteatosis in HF-fed mice. The lipid-reducing effect of matrine was attributed to a suppression of the lipid synthesis pathway and increased fatty acid oxidation. Unlike metformin, the

molecular action of matrine involved an up-regulation of heat shock protein 72 (HSP72) and an inhibition of the elevated expression of TNFα in the liver. Collectively, our results suggest that matrine may be a promising novel therapeutic for T2D with favourable effects on the associated fatty liver disease.

Methods

Animal model

Ten-week-old male C57BL/6J mice (24.5 ± 0.3 g), purchased from the Animal Resources Centre (Perth, Australia), were kept in a temperature-controlled room (22 ± 1°C) on a 12 h light/dark cycle with free access to food and water. After 1 week of acclimatization, mice were randomly assigned to receive a standard lab chow diet (CH; Specialty Feeds, Australia, 3.4 kcal·g^{-1}) or a HF diet (45% calories from fat, 0.2% w w^{-1} cholesterol, 4.9 kcal·g^{-1}) for 10 weeks. One group of CH or HF mice was then randomly selected to receive matrine (100 mg·kg^{-1}·day^{-1}; purchased by Professor Li-Hong Hu from Sigma-Aldrich, Shanghai, China) or metformin (250 mg·kg^{-1}·day^{-1}; Sigma-Aldrich, St. Louis, MO, USA) as a food additive for 4 weeks while the rest of the mice remained on the CH or HF diet. Following 5–7 h of fasting at the end of the study, blood samples were collected from the tail tip. Tissue samples were immediately freeze-clamped after mice were killed by cervical dislocation. All experiments were approved by the Animal Ethics Committee of RMIT University (#1012; #1208), following the guidelines issued by the National Health and Medical Research Council of Australia. All studies involving animals are reported in accordance with the ARRIVE guidelines (Kilkenny *et al.*, 2010; McGrath *et al.*, 2010).

Blood glucose and insulin, and glucose tolerance

Blood glucose levels were measured with a glucometer (Accu-Check II; Roche, Castle Hill, Australia). Glucose tolerance tests (GTTs; glucose load 1 g·kg^{-1} body weight, i.p.) were performed after 5–7 h of fasting at week 8 or after 2 weeks of drug treatment. Blood insulin levels were determined by radioimmunoassay (Linco/Millipore, Billerica, MA, USA).

Metabolic measurements

The acute effect of matrine on whole-body metabolic rates was assessed using an indirect calorimeter (Oxymax; Columbus Instruments, Columbus, OH, USA) (Tan *et al.*, 2008). Briefly, 12-week-old CH-fed mice were acclimatized in the Oxymax system for 2 h. O_2 consumption and CO_2 production were continuously monitored for 7 h after mice received matrine (100 mg·kg^{-1}, dissolved in 0.5% methylcellulose) or metformin (250 mg·kg^{-1}, dissolved in 0.5% methylcellulose) by oral gavage.

Immunoblotting

Liver lysates were resolved by SDS-PAGE and immunoblotted with specific antibodies (Chan *et al.*, 2013). Antibodies for HSP72 (Catalogue No. C92F3A-5, 1:1000 dilution) and HSP90 (ADI-SPA-840HRP, 1:1000) were purchased from Enzo Life Sciences, Farmingdale, NY, USA; ACOX1 (sc-98499, 1:100), IKKα/β (sc-23470-R, 1:100), p-IKKα/β (Ser$^{180/181}$) (sc-7607, 1:100), SREBP-1 (sc-367, 1:100) and UCP2 (sc-6525, 1:100) were from Santa Cruz, Dallas, TX, USA; ACC (3662, 1:1000), p-ACC (Ser^{79}) (3661, 1:1000), α-tubulin (3873, 1:1000), AMPK (2532, 1:1000), p-AMPK (Thr^{172}) (2535, 1:1000), ERK1/2 (4695, 1:1000), p-ERK1/2 (Thr^{202}/Tyr^{204}) (4370, 1:1000), FAS (3180, 1:1000), GSK-3β (27C10) (9315, 1:1000), p-GSK-3β (Ser^{9}) (9323, 1:1000), HSF1 (4356, 1:1000), JNK (9252, 1:1000), p-JNK (Thr^{183}/Tyr^{185}) (9251, 1:1000), PKCϵ (2683, 1:1000), SCD-1 (2794, 1:1000) and SP1 (5931, 1:1000) were from Cell Signaling, Danvers, MA, USA. p-PKCε (Ser^{729}) (06–821, 1:1000) was from Merck Millipore, Billerica, MA, USA. Densitometry analysis was performed using Image Lab (version 4.1; Bio-Rad Laboratories, Hercules, CA, USA).

Real-time PCR

Specific genes in the liver were amplified by real-time PCR (Zeng *et al.*, 2012b). The primer sequence (5′ to 3′) of 18S is: CGCCGCTAGAGGTGAAATTCT (sense) and CGAACCTCCG ACTTTCGTTCT (antisense); TNFα: CACAAGATGCTGGGA-CAGTGA (sense) and TCCTTGATGGTGGTGCATGA (antisense); IL-1β: CAACCAACAAGTGATATTCTCCATG (sense) and GATCCACACTCTCCAGCTGCA (antisense); IL-6: CTG-GTGACAACCACGGCCTTCCCTA (sense) and ATGCTTAGG-CATAACGCACTAGGTT (antisense).

Biochemical metabolic parameters

Liver or muscle triglycerides were extracted by the method of Folch and determined using a colorimetric assay kit (Triglyceride GPO-PAP; Roche Diagnostics, Indianapolis, IN, USA) (Zeng *et al.*, 2012b). Plasma alanine transaminase and aspartate transaminase were measured according to the manufacturer's instruction (Stanbio Laboratory, Boerne, TX, USA). Plasma adiponectin was measured using a commercial ELISA kit (Adipogen International, San Diego, CA, USA). Plasma leptin was determined by radioimmunoassay (Merck Millipore, USA).

Enzyme activity measurements

Liver samples were homogenized in 175 mM KCl and 1.98 mM EDTA-containing buffer (pH 7.4) with a glass homogenizer and subjected to three freeze-thaw cycles. Citrate synthase (CS) and β-hydroxyacyl-CoA dehydrogenase activities were determined with a Flexstation 3 plate reader (Molecular Devices, Sunnyvale, CA, USA) (Molero *et al.*, 2006).

Histological analysis

Liver tissues fixed in 10% neutral-buffered formalin were embedded in paraffin, cut into 5 µm sections and stained with haematoxylin-eosin (H&E) for microscopic examination. Five non-overlapping fields (magnification ×400) were randomly selected per liver sample for the quantification of ballooning injury (score 0–2) (Kleiner *et al.*, 2005).

Respiration measurements

Respiration of mitochondria isolated from the quadriceps muscle of 10-week-old C57BL/6J mice was measured at 37°C with a Clark-type oxygen electrode (Strathkelvin Instruments, North Lanarkshire, Scotland) (Turner *et al.*, 2008).

Fatty acid oxidation assay

Palmitate oxidation was measured by incubating liver homogenates with a respiration medium containing [1-^{14}C]-palmitate (Chan *et al.*, 2013). Palmitate oxidation rates were determined by counting the ^{14}C radioactivity of captured CO_2 and acid-soluble metabolites.

Molecular docking simulation

Matrine or metformin was docked to a series of selected targets, including HSP90 (PDB Code: 3T0Z), HSF1 (IL-6; 2LDU), HSP72 (2LDU) and SREBP-1c (1AM9). Molecular docking simulation was performed using the default setting unless stated otherwise. Briefly, the X-ray crystal structures were firstly prepared with the 'Protein Preparation Wizard' in the Schrödinger suite. The unwanted water molecules as well as cofactors were removed from the target protein, and hydrogen atoms were added and optimized in order to obtain a better hydrogen bond assignment. The grid box centre of each protein (size 20 × 20 × 20 Å^3) was set according to its co-crystal ligand. The coordinates and ionization states of matrine and metformin were built by Epik (version 2.2; Schrödinger LLC, New York, NY, USA) (Shelley *et al.*, 2007) with pH 7.0 and OPLS_2005 force field. The molecular docking simulations were performed using Glide (5.5; Schrödinger LLC) (Friesner *et al.*, 2004). To each selected target, top 10 scored binding poses of matrine and metformin were conserved respectively, and followed by visual inspection to select the most reliable binding pose according to the pharmacophore matching, formation of favourable molecular interaction, such as hydrogen bonds and hydrophobic contacts.

Statistical analyses

Data are expressed as mean ± SEM. For calculation of statistical differences among CH or HF mice treated with or without matrine, a two-way ANOVA was performed followed by *post hoc* Fisher's protected least significant difference test to compare two appropriate individual groups. Student's two-tailed *t*-test was used to compare metformin-treated HF mice with corresponding vehicle- or matrine-treated HF mice. Pearson's two-sided correlation test was used for correlation analyses. All statistical analyses were performed using GraphPad Prism 6.0 (GraphPad Software Inc., La Jolla, CA, USA). Differences at $P < 0.05$ were considered to be statistically significant.

Results

Glucose tolerance in HF-fed mice

A mouse model of obesity and insulin resistance was first induced by feeding a HF diet. The HF diet induced a ~30% increase in body weight at week 10 compared with the CH diet. HF mice were glucose intolerant as indicated by a 45% increase of incremental AUC (iAUC) during a GTT (1473 ± 52 vs. 1017 ± 130 mM × min compared with CH mice, $P < 0.05$) at week 8. Both matrine and metformin significantly reduced body weight gain and adiposity without affecting caloric intake (Table 1). Matrine reduced both basal blood glucose and plasma insulin levels (Figure 1B,D). In contrast, metformin was less effective than matrine in reducing basal blood glucose and plasma insulin in HF mice (Figure 1B,C). Furthermore, matrine improved glucose tolerance (~15%

Table 1

Metabolic characteristics of HF-induced insulin resistance in mice

	CH	CH matrine	HF	HF matrine	HF metformin
BW before treatment	29.8 ± 0.9	29.3 ± 0.5	38.0 ± 1.8**	37.6 ± 1.1	37.3 ± 1.6
BW after treatment	30.2 ± 1.0	28.2 ± 0.5	39.8 ± 2.0**	32.5 ± 0.5††	35.4 ± 1.4
BW gain during treatment (g)	0.4 ± 0.2	–1.2 ± 0.3**	1.7 ± 0.4*	–5.1 ± 0.8††	–1.8 ± 0.5††
Caloric intake (kcal per mouse day^{-1})	12.1 ± 0.5	12.0 ± 0.6	15.0 ± 0.9	14.6 ± 0.2	14.5 ± 0.7
Liver/BW (%)	4.0 ± 0.2	3.8 ± 0.2	3.9 ± 0.2	3.8 ± 0.2	3.7 ± 0.2
EPI/BW (%)	1.9 ± 0.2	1.1 ± 0.1**	5.0 ± 0.5**	3.2 ± 0.5†	4.3 ± 0.3
Plasma trig (mM)	0.9 ± 0.0	0.8 ± 0.0	0.8 ± 0.1	0.9 ± 0.1	0.8 ± 0.1
Plasma adiponectin (μg·mL^{-1})	16.8 ± 0.9	17.9 ± 1.3	19.4 ± 1.9	27.7 ± 6.5	18.0 ± 1.0
Plasma leptin (ng·mL^{-1})	35.5 ± 0.5	34.7 ± 0.4	42.3 ± 2.9*	36.6 ± 0.7†	36.1 ± 0.8†
Plasma AST (U·L^{-1})	19.5 ± 2.4	22.1 ± 2.2	21.7 ± 1.9	17.6 ± 5.8	18.7 ± 0.8
Plasma ALT (U·L^{-1})	11.0 ± 2.3	12.2 ± 2.4	12.7 ± 2.0	7.5 ± 1.9	5.9 ± 0.7†
Hepatocyte ballooning	0.4 ± 0.2	0.0 ± 0.0*	1.6 ± 0.2**	0.7 ± 0.1††	1.4 ± 0.2**

C57BL/6J mice were fed either a CH or a HF diet for 14 weeks. Matrine (100 mg·kg^{-1}·day^{-1}) or metformin (250 mg·kg^{-1}·day^{-1}) was administered as a food additive for the last 4 weeks of the study. Body weight was measured before the administration of drugs (week 11) and at the end of the study (week 14). The caloric intake is calculated based on the energy density of the corresponding diet and the food intake, which was monitored twice a week. Plasma was collected before the tissue collection at week 14 for the subsequent analysis of triglyceride, adiponectin, leptin, AST and ALT. Liver and epididymal fat were collected and weighted during the tissue. Data are mean ± SEM.
*$P < 0.05$; **$P < 0.01$ versus CH. †$P < 0.05$; ††$P < 0.01$ versus HF.
#$P < 0.05$; ##$P < 0.01$ versus HF mice treated with matrine, $n = 8$ per group.

reduction in iAUC) (Figure 1D,F) and lowered plasma insulin levels during the GTT (Figure 1D,G), while metformin only mildly reduced plasma insulin levels in HF mice (Figure 1D–G). When expressed as blood glucose iAUC × plasma insulin AUC, both matrine and metformin significantly ameliorated whole-body insulin resistance (Figure 1H).

Liver triglyceride, morphology and inflammation

Ectopic lipid accumulation is closely linked to insulin resistance (Samuel and Shulman, 2012). HF feeding increased the level of triglyceride by twofold in both liver and muscle. Matrine, in contrast to metformin, resulted in an apparent reduction (~25%) of triglyceride level in the liver (but not muscle) of HF mice (Figure 2A). Plasma triglyceride level was not affected by HF feeding, matrine or metformin treatment (Table 1). It has been proposed that lipid intermediates, such as DAG, are directly responsible for insulin resistance by the inhibitory serine phosphorylation of IRS1 through the activation of PKC (Muoio, 2010; Samuel and Shulman, 2012). We measured the phosphorylation of PKCε in the liver, and observed a small trend of reduction (~20%) in HF mice treated with matrine compared with untreated HF mice (P = 0.08) (Figure 2A). However, further studies are warranted for the direct measurement of these lipid intermediates. H&E staining showed that HF feeding resulted in a dramatic increase of liver cells with swollen and rarefied cytoplasm (ballooning), which was significantly reduced after matrine (but not metformin) treatment (Figure 2B; Table 1). Hepatic gene expressions of inflammation markers, including *Tnfa* and *Il1b*, were increased onefold by HF feeding, while *Il6* remained unchanged. Matrine normalized the gene expression of *Tnfa*, but not *Il1b*, in HF-fed mice (Figure 2C–E). Phosphorylation of JNK, but not IKKα/β, was increased by 40% after HF feeding. However, neither matrine nor metformin significantly suppressed the phosphorylation of JNK (Figure 2F).

Lipid synthesis pathway in the liver

To determine whether the matrine-mediated reduction of hepatic lipid accumulation was a result of reduced lipid synthesis, we measured the expression level of related proteins in the liver. HF feeding induced mark increases of mature form of SREBP-1c (mSREBP-1c, 2.5-fold) and SCD-1 (threefold) compared with CH diet. Matrine reduced ACC, FAS and SCD-1 protein levels in CH-fed mice, and normalized the elevated level of SCD-1 in HF mice. Metformin had no effect on ACC or FAS in HF mice, but normalized SCD-1 to a level similar to matrine (Figure 2G,H). These data suggested the lipid-lowering effect of matrine might be a result of suppressed lipid synthesis pathway.

Energy expenditure and fatty acid oxidation

Increased energy expenditure and fatty acid oxidation can contribute to the reduction of hepatic lipid accumulation and adiposity. To test this possibility, we examined the protein expression or activity of protein markers related to energy metabolism. Matrine significantly increased the hepatic protein expression of UCP2 in both CH and HF mice (Figure 3A), and mildly (P = 0.09) increased the activity of CS, but not β–HAD, in HF mice (Figure 3B,C). Matrine also up-regulated UCP1 protein expression in the epididymal fat of HF mice (Supporting Information Fig. S2). In contrast to matrine, metformin had no effect on the protein expression or activities of these markers (Figure 3A–C). With the use of [1-^{14}C]-palmitate, matrine was shown to increase palmitate oxidation in a concentration-dependent manner (up to 15% at 100 μM) (Figure 3D). Furthermore, oral administration of matrine significantly increased whole-body energy expenditure (10% increase of VO_2) and fat oxidation (4% decrease of respiratory exchange ratio, RER) for at least 7 h in CH-fed mice (Figure 3E). In contrast to matrine, metformin increased VO_2 without altering RER compared with CH mice, indicating metformin increased energy expenditure without promoting fatty acid as the predominant fuel for energy (Figure 3F).

Effects on AMPK or PPARα

To investigate whether the anti-diabetic effects of matrine involved activation of AMPK through suppressing the mitochondrial respiratory chain, we measured mitochondria respiration with isolated mitochondria, as well as the phosphorylation of AMPK and ACC in livers of matrine-treated mice. In contrast to berberine, which inhibited the mitochondrial complex I in a concentration-dependent manner, matrine did not affect the oxygen consumption rate at a concentration of up to 30 μM (Figure 4A). Consistent with the unimpaired mitochondrial respiration, the phosphorylation status of AMPK and its substrate ACC was unaltered in the liver of matrine-treated mice (Figure 4B).

Previous studies including ours (Ye *et al.*, 2001; Chan *et al.*, 2013) demonstrated that activation of PPARα results in similar phenotypes in mice as observed in this study, including increased fatty acid oxidation and reduced liver triglyceride, and improves insulin resistance. Oxymatrine, an analogue of matrine, is suggested to improve hepatic steatosis via activation of PPARα (Shi *et al.*, 2013). However, the hepatic protein level of ACOX1, a downstream target of PPARα, remained unchanged (Figure 4C) and no hepatomegaly (Table 1) was observed in the matrine-treated mice, indicating the PPARα pathway was not activated.

Effects on HSP72

HSP72 has been suggested as a potential therapeutic target for T2D (Soti *et al.*, 2005). Interestingly, HSP90, a negative regulator of HSP72 (Anckar and Sistonen, 2011), is shown to be essential for the progression of viral hepatitis (Hu and Seeger, 1996; Okamoto *et al.*, 2006), which is treated by matrine clinically. To assess whether HSP72 or HSP90 might be involved in the therapeutic effects of matrine, we first used molecular docking simulation to estimate the binding strength between matrine and a number of protein targets. Docking simulation revealed that the predicted binding affinity of matrine to HSP90 is much stronger than HSF1, HSP72 or SREBP-1c (Table 2). Meanwhile, matrine was predicted to interact with HSP90 with a binding affinity similar to that of ganetespib (a reported HSP90 inhibitor; Neckers and Workman, 2012) (–6.7 vs. –7.0 kcal·mol^{-1}). From the predicted binding pose, we could find two hydrogen bonds between matrine and two residues of HSP90 (T184 and G108), and two water bridged hydrogen bonds between the

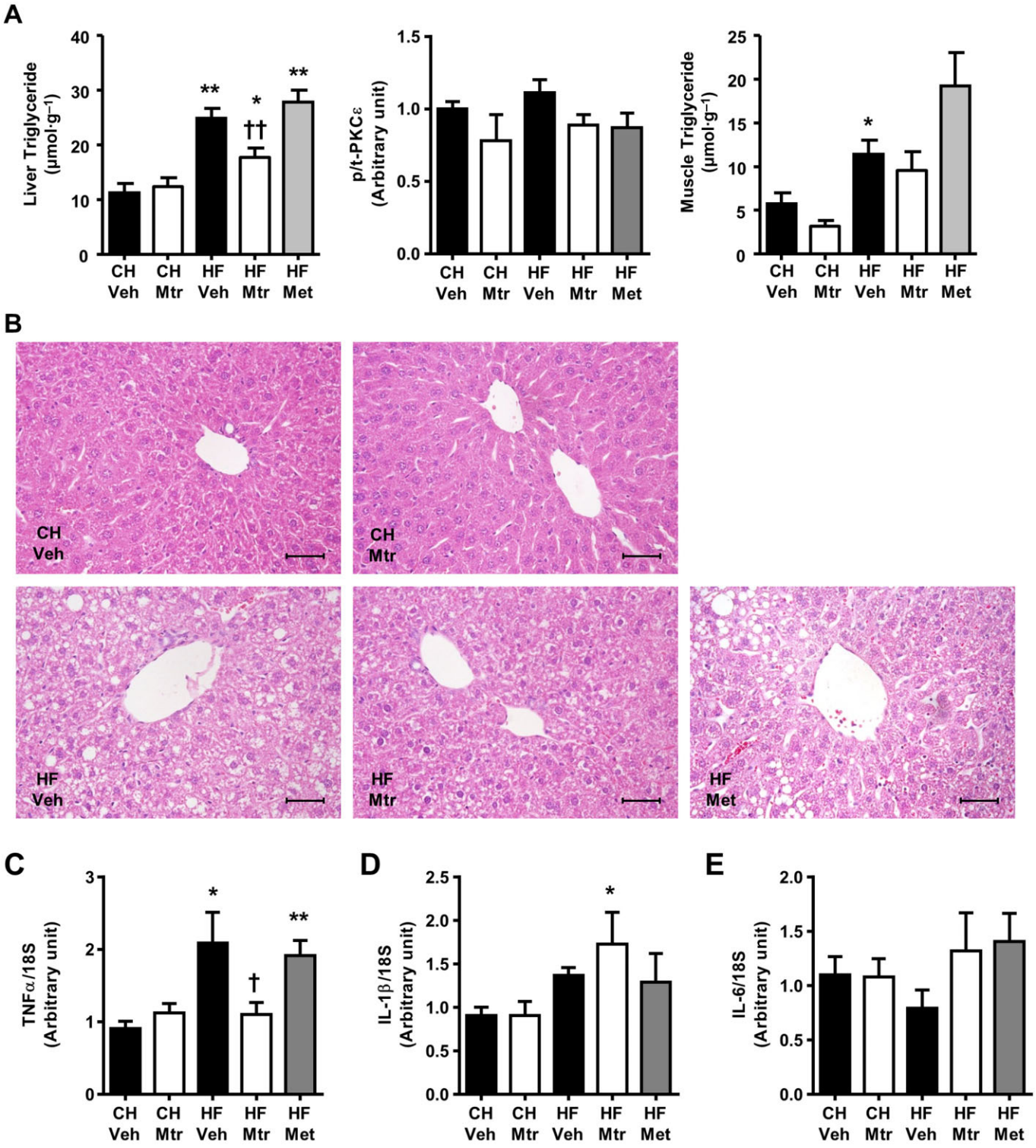

Figure 2

Matrine reduced lipid accumulation and inflammation in the liver with a suppression of the lipid synthesis pathway. Liver and muscle triglyceride levels and the phosphorylation status of PKCε in the liver were measured after drug treatment (A). Representative H&E staining (400×) images of liver sections. Scale bar, 150 μm (B). Gene expressions of TNFα (C), IL-1β (D) and IL-6 (E) in the liver. The phosphorylation status of JNK and IKKα/β (F). Protein expression of SREBP-1 (G) in the nuclear fraction of the liver. Protein levels of ACC, FAS and SCD-1 (H) in the liver. Data are mean ± SEM. *$P < 0.05$, **$P < 0.01$ versus CH-Veh; †$P < 0.05$, ††$P < 0.01$ versus HF-Veh, $n = 8$ per group.

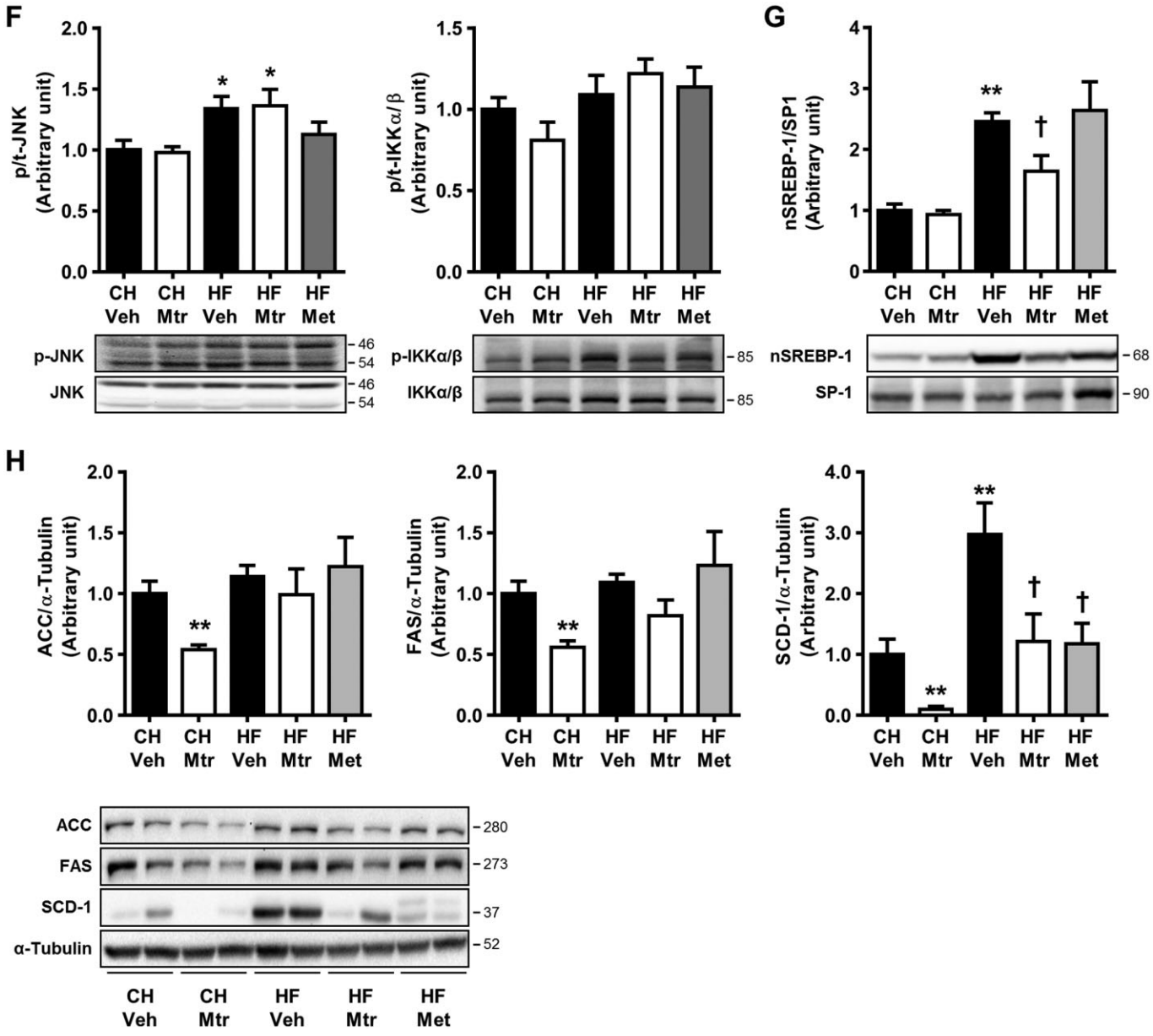

Figure 2
Continued

carbonyl group of matrine and residues D93 as well as G97 on HSP90. In addition, matrine also formed hydrophobic contacts with residues F138, M98 and L107 (Figure 5A). In contrast to the strong binding affinity between matrine and HSP90, the energy required for metformin to bind to selected protein targets was predicted to be much greater, rendering the direct interaction less likely to happen (Table 2).

To determine whether the possible interaction between matrine and HSP90 would have any effect on the expression of HSP72, we measured the hepatic protein expression of HSP72. Consistent with previous reports, HSP72 expression was blunted (40% reduction compared with CH, $P < 0.05$) by HF feeding (Chung *et al.*, 2008). Matrine elevated (~50% increase, $P < 0.05$) the protein expression of HSP72 in both CH and HF mice, but had no effect on the protein level of HSP90 and HSF1 (transcription regulators of HSP72), and the phosphorylation of two HSF1 negative regulators (ERK and GSK3β) (He *et al.*, 1998). In contrast to matrine, metformin failed to restore the reduced HSP72 level in HF mice (Figure 5B). Furthermore, the up-regulation of hepatic HSP72 expression inversely correlated with the liver triglyceride level ($r = –0.487$, $P = 0.05$) and glucose iAUC ($r = –0.427$, $P < 0.05$) (Figure 5C). As adiponectin has been suggested to contribute to the beneficial effect of HSP72 in HF-fed mice (Chung *et al.*, 2008), we next examined the plasma level of adiponectin and leptin. Matrine treatment, which up-regulated HSP72, had no effect on the plasma adiponectin level (Table 1). Such discrepancy in adiponectin level after

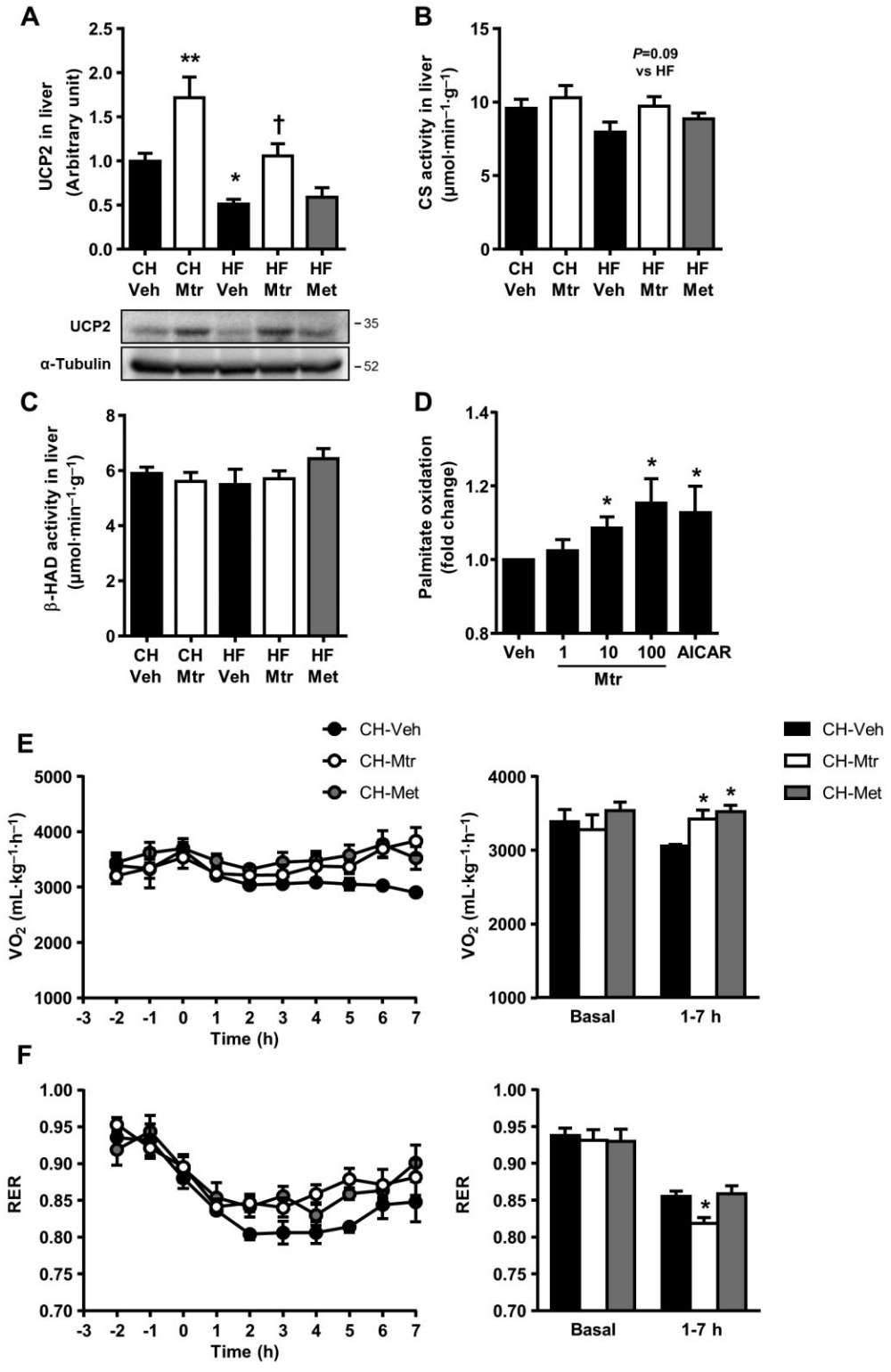

Figure 3

Matrine increased energy expenditure and promoted fatty acid utilization as the energy source. Protein expression of UCP2 in the liver of CH- or HF-fed mice treated with matrine or metformin (A). Activities of CS and β-hydroxyacyl-CoA dehydrogenase (β-HAD) isolated from the liver. Data are mean ± SEM. *$P < 0.05$ versus CH-Veh; †$P < 0.05$ versus HF-Veh, $n = 8$ per group (B,C). Palmitate oxidation in liver homogenate from CH-fed mice was determined with [1-^{14}C]-palmitate. *$P < 0.05$ versus vehicle, $n = 2$–4 per group from four independent experiments (D). Oxygen consumption (E) and RER (F) of CH-fed mice were monitored for 7 h after administration of vehicle (0.5% methylcellulose), matrine or metformin by oral gavage. *$P < 0.05$ versus CH-Veh, $n = 5$–9 per group.

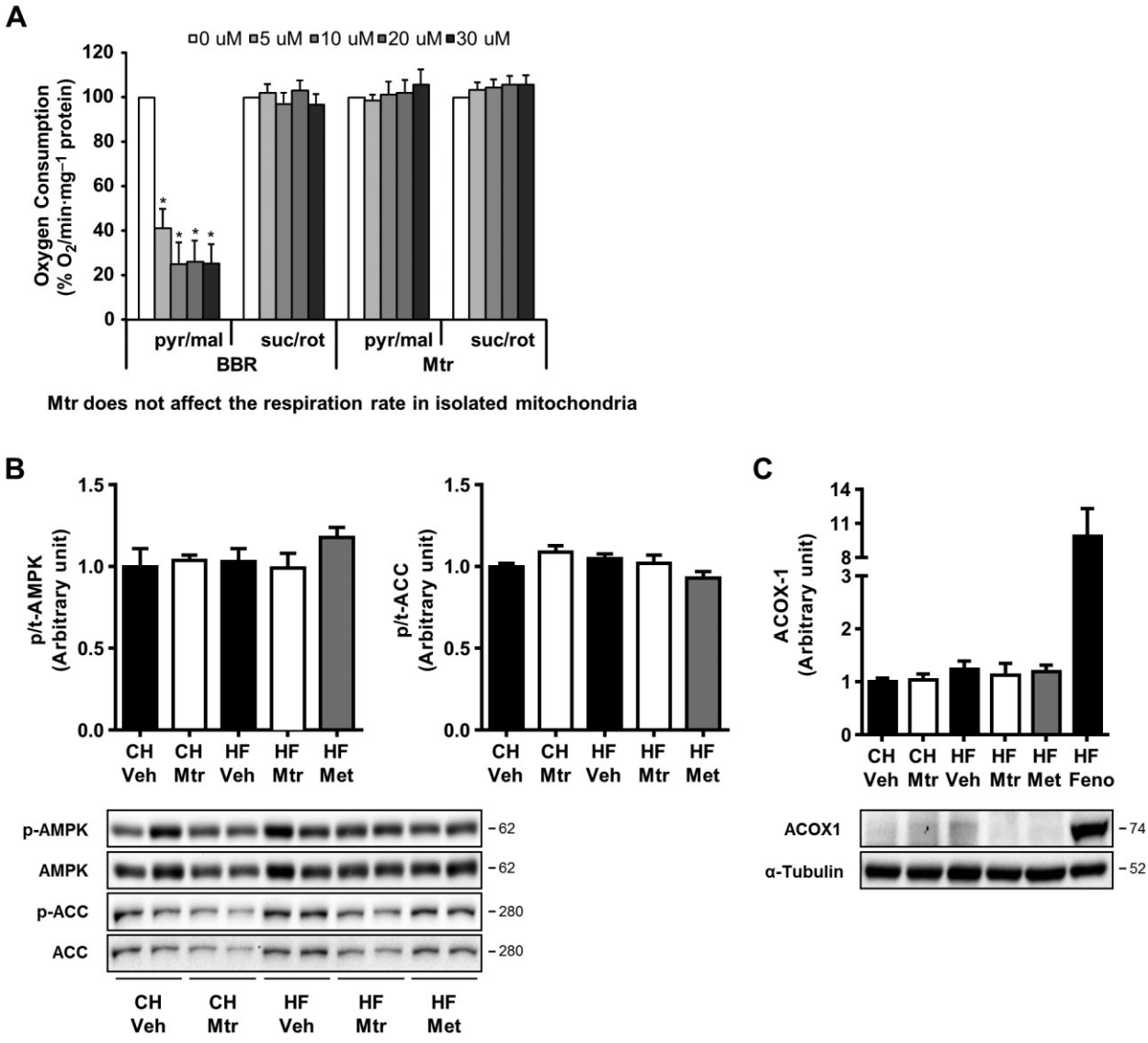

Figure 4

Effects of matrine did not involve altered mitochondria electron transport chain, or activation of AMPK or PPARα. Oxygen consumption rates were measured in isolated mitochondria from CH-fed mice at 37°C. $n = 3$ per group (A). The phosphorylation status of AMPK and ACC (B) and the protein expression of ACOX1 (C) in the liver after drug treatment. Data are mean ± SEM. $^*P < 0.05$ versus CH-Veh, $n = 8$ per group.

Table 2

Docking results of matrine and metformin to selected protein targets

Target	Target name	HSP90	HSF1	HSP72	SREBP-1c
	PDB code	3T0Z	2LDU	3ATU	1AM9
Ligand scoring (kcal·mol^{-1})	Matrine	–6.743	–2.419	–2.878	–1.902
	Metformin	–4.315	–2.480	–4.943	–1.853
	Ganetespib	–7.000	n.d.	n.d.	n.d.

The interactions between matrine or metformin and a series of selected protein targets were simulated by software Schrödinger suite (Schrödinger LLC). X-ray crystal structures of protein targets with relatively high resolution and complete structure were acquired from the Protein Data Bank (http://www.rcsb.org/pdb/). Ganetespib is a reported HSP90 inhibitor. n.d., not determined.

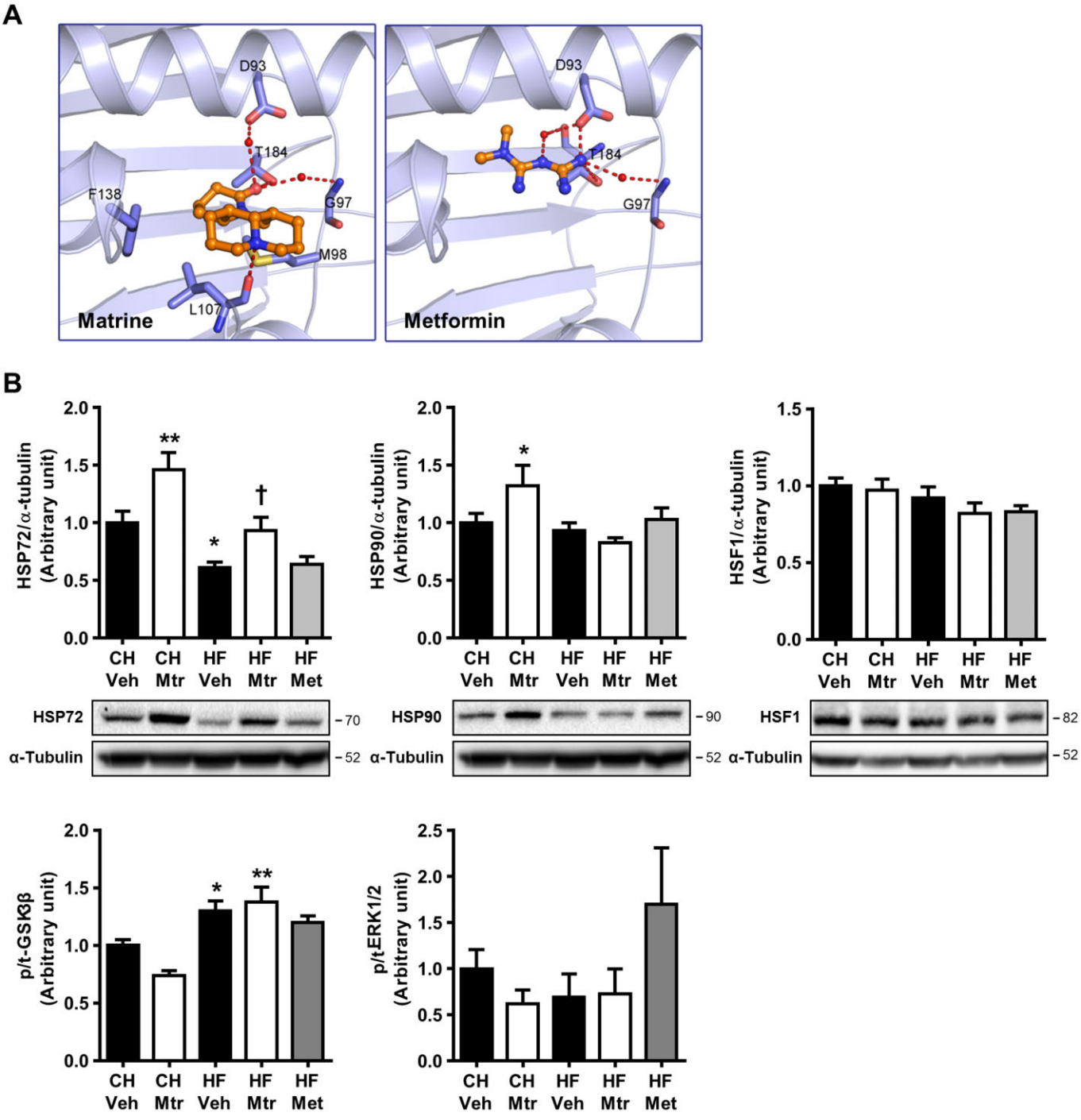

Figure 5

Matrine up-regulated hepatic HSP72 expression, which is associated with liver triglyceride and glucose tolerance. The binding pose of matrine or metformin to HSP90 was predicted by molecular docking simulation. The lilac cartoon represents HSP90, the violet stick models are important amino acid residues, red spheres are conserved water molecules, the dash lines represent hydrogen bonds and the orange stick-ball models are matrine or metformin (A). Protein levels of HSP72, HSP90, HSF1, GSK3β and ERK in the liver after drug treatment (B). Correlation of hepatic HSP72 expression with liver triglyceride or iAUC of blood glucose (C). Illustration of matrine-mediated reduction of lipid accumulation and improvement of glucose tolerance (D). Data are mean ± SEM. *$P < 0.05$ versus CH-Veh; †$P < 0.05$ versus HF-Veh, $n = 8$ per group.

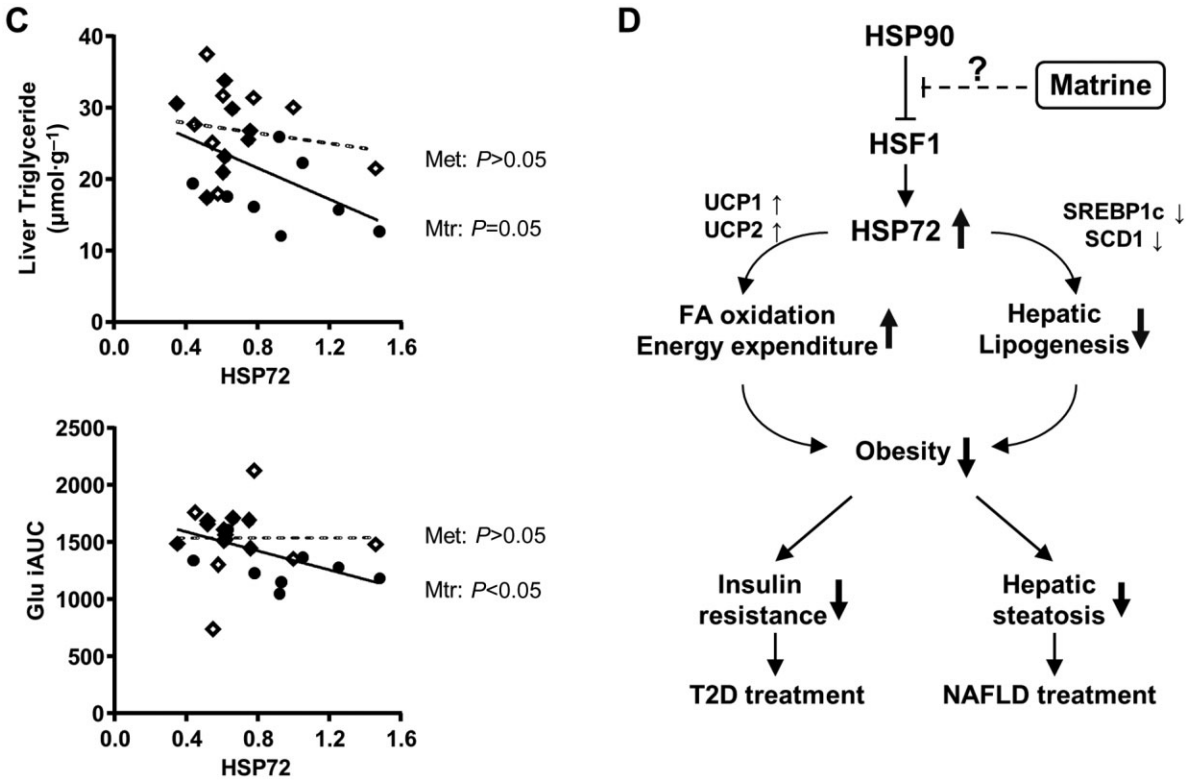

Figure 5
Continued

HSP72 up-regulation might be a result of the difference in the mean (transgenic vs. pharmacological), location (muscle vs. liver) and extent of HSP72 up-regulation in these two studies. Both matrine and metformin normalized the HF-induced increase of plasma leptin level (Table 1). These data suggest that neither adiponectin nor leptin is likely to be a mediator for the effect of matrine on HSP72 expression.

Discussion

The present study demonstrated that matrine, a drug used clinically for viral hepatitis and hepatic carcinoma, ameliorated visceral adiposity and glucose intolerance as effectively as metformin in HF-fed mice, with additional effects to reduce hepatic steatosis. The metabolic effect of matrine was associated with an up-regulation of HSP72, suggesting matrine might be a novel drug for hepatic steatosis and T2D by a mechanism different from the current anti-diabetic drugs.

The liver is a key tissue for the homeostasis of blood glucose and lipids in the whole body. Recent studies demonstrate that hepatic steatosis is strongly associated with insulin resistance in the liver (Petersen *et al.*, 2005; Fabbrini *et al.*, 2009), and patients with benign hepatosteatosis might progress to more severe steatohepatitis (Cohen *et al.*, 2011). Similar to metformin, of which the main site of action is in the liver (Rena *et al.*, 2013), matrine has a high tissue distribution in the liver compared with other organs (e.g. muscle and fat) after an oral administration (Gao and Law, 2009). Importantly, 4 week matrine treatment did not induce liver injuries as indicated by unchanged plasma AST and ALT levels (Table 1). In the current study, we demonstrated that matrine reduced lipid accumulation in the liver, but not muscle, indicating the liver might be the major target tissue of matrine. Our results further showed that the lipid-lowering effect of matrine was associated with an inhibition of the lipid synthesis pathway (as indicated by a reduction of mature SREBP-1 and SCD-1) as well as a stimulation of energy expenditure and fatty acid oxidation (as indicated by increased VO_2 and reduced RER, and increased *ex vivo* palmitate oxidation).

To determine the upstream target of matrine, we examined its effects on AMPK and PPARα, activation of which have been shown to eliminate insulin resistance and fatty liver in rodents via suppression of lipid synthesis and/or stimulation of fatty acid oxidation (Chan *et al.*, 2013; Hardie, 2013). Metformin is believed to exert its therapeutic efficacy partly via the activation of AMPK as a result of suppressed mitochondrial complex I (Rena *et al.*, 2013). While acute injection of metformin has been reported to increase AMPK phosphorylation in the liver (Fullerton *et al.*, 2013), we did not observe this increase in metformin-treated HF mice, probably due to different doses and administration routes used. In this study, matrine improved glucose tolerance and fasting glucose homeostasis as effectively as metformin in HF-fed mice.

Importantly, matrine exerts these metabolic effects through a distinct pathway from metformin. Matrine neither activated the AMPK pathway (Figure 4B and confirmed by data in Supporting Information Fig. S1) nor disturbed the mitochondria electron transport chain. Furthermore, the lack of effect of matrine on ACOX1 and liver size ruled out the possible involvement of PPARα stimulation.

The neutral effect of matrine on mitochondrial respiration is an attractive pharmaceutical property, as inhibition of mitochondrial respiration might lead to lactic acidosis, which limits the use of metformin in T2D patients with chronic renal disease (Inzucchi *et al.*, 2012; Rena *et al.*, 2013). Furthermore, metformin is unable to improve liver histology such as histological steatosis and fibrosis (Musso *et al.*, 2012), despite liver is its target organ for T2D. Therefore, metformin is not regarded as an effective treatment for non-alcoholic fatty liver disease (NAFLD) (Chalasani *et al.*, 2012). In comparison, matrine appeared to display a favourable therapeutic profile for key elements of NAFLD (reduction of hepatosteatosis and TNFα expression). In addition, matrine is reported to protect the liver from CCl_4-induced fibrosis in rats (Zhang *et al.*, 2001). These findings together raise the possibility that matrine might offer additional therapeutic effects to manifestations of metabolic syndrome in the liver.

Molecular docking simulation revealed that matrine might interact with a number of amino acid residues (Figure 5A) of HSP90 in an N-terminal nucleotide-binding pocket (residues 9–232), which is also the binding domain for HSP90 inhibitors (Stebbins *et al.*, 1997; Neckers and Workman, 2012) and substrates (Prodromou *et al.*, 1997). HSP90 is reported to implicate in the development of a number of diseases, including hepatic viral infection (Csermely *et al.*, 1998). HSP90 is essential for the formation of complexes that are critical for the replication of hepatitis B or C virus (Hu and Seeger, 1996; Okamoto *et al.*, 2006), and inhibitors of HSP90 (e.g. geldanamycin) have been shown to inhibit the progression of hepatitis C viral infection (Okamoto *et al.*, 2006). Furthermore, HSP90 has been reported to interact with HSF1 (a transcription factor for HSP72; Anckar and Sistonen, 2011) and inhibit its transcription activity (Winklhofer *et al.*, 2001). To test whether the expression of HSP72 is affected by matrine treatment, we next examined its expression level in different tissues.

HSP72 expression has been shown to be reduced in the skeletal muscle of patients with obesity/insulin resistance (Chung *et al.*, 2008) or T2D (Kurucz *et al.*, 2002), and heat treatment-induced HSP72 expression in both muscle and liver is blunted by HF feeding in mice (Chung *et al.*, 2008). Consistent with these reports, a similar reduction of HSP72 expression was observed in the liver of HF-fed mice. While HSP72 expression in the skeletal muscle correlates with insulin resistance (Kurucz *et al.*, 2002), a similar correlation between the hepatic HSP72 expression and hepatosteatosis/glucose tolerance was observed in this study, suggesting HSP72 might be a key mediator of the metabolic effect of matrine. In support of this notion, heat treatment was recently reported to improve glucose tolerance and prevent skeletal muscle insulin resistance in HF-fed rats with associated up-regulation of HSP72 (Gupte *et al.*, 2009). A number of potential anti-diabetic compounds, including resveratrol (Putics *et al.*, 2008) and BGP-15 (Chung *et al.*, 2008), were shown to induce HSP72 expression *in vitro* and *in vivo*. More importantly, transgenic overexpression of HSP72 prevents HF-induced body weight gain, and improves glucose tolerance and insulin sensitivity (Chung *et al.*, 2008). However, further investigations are warranted to investigate the exact mechanism by which matrine up-regulates HSP72.

Matrine is reported to possess several other pharmacological properties, protecting against viral infection (Liu *et al.*, 2003), toxin-induced liver damage (Zhang *et al.*, 2001), inflammation (Zhang *et al.*, 2011) and tumour development (Liang *et al.*, 2012). These properties were attributed to its effects to reduce the expression of inflammatory mediators (Zhang *et al.*, 2001; 2011) and induce apoptosis in carcinoma cells (Liang *et al.*, 2012; Zhou *et al.*, 2014). Intriguingly, modulations of the HSP pathway have been shown to suppress canonical TGF-β and NFκB signalling (Anderson *et al.*, 2014; Tomcik *et al.*, 2014) and induce apoptosis (Liossis *et al.*, 1997; Yang *et al.*, 2011). These observations raise an exciting possibility that a unifying mechanism underlying these seemingly disparate effects of matrine might exist and involve the modulation of the HSP pathway. However, this hypothesis and the exact role of HSP72 in the metabolic effect of matrine require further investigation.

Apart from the associated effects of matrine in promoting oxidative capacity of the liver (as shown in Figure 3), we further investigated whether adipose tissue may also be a possible site contributing to the decreased obesity after matrine treatment because epididymal fat mass was reduced by more than 40%. We found that UCP1 protein level was up-regulated in HF mice by approximately 50% in white adipose tissue as compared with untreated HF mice (Supporting Information Fig. S2). UCP1 is a hallmark of brown adipose tissue and an increase in its expression can promote energy expenditure as heat production. These results suggest a possibility of 'browning' of white adipose tissue in HF mice treated with matrine as another contributor to the observed reduction in obesity. The inhibition of HSP90 has been shown to destabilize PPARγ and attenuate adipogenesis (Nguyen *et al.*, 2013). However, further studies are required to determine whether the observed up-regulation of UCP1 results from matrine's direct effect at HSP90 of adipose tissue or a secondary effect of hepatokines such as FGF21 (Fisher *et al.*, 2012).

In summary, the present study has identified the hepatoprotective drug matrine as a promising novel anti-diabetic drug with liver as an important target organ. Our data demonstrated that matrine reduced obesity and glucose intolerance as effectively as metformin in insulin-resistant HF-fed mice. Importantly, matrine exerts distinct mechanisms with HSP72 as a possible upstream mediator (Figure 5D). Given that matrine is well tolerated in humans during chronic use, it has a good chance to be used clinically for the metabolic syndrome with additional favourable effects on the associated NAFLD. Our findings provide a strong rationale for future clinical trials for the new application of matrine for these metabolic diseases.

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Author contributions

J. M. Y., X. Y. Z. and L. H. H. conceived and designed the experiments. X. Y. Z., X. Z. H. W., L. P. R., S. P. L., R. Q. S., J. M. Y. and F. B. performed the experiments. J. M. Y., X. Y. Z., L. P. R. and H. L. J. analysed the data. J. M. Y., H. L. J., L. H. H. and C. L. X. contributed reagents/materials/analysis tools. X. Y. Z. and J. M. Y. wrote the manuscript. C. C. L. and L. H. H. revised the paper.

Conflict of interest

The authors declare that there is no duality of interest associated with this manuscript.

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Supporting information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

http://dx.doi.org/10.1111/bph.13209

Figure S1 Effects of matrine on the AMPK and ACC *in vitro*.
Figure S2 Effects of matrine on the UCP1 expression in white adipose tissue.