Berberine Attenuates Hepatic Steatosis and Enhances Energy Expenditure in Mice by Inducing Autophagy and Fibroblast Growth Factor 21

Yixuan Sun¹,², Mingfeng Xia¹,², Hongmei Yan¹,², Yamei Han³, Feifei Zhang³, Zhimin Hu³, Aoyuan Cui³, Fengguang Ma³, Zhengshuai Liu³, Qi Gong³, Xuqing Chen³, Jing Gao³, Hua Bian¹,², Yi Tan⁴,⁵, Yu Li*, Xin Gao¹,²*

¹Department of Endocrinology and Metabolism, Zhongshan Hospital, Fudan University, Shanghai, China;
²Fudan Institute for Metabolic Diseases, Shanghai, China;
³CAS Key Laboratory of Nutrition and Metabolism, Institute for Nutritional Sciences, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences, University of Chinese Academy of Sciences, Shanghai 200031, China;
⁴Chinese-American Research Institute for Diabetic Complications, School of Pharmaceutical Science, Wenzhou Medical University, Wenzhou, Zhejiang 325035, China;
⁵Pediatric Research Institute at the Department of Pediatrics, Wendy L. Novak Diabetes Care Center, University of Louisville, Louisville, KY 40202, USA;

Running Title: Berberine regulates hepatic steatosis and energy metabolism

*Correspondence to:
Yu Li, PhD
Professor
Institute for Nutritional Sciences
Shanghai Institutes for Biological Sciences
Chinese Academy of Sciences
320 Yue Yang Road
Life Science Research Building A1816
Shanghai 200031, China
Phone: +86 (21) 5492 0753
Email: liyu@sibs.ac.cn

Xin Gao, MD
Professor
Department of Endocrinology and Metabolism
Zhongshan Hospital, Fudan University
180 Fenglin Road
Shanghai 200032, China
Phone: +86 (21) 6443 9025
Email: happy20061208@126.com and gao.xin@zs-hospital.sh.cn

Funding: This work was supported by grants from the National Key Basic Research Program of China (2012CB524906) and the National Natural Science Foundation of China (81270933, 81570718) to X.G. This work was also supported by grants from the National Key R&D Program of China (2017YFC0909601), the National Natural Science Foundation of China (31471129, 31671224) and the Chinese Academy of Sciences (ZDBS-SSW-DQC-02, 2013OHTP04) to Y.L.
Author Contribution:
Y.S., X.G. and Y.L. contributed to experiment design; Y.S., M.X., H.Y., Y.H., F.Z., Z.H., A.C., F.M., Z.L., X.C., Q.G., J.G., and H.B. contributed to the acquisition and analysis of data; Y.T. reviewed the manuscript; X.G. and Y.L. obtained the funding; Y.S., X.G. and Y.L. wrote the manuscript.

### Tables of Links

<table>
<thead>
<tr>
<th>TARGETS</th>
<th>Target ID</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Enzymes</strong>&lt;sup&gt;a&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>SIRT1, sirtuins 1</td>
<td>2707</td>
</tr>
<tr>
<td><strong>Transporters</strong>&lt;sup&gt;b&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>UCP1</td>
<td>1066</td>
</tr>
<tr>
<td><strong>Ligands</strong></td>
<td></td>
</tr>
<tr>
<td>Chloroquine</td>
<td>5535</td>
</tr>
<tr>
<td>EX527, selisistat</td>
<td>8100</td>
</tr>
<tr>
<td>Palmitate, palmitic acid</td>
<td>1055</td>
</tr>
</tbody>
</table>

### Abstract

**Background and Purpose** Berberine, a compound from rhizome coptidis, is traditionally used to treat gastrointestinal infections, such as bacteria diarrhea. Recently, hypoglycemic and hypolipidemic efficacies of berberine were identified. We investigate mechanisms by which berberine regulates hepatic lipid metabolism and energy expenditure in mice.

**Experimental approach**
Liver-specific SIRT1 knockout (SIRT1 LKO) mice and their wild-type littermates were fed on a HFHS diet, and then treated with berberine by intraperitoneal injection for five weeks. Mouse primary hepatocytes and human HepG2 cells were treated with berberine, and then subject to immunoblotting analysis and Oil Red O staining.

**Key Results**
Berberine attenuates hepatic steatosis and controls energy balance in mice by inducing autophagy and fibroblast growth factor 21. The beneficial effects of berberine to induce autophagy and ameliorate hepatic steatosis are abrogated by the nutrient sensor SIRT1 deficiency in the liver of high-fat, high-sucrose (HFHS) diet-fed obese mice and in mouse primary hepatocytes. SIRT1 is essential for berberine to potentiate autophagy and inhibit lipid storage in mouse livers in response to fasting. Mechanically, berberine stimulates SIRT1 deacetylation activity and induces autophagy in an Atg5-dependent manner. Moreover, administration of berberine is sufficient to promote hepatic gene expression and circulating levels of FGF21 and ketone bodies in mice in a SIRT1 dependent manner.
Conclusions & Implications
Berberine acts in the liver to regulate lipid utilization and maintain whole-body energy metabolism, and berberine-mediated autophagy and FGF21 activation have the therapeutic potential for treating metabolic defects under nutrition overload, such as fatty liver diseases, type 2 diabetes and obesity.

Key Words: Berberine, FGF21, Obesity, SIRT1, Autophagy, Hepatic Steatosis

Abbreviations: mRNA, messenger RNA; FGF21, fibroblast growth factor 21; BBR, berberine; SIRT1, a mammalian ortholog of Sir2 (silent information regulator 2); Atg5, autophagy protein 5; LKO, liver-specific knockout; WAT, white adipose tissue; BAT, brown adipose tissue; KO, knockout; WT, wild-type.

Highlights
- Administration of berberine enhances systemic energy expenditure and ameliorates obesity;
- Hepatic SIRT1 is required for berberine-induced production of FGF21;
- Berberine stimulates autophagy to ameliorate hepatic steatosis in mice;
- SIRT1 is required for berberine’s effects on activation of autophagy in mice under diet-induced obesity or in response to fasting;

Introduction
The liver is the central metabolic organ that regulates lipid homeostasis such as de novo lipogenesis, fatty acid β oxidation in mitochondria, lipoprotein uptake and secretion in response to nutritional and hormonal signals(Cohen et al., 2011). Autophagy is a cellular quality control pathway that degrades cytoplasmic contents in the lysosomes(Levine et al., 2008; Mizushima et al., 2008). Autophagy-related (Atg) proteins regulate different steps of autophagy, including the expansion of the isolation membrane and autophagosomes production that sequester cytoplasmic cargo for lysosomal degradation(Settembre et al., 2014). Lipophagy is a specific degradation of lipids by autophagy(Singh et al., 2009a), in which lipid droplets are hydrolysed to release free fatty acids for mitochondrial oxidation and the TCA cycle to produce energy(Kaur et al., 2015). Lipophagy has been shown to regulate intracellular lipid homeostasis in response to various nutrient signals, and impaired lipophagy can cause excessive lipid accumulation in the liver(Liu et al., 2013).
Berberine, a commonly used drug in traditional Chinese medicine, has drawn increasing attention for its therapeutic potential for treating hepatic steatosis, dyslipidemia and diabetes (Jun et al., 2008). Berberine or its derivative was shown to decrease hepatic steatosis in HepG2 cells (Brusq et al., 2006), in high fat diet-fed rats (Chang et al., 2010; Turner et al., 2008; Xia et al., 2011; Yuan et al., 2015), and in a randomized, placebo-controlled trial in patient with non-alcoholic fatty liver disease (NAFLD) (Yan et al., 2015), and to lower serum LDL-cholesterol in hypercholesterolemic patients (Kong et al., 2004). Although the beneficial effects of berberine appear to be partially mediated by activating AMPK (Hardie, 2011; Lee et al., 2006) or upregulating LDLR expression (Kong et al., 2004), the underlying mechanism of berberine on hepatic lipid metabolism remains incompletely understood.

The NAD⁺-dependent deacetylase SIRT1, which induces longevity in yeast, worms and flies, and may mediate the beneficial effects of caloric restriction (Haigis et al., 2006; Michan et al., 2007), has emerged as a critical regulator of hepatic lipid homeostasis. Hepatic specific SIRT1 deficiency caused hepatic steatosis in fasted and diet induced obese (DIO) mice (Purushotham et al., 2009). Hepatic overexpression of SIRT1 represses ER stress and insulin resistance in DIO and ob/ob mice (Li et al., 2011a). Resveratrol, a pharmacological SIRT1 activator, represses hepatic lipid accumulation and protects liver function in DIO mice (Baur et al., 2006). In addition, fibroblast growth factor 21 (FGF21), a liver derived hormone, has emerged as a key metabolic regulator. FGF21 improves hepatic insulin sensitivity and maintain systemic glucose homeostasis in insulin resistant mice (Gong et al., 2016; Kharitonenkov et al., 2005), which is similar to the observations that another FGF family member FGF1 acts as an insulin sensitizer and lowers blood glucose levels in diabetic mice (Ahmadian et al., 2013; Suh et al., 2014). Interestingly, FGF21 mediates SIRT1’s salutary effects on alleviating hepatic steatosis and inducing energy expenditure (Li et al., 2014b). However, the relative contribution of SIRT1 or FGF21 to berberine’s effects on hepatic lipid and energy metabolism has not been investigated.
Our recent studies demonstrate that berberine ameliorates hepatic steatosis in rodents and patients with NAFLD (Chang et al., 2010; Yan et al., 2015) and hepatic SIRT1 is a key regulator for hepatic lipid metabolism (Li et al., 2011a). The present study characterizes SIRT1 as a critical regulator that mediates berberine’s salutary effects on improving hepatic steatosis by inducing autophagy, and promoting energy expenditure by stimulating FGF21. These in vivo and in vitro studies indicate that (1) Administration of berberine enhances systemic energy expenditure and ameliorates obesity; (2) Hepatic SIRT1 is required for berberine-induced production of FGF21; (3) Berberine stimulates autophagy to ameliorate hepatic steatosis in mice; (4) SIRT1 is required for berberine’s effects on activation of autophagy in mice under diet-induced obesity or in response to fasting.

**Materials and Methods**

**Animals.** All animal experimental protocols were approved by Institutional Animal Care and Use Committee at the Institute for Nutritional Sciences, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences. Animal studies are reported in compliance with the ARRIVE guidelines (Kilkenny et al., 2010; McGrath et al., 2015). Hepatocyte-specific deletion of SIRT1 (SIRT1 LKO) mice were generated by crossing albumin-Cre recombinase transgenic mice with floxed SIRT1 mice containing the deleted SIRT1 exon4 as described previously (Chen et al., 2008). Eight-week-old male C57BL/6 mice were purchased from Shanghai Laboratory Animal Co. Ltd, China. Mice were sacrificed under isoflurane anesthesia. Tissues were rapidly taken, freshly frozen in liquid nitrogen, and stored at -80 °C until needed for immunoblots. Other parts of tissues were fixed for histological analysis. The mice were housed in the research animal facility at Shanghai Institutes for Biological Sciences with proper husbandry care, and maintained at 23 °C under a 12:12-h light/dark. Mice were allowed access to the diets and water ad libitum. The experimenters were blind to group assignment and outcome assessment. Diet-induced or fasted mice are routinely used animal models to study the pathophysiological mechanisms of metabolic diseases such as
hepatic steatosis (Li et al., 2014b; Li et al., 2011b). The data obtained through these mice have the translational potential for human intentions.

**Experimental procedures.** Mice were fed without or with on high-fat, high-sucrose diet (D12327, Research Diets, NJ) consisting of 40% fat, 40% carbohydrate and 20% protein, randomly divided into groups as indicated, and then treated with berberine (5 mg/kg/day, Sigma-Aldrich, MO) or vehicle (PBS) by intraperitoneal injection once-daily for five weeks. In vivo autophagy inhibition assay were performed as described previously (Ding et al., 2010), the mice were intraperitoneally injected with chloroquine (60 mg/kg, Sigma-Aldrich, MO) or vehicle (PBS) for 24 hours. Fasting analysis was performed in mice as described previously (Chen et al., 2016; Li et al., 2014b). Mice were treated with berberine (5 mg/kg/day) or vehicle (PBS) by intraperitoneal injection once-daily for four weeks, and then randomly divided into groups: fed, fasted and refed. The fed group was placed on a normal chow diet, and the fasted group was fasted for 24 hours, and the refed group was fasted for 24 hours and then refed for 6 hours before the end of experiments.

**Liver histological analysis.** Livers were fixed in 10% phosphate-buffered formalin acetate at 4°C overnight and embedded in paraffin wax. Paraffin sections (5 μm) were cut and mounted on glass slides for hematoxylin and eosin (H&E) staining as previously described (Li et al., 2013; Li et al., 2011b). Livers embedded in optimum cutting temperature compound (Tissue-Tek, Laborimpex) were used for Oil Red O staining for the assessment of hepatic steatosis according to the manufacturer’s instructions (American MasterTech, Lodi, CA).

**small interfering RNA (siRNA) knockdown.** Knockdown experiments of Atg5 in human HepG2 cells were performed using siRNA oligonucleotides from GenePharma, China. The sense sequences of the siRNA oligos are as follows: siAtg5-938, GACCUUUCAUUCAGAAGCUTT; siAtg5-695, GUCCAUCUAAGGAUGCAAUTT; Negative control siRNA, UUCUCCGAACGUGUCACGUTT. Cells were transfected with siRNAs using Lipofectamine 2000 (Life Technologies).
In vitro lipid accumulation. Lipid accumulation in hepatocytes was described previously (Gómez-Lechón et al., 2007). The HepG2 hepatocytes are starved in serum-free DMEM (with 1% Penicillin/Streptomycin) for 24 hours and treated for an additional 20 hours in DMEM containing 100 μM palmitate, without or with berberine (10 μM), EX527 (5 μM) or chloroquine (50 μM) as indicated. The accumulation of lipid droplets are visualized by Oil Red O staining or quantified by chloroform/methanol extraction analysis.

Cell treatment. Primary mouse hepatocytes, human HepG2, HEK293 and HeLa cells, SIRT1+/+ or SIRT1−/− hepatocytes and MEFs were cultured and treated as described previously (Chen et al., 2016; Gong et al., 2016; Li et al., 2011b). SIRT1+/+ or SIRT1−/− hepatocytes were isolated from WT or SIRT1 LKO mice. MEFs were isolated from floxed SIRT1 mice containing the deleted SIRT1 exon4, and then infected with adenoviral Cre or GFP control for 72 h.

Statistical analysis. The data and statistical analysis comply with the recommendations on experimental design and analysis in pharmacology (Curtis et al., 2015). Data are expressed as mean±SEM. Statistical significance was evaluated using the unpaired two-tailed Student’s t-test, and among more than two groups by analysis of one-way ANOVA with Bonferroni’s post hoc test. Post hoc test was run only if F achieved p < 0.05 and there was no significant variance in homogeneity. Data were analyzed with GraphPad Prism software, version 5.0 (La Jolla, CA).

Nomenclature of Targets and Ligands. Key protein targets and ligands in this article are hyperlinked to corresponding entries in http://www.guidetopharmacology.org/, the common portal for data from the IUPHAR/BPS Guide to PHARMACOLOGY (Southan et al., 2016), and are permanently archived in the Concise Guide to PHARMACOLOGY 2017/18 (Alexander et al., 2015a; Alexander et al., 2015b).

Results

Hepatic specific deletion of SIRT1 abrogates berberine’s effects on lowering hepatic steatosis and body weight in HFHS diet-fed mice.
To determine whether SIRT1 plays a role in berberine’s effects on hepatic steatosis and obesity, wild type littermates (WT) or liver-specific SIRT1 deficient mice were fed on a diet composed of high-fat, high-sucrose (HFHS) (Gong et al., 2016; Li et al., 2011b), followed by treatment with berberine or vehicle. In the WT mice, administration of berberine caused a potent reduction of hepatic steatosis as evidenced by significant reduction of liver triglyceride levels, H&E and Oil Red O staining, and decreased stained areas (Fig. 1A-1D), suggesting a critical role of berberine in regulating hepatic lipid metabolism. Notably, hepatic cholesterol levels were comparable between these groups. Moreover, berberine-treated mice had reduced body weight without significant affecting liver weight (Fig. 1E and S1A). These results are consistent with amelioration of hepatic steatosis and reduction of body weight by treatment with berberine or its derivative in obese rodents and hepatocytes (Brusq et al., 2006; Chang et al., 2010; Turner et al., 2008). Strikingly, compared with WT mice, the beneficial effects of berberine on lowering hepatic steatosis and body weight were largely diminished in SIRT1 LKO mice, suggesting a critical role of SIRT1 in mediating berberine’s effects on hepatic lipid metabolism and body weight gain.

**Berberine stimulates autophagy activation in a SIRT1-dependent manner in the liver of HFHS diet-fed mice.**

Recently studies demonstrated that berberine induces the nutrient sensor SIRT1 to promote muscle function (Gomes et al., 2012), and stimulates autophagy to regulate cellular apoptosis (Wang et al., 2010; Yu et al., 2014). To determine whether SIRT1 plays a role in mediating berberine’s effects on autophagy in the liver, the immunoblotting analysis was performed in the liver of mice fed with HFHS diet. As shown in Fig.1F, administration with berberine induced a significant elevation of autophagy as evidenced by the increase of membrane-associated, PE-conjugated form of LC3 (LC3-II). Strikingly, the ability of berberine to induce autophagy was abrogated by SIRT1 deficiency as evidenced by significant reduction of LC3-II in the liver of SIRT1 LKO mice-fed with HFHS diet. Importantly, electron microscopic (EM) analysis indicated that cytosolic autophagic vacuoles (AVs) as evidenced
by double membrane structures containing undigested cytoplasmic contents were induced in the berberine-treated mouse livers, which was diminished by hepatic SIRT1 deficiency (Fig. 1G and 1H).

To determine whether berberine induces autophagic flux in vivo, administration of the lysosome inhibitor chloroquine in mice was performed. As shown in Fig. 2A, administration of 60 mg/kg chloroquine induced the expression levels of LC3-II in the liver of mice which is consistent with previous observation (Ding et al., 2010). Strikingly, the stimulating effects of berberine on LC3-II were further increased in the liver when lysosome-mediated protein degradation was inhibited by chloroquine treatment, consistent with the EM analysis showing berberine-induced AVs (Fig. 2B). Given that a significant induction of LC3-II or AVs stimulated by berberine in the presence of chloroquine, these data suggest that berberine is sufficient to promote the synthesis of autophagy in the liver of mice fed with HFHS diet.

**Berberine is sufficient to induce autophagy and repress lipid accumulation in hepatocytes.**

To investigate whether berberine is a driving force to induce autophagy and improve hepatic steatosis in vitro, effects of berberine treatment were assessed in multiple hepatocytes. As shown in Fig. 3A, berberine treatment caused an induction of LC3-II in a dose dependent manner in HepG2 cells, suggesting increased autophagy by berberine. Notably, expression levels of LC3-II were induced by serum starvation. Importantly, effects of berberine on stimulating autophagy were confirmed in mouse primary hepatocytes as evidenced by increased LC3-II expression (Fig. 3B), and in HeLa cells showing increased cytosolic redistribution of GFP-LC3 fusion protein to form puncta (Fig. 3C).

To further test the functional consequence of berberine on hepatic steatosis *in vitro*, hepatocytes were treated with fatty acid palmitate to induce intracellular lipid accumulation, which has been showed to mimick hepatic steatosis in HepG2 and Huh7 cells (Liu *et al.*, 2011; Wobser *et al.*, 2009). Strikingly, treatment of berberine repressed palmitate-induced lipid accumulation in a dose dependent manner as evidenced by triglyceride levels measured by
chloroform/methanol extraction method (Fig. 3D), and by a reduction of lipid staining using Oil Red O staining (Fig. 3E). Consistent with the in vivo results, these observations suggest that berberine is sufficient to induce autophagy and repress lipid accumulation in hepatocytes.

**SIRT1 is necessary for berberine to induce autophagy and inhibit lipid accumulation in hepatocytes.**

We next elucidated a causal relationship between SIRT1 and autophagy and their effects on lipid accumulation in SIRT1-deficient hepatocytes. Consistent with the in vivo results, SIRT1 deletion caused a profound reduction LC3-II in mouse embryonic fibroblasts (Fig. 4A) and of berberine-induced LC3-II in primary hepatocytes (Fig. 4B). Notably, SIRT1 deficiency was confirmed by immunoblotting showing the expected deletion mutant protein in SIRT1 deficient hepatocytes, which migrated slightly faster than that in WT cells.

To test the functional consequence of SIRT1 inhibition on the lipid lowering effects of berberine hepatocytes, lipid quantification was measured in HepG2 cells treated with palmitate. Strikingly, the reduction of lipid accumulation caused by berberine was largely blocked by treatment with specific SIRT1 inhibitor EX527 (Rodgers et al., 2005), as evidenced by triglyceride levels (Fig. 4C) and Oil Red O staining (Fig. 4D). Together, these data indicate that SIRT1 is required for berberine to induce autophagy and inhibit lipid accumulation in vitro.

**Berberine alleviates hepatic steatosis in an autophagy dependent manner.**

Next, the mechanism by which berberine regulates SIRT1-dependent activation of autophagy and its effects on hepatic steatosis were rigorously explored in vitro. As shown in Fig. 5A, treatment with berberine decreased acetylation levels of PGC-1α, a key downstream substrate of SIRT1, in HEK293 cells stably expressing human FLAG-tagged PGC-1α (St-FLAG-PGC-1α), suggesting increased SIRT1 activity by berberine treatment. Notably, treatment with SIRT1 specific inhibitor EX527 (Peck et al., 2010) increased PGC-1α acetylation.
Recently, SIRT1 was shown to regulate autophagy through deacetylating Atg5 and Atg7 (Lee et al., 2008). Given that SIRT1 is necessary for berberine-induced autophagy, we hypothesize that berberine regulates autophagy through SIRT1-mediated deacetylation of Atg proteins. As expected, treatment with berberine decreased acetylation levels of Atg5 in HEK293 cells (Fig. 5B). These data suggest that SIRT1-dependent deacetylation of Atg5 may contribute to berberine-induced autophagy activation. We next determined whether Atg5 is necessary for berberine to induce autophagy in HepG2 cells. The small interfering RNAs (siRNA)-mediated knockdown was examined. As shown in Fig. 5C and 5D, knockdown of Atg5 by small interfering RNAs (siRNA) that was used to block autophagy in hepatocytes (Singh et al., 2009a) resulted in a reduction of berberine-induced LC3-II accumulation.

Next, an autophagy inhibitor chloroquine was used to test the functional consequence of autophagy inhibition on lipid lowering effects of berberine in HepG2 cells treated with palmitate. Strikingly, administration with chloroquine largely ablated berberine’s effects on lipid accumulation under palmitate condition in HepG2 cells as evidenced by Oil Red O staining (Fig. 5E). Taken together, these studies indicate that berberine stimulates autophagy and improves hepatic steatosis in hepatocytes.

**Berberine increases fasting-induced autophagy through hepatic SIRT1 in mice.**

To determine whether autophagy is regulated by nutritional status, immunoblotting analysis was performed in the liver of mice under fed, fasted or refed conditions. Consistent with previous observation showing increased autophagy in fasted mice (Ezaki et al., 2011a), fasting for twenty four hours caused a profound induction of autophagy in mouse livers as evidenced by induced conversion of LC3-I to LC3-II (Fig. 6A). As shown in Fig. 6B and 6C, compared with the fed state, fasting caused induction of lipid deposition in mouse livers as evidenced by Oil Red O staining, which is consistent with our recent observation in mice (Chen et al., 2016). Strikingly, berberine treatment caused an additional induction of LC3-I to LC3-II ratio, and reduced p62 expression in fasted mice liver comparing with that in
the control group, which is correlated with attenuation of fasting-induced hepatic steatosis, suggesting that berberine lowers fasting-induced lipid accumulation probably through stimulating autophagy. To investigate whether SIRT1 is required for fasting-induced autophagy in the liver, immunoblotting analysis was performed using mouse livers. Consistent with the effects of SIRT1 deficiency in the liver of HFHS diet-fed mice, berberine-stimulated autophagy was abrogated in SIRT1 LKO mice in response to fasting as evidenced by a significant reduction of the conversion of LC3-I to LC3-II, not obvious changed p62 expression (Fig. 6D), as well as the reduced cytosolic autophagic vacuole numbers (Fig. 6E-F). The mRNA levels of autophagic markers, such as BECN1 for initiation and p62 for degradation were measured (Fig. 6G). Compared with vehicle, no significant changes were observed in mouse livers treated with berberine, suggesting that berberine does not regulate autophagy at the transcriptional level. Future studies using the tandem sensor RFP-GFP will be used to determine in depth the autophagy flux. Moreover, these results are correlated with the ablation berberine’s effects on improving steatotic phenotypes by hepatic SIRT1 deficiency as evidenced by Oil Red O staining (Fig. S1B and S1C). Together, these data indicate that SIRT1 is indeed necessary for berberine-induced autophagy and lipid lowering effects in the liver of fasted mice.

**Production of FGF21 and whole-body energy expenditure is increased by berberine.**

Our recent study indicates that SIRT1 induces the key metabolic regulator FGF21 and promotes energy expenditure and weight loss in mice, suggesting a potential role of FGF21 in mediating berberine’s body weight lowering effects. We next determine whether berberine affects expression levels of FGF21 in mice in response to fasting. As shown in Fig. 7A, messenger RNA levels of FGF21 were increased robustly 8-fold in livers of vehicle-treated mice upon fasting and decreased upon refeeding. Consistently, circulating levels of FGF21 was increased 8-fold in vehicle-treated mice upon fasting, and this induction was decreased upon refeeding. These data are consistent with previous observations (Inagaki et al., 2007). Interestingly, compared with vehicle, treatment with berberine caused an additional induction...
of the production and secretion of FGF21 in mice. Notably, an induction of plasma β-hydroxybutyrate concentrations in berberine-treated mice was observed (Fig. 7B), which is consistent the essential role of FGF21 in ketogenesis (Badman et al., 2007; Inagaki et al., 2007). We next investigate whether SIRT1 is necessary for berberine induced FGF21 expression. As shown in Fig. 7C and Fig. 7D, berberine-induced expression and secretion of FGF21 was abrogated by SIRT1 deficiency or SIRT1 inhibitor EX527 in hepatocytes respectively, suggesting a cell-autonomous regulation of FGF21 by treatment with berberine.

To understand the mechanisms by which berberine-treated mice are resistant to developing obesity, the major components of energy metabolism such as oxygen consumption and energy expenditure were examined. As shown in Fig. 7F, comprehensive metabolic cage studies indicated that the rates of oxygen consumption (VO$_2$) and the calculated energy expenditure were increased in response to berberine treatment during the light and dark phases of the fed and fasted mice. Consistent with the rates of VO$_2$ and energy expenditure, VCO$_2$ rates were significantly higher in the mice treated with berberine. Moreover, the respiratory quotients (respiratory quotient=VCO$_2$/VO$_2$) appeared indistinguishable in vehicle and berberine treated mice in fed and fasted conditions, likely due to the combined increase in both VO$_2$ and VCO$_2$. These results indicated that relatively equal use of carbohydrates versus lipids as an energy source in whole body. Notably, no significant alterations in daily food intake or physical activity were observed between berberine- and vehicle-treated mice (Fig.7E and Fig. S2).

Given that FGF21 regulates browning of white adipose tissue in adaptive thermogenesis (Fisher et al., 2012), we further investigated whether berberine alters transcription of browning genes in white adipose tissue (WAT) or thermogenic genes in brown adipose tissue (BAT). Strikingly, administration of berberine caused a significant induction of key genes related to the browning and thermogenesis, such as uncoupling protein 1 (UCP1), and the BAT program coactivator PRD1-BF1-RIZ1 homologous domain containing 16 (PRDM16), as well as expression of other BAT markers, type II iodothyronine
deiodinase (DIO2) (Fig. 7G). Taken together, it appears to suggest that berberine ameliorates adiposity of HFHS diet-fed mice, possibly through increased FGF21 signaling, improved whole body energy expenditure and browning of white adipose tissue.

**Discussion**

Although pharmacological studies have demonstrated therapeutic actions of berberine on hepatic steatosis, insulin resistance and obesity, the downstream signaling pathways mediating these activities remains poorly understood. Administration of berberine or berberine’s derivative protects against hepatic steatosis in hepatocytes (Brusq et al., 2006), rodents (Chang et al., 2010; Turner et al., 2008), and patients with NAFLD (Yan et al., 2015) suggesting an obligatory role of liver in berberine-mediated lipid homeostasis. In the present study, we provide both in vivo and in vitro evidence showing that berberine induces autophagy to ameliorate hepatic steatosis in a SIRT1 dependent manner in the liver of mice under nutrient overload or in response to fasting. Moreover, berberine’s regulation of FGF21 and energy expenditure may represent a molecular mechanism by which pharmacological administration of berberine protects against obesity (Fig. 7H).

**The liver is a direct target tissue in mediating berberine’s effects on regulating autophagy and lipid metabolism**

In vivo and in vitro approaches were utilized in the present study to demonstrate that berberine induces autophagy in the liver. First, administration of berberine induced conversion of LC3-I to LC3-II in livers of mice fed with HFHS diet or mice in response to nutrient deprivation. Second, the data from pharmacological administration of berberine and genetic manipulation of autophagy signaling confirmed the important role of hepatocytes in berberine’s effects on activation of autophagy in HepG2 and primary mouse hepatocytes. The efficacy of berberine on autophagy augmentation in the liver are consistent with the findings showing that berberine treatment induces autophagy to regulate apoptosis in a number of carcinoma cell lines (Wang et al., 2010; Yu et al., 2014), protects against cardiac hypertrophy in transverse aortic contraction (TAC)-treated rats (Li et al., 2014a), and inhibits
macrophage inflammation and its potential roles in alleviating atherosclerosis (Fan et al., 2015). Importantly, treatment with an autophagy inhibitor, chloroquine, to block autophagy degradation in the lysosome largely abrogated berberine’s effects on lowering lipid accumulation in HepG2 cells, suggesting that autophagy augmentation mediates berberine’s effects on improving hepatic steatosis. These results are consistent with recent findings showing that genetic manipulation- or nutrient deprivation-stimulated autophagy induces lipid droplets hydrolysis in the liver to release free fatty acid for mitochondrial oxidation, leading to attenuated hepatic steatosis in obese ob/ob or fasted mice (Liu et al., 2013; Singh et al., 2009a; Yang et al., 2010). Another study reported that berberine inhibits autophagy in 3T3-L1 adipocytes. This may therefore reflect the deregulation of autophagic signaling in the adipose tissue under these conditions or differential signaling in these cells.

Moreover, autophagy is an adaptive process for intracellular component degradation and recycling, especially when cell is challenged with disordered nutrient status. Cellular metabolism is tightly correlated to autophagy. Nutrient deprivation induces autophagy, contributing to maintain the energy balance by recycling the proteins, lipids and glycogen (Ezaki et al., 2011b; Singh et al., 2009b). Our results show that autophagy is induced in mouse livers by fasting, which is consistent with the previous observations (Lee et al., 2014). Importantly, administration of berberine potentiates fasting-induced autophagy synergistically, suggesting that berberine is sufficient to stimulate autophagy and maintain cellular metabolism in the liver during the physiological condition.

**Berberine regulates lipid metabolism in the liver during nutrient overload condition in a SIRT1 dependent manner**

We are the first to identify that the identification of cross-talk between berberine and the nutrient sensor SIRT1 directly in the liver. The fact that the abrogation of berberine’s augmentation of autophagy and amelioration of steatotic phenotypes in liver-specific SIRT1 knockout (SIRT1 LKO) mice suggests a critical role of SIRT1 in mediating berberine’s actions in the liver. Moreover, SIRT1 loss-of-function approaches in numerous cells, including
primary hepatocytes, HepG2 and MEFs, further confirmed that SIRT1 is necessary for berberine to induce autophagy and inhibit lipid accumulation in a cell-autonomous manner. Mechanically, we demonstrate that berberine deacetylates the key autophagy gene Atg5 to enhance autophagy probably through SIRT1 activation, which is consistent with previous observations that SIRT1 positively regulates autophagy in mouse livers and numerous cell lines, including HeLa, HEK293, embryonic stem cells and liver (Dong et al., 2013; Lee et al., 2008; Lin et al., 2017a; Ou et al., 2014; Qin et al., 2016a). It was reported that the efficacy of berberine to induce autophagy and regulate liver ischemia-reperfusion injury was attenuated by SIRT1 inhibitor Sirtinol or EX527 (Lin et al., 2017b; Qin et al., 2016b). However, the SIRT1 inhibitor Sirtinol or EX527 is known to be off-targeted and inhibit sirtuin family deacetylase activity, such as SIRT2 (Grozinger et al., 2001; Peck et al., 2010). Moreover, pharmacological administration of Sirtinol or EX527 causes systemic effects indirectly through other tissues not related to the liver, which may contribute to the net hepatic phenotypes. Our study using liver-specific SIRT1 knockout mice and SIRT1−/− hepatocytes define berberine as an activator of SIRT1, and demonstrate an essential role of SIRT1 in mediating berberine’s reduction of hepatic fat storage.

In support our results, recent studies indicate that berberine improves muscle mitochondrial function and insulin sensitivity through SIRT1 (Gomes et al., 2012), although other studies support a role of mitochondrial inhibition by berberine (Jun et al., 2008). Taken together, in addition to improving insulin sensitivity and regulates glucose metabolism via activation of AMPK (Jun et al., 2008), hepatic SIRT1 is critical for berberine to induce autophagy flux and control hepatic lipid metabolism in the liver and hepatocytes.

Regulation of browning of white adipose tissue by berberine-induced FGF21

The most important finding of the current paper is the identification that treatment of berberine induces production and secretion of FGF21, and contributes to enhanced energy expenditure and weight loss likely through promoting white fat browning. Given that FGF21 acts as a key regulator in energy metabolism (Fisher et al., 2012; Owen et al., 2014; Xu et al.,...
2009), and SIRT1 loss-of-function approaches blocked berberine’s effects on inducing FGF21, our results suggest that berberine’s effects on controlling energy metabolism and weight loss are likely mediated via SIRT1 activation. These results are consistent with recent observations that berberine induces the development of brown-like adipocytes in the white adipose tissue and enhances energy metabolism in obese db/db mice (Zhang et al., 2014). Our findings of berberine regulation of FGF21 establish a cross-talk between the liver and adipose tissue, which may represent a therapeutic strategy for the development of drugs to combat metabolic disorders during obesity.

In conclusion, the ability of berberine to prevent the deleterious effects of excess caloric intake on hepatic steatosis and obesity suggests that berberine and its derivative with similar properties might be a promising new approach for treating these metabolic disorders. Given that berberine administration is safe in humans, these studies also highlight the therapeutic relevance of targeting hepatic SIRT1-autophagy axis to reverse hepatic lipid deregulation and improve lipid homeostasis in obesity and type 2 diabetes.

**Acknowledgments**

This work was supported by grants from the National Key Basic Research Program of China (2012CB524906) and the National Natural Science Foundation of China (81270933, 81570718) to X.G. This work was also supported by grants from the National Key R&D Program of China (2017YFC0909601), the National Natural Science Foundation of China (31471129, 31671224) and the Chinese Academy of Sciences (ZDBS-SSW-DQC-02, 2013OHTP04) to Y.L. We thank Drs. Leonard Guarente at Massachusetts Institute of Technology and Mengwei Zang at Boston University for SIRT1 LKO mice, Drs. Jiandie Lin and Chang Liu for providing FLAG-tagged PGC1α plasmid, Dr. Chuangui Wang for providing FLAG-tagged Atg5 plasmid, and Dr. Zhixue Liu for providing pCDH-CMV-3xFLAG-SBP and pEGFP-C1-LC3 plasmids. We would like to thank Fengling Qin for electron microscopy analysis, Dr. Yi Yang for body composition analysis, and Zhonghui Weng for animal studies.

**Conflict of interest**

This article is protected by copyright. All rights reserved.
The authors declare no conflicts of interest.

**Declaration of transparency and scientific rigour**

This Declaration acknowledges that this paper adheres to the principles for transparent reporting and scientific rigour of preclinical research recommended by funding agencies, publishers and other organizations engaged with supporting research.

**Reference**


This article is protected by copyright. All rights reserved.


This article is protected by copyright. All rights reserved.


This article is protected by copyright. All rights reserved.


This article is protected by copyright. All rights reserved.


This article is protected by copyright. All rights reserved.
The beneficial effects of berberine on body weight and hepatic steatosis are compromised by hepatic specific deletion of SIRT1 in HFHS diet-fed mice.

Wild type (WT) and SIRT1 LKO male mice at sixteen-week-old were fed on a HFHS diet for 12 weeks, and then treated with berberine (5 mg/kg/day) or vehicle (PBS) by intraperitoneal injection once-daily for five weeks. (A-B) Liver (A) and plasma (B) triglyceride and cholesterol levels were assessed in mice. (C-D) Representative H&E and Oil Red O staining, and quantification of Oil Red O stained areas are shown, n=6. (E) The body weight was measured, n=6. (F-G) SIRT1 is necessary for berberine-induced autophagy in the liver of HFHS diet-fed mice. (F) Berberine-stimulated conversion of LC3-I to LC3-II is reduced in SIRT1 LKO mice fed with HFHS diet. Band intensity of LC3-II was quantified by densitometry and normalized.
to the levels of LC3-I for unwanted sources of variation, and presented as the mean±SEM, n=6. *p<0.05, versus WT and vehicle; #p<0.05, versus WT mice treated with BBR. (G-H) Electron micrographs of the liver tissue (G). Images 1-4 showing autophagic vacuoles (AVs) are high magnifications of the “white box” area indicated in the images of berberine-treated WT or SIRT1 LKO mice fed with HFHS diet. Arrows denote autophagic vacuoles. N, nucleus; LD, lipid droplet. The average numbers of AVs were quantified from a randomly selected pool of 5 fields under each condition (H). The data are presented as the mean±SEM, n=5. *p<0.05, versus WT and vehicle; #p<0.05, versus WT mice treated with BBR.
Fig. 2. Berberine is sufficient to induce autophagic flux in the livers of HFHS diet-fed mice. (A)

Eight-week-old male C57BL/6 mice were fed on a HFHS diet for 9 weeks, and then treated with berberine (5 mg/kg/day) once-daily for four weeks, followed by treatment with chloroquine (60 mg/kg) intraperitoneally for 24 hours before the sacrifice. (A) Berberine stimulates conversion of LC3-I to LC3-II under chloroquine treatment in the liver of mice fed with HFHS diet. Band intensity of LC3-II was quantified by densitometry and normalized to the levels of LC3-I for unwanted sources of variation, and presented as the mean±SEM, n=5. *p<0.05, versus WT and vehicle; #p<0.05, versus WT mice treated with BBR. (B) Electron micrographs of the liver tissue. Images 1-4 showing autophagic vacuoles (AVs) are high magnifications of the “white box” area indicated in the images of berberine- or chloroquine-treated mice fed with HFHS diet. Arrows denote autophagic vacuoles. N, nucleus.

The average numbers of AVs were quantified from a randomly selected pool of 5 fields under each condition. The data are presented as the mean±SEM, n=5. *p<0.05, versus vehicle; #p<0.05, versus vehicle and chloroquine.
Fig. 3. Berberine stimulates autophagy to repress lipid accumulation in hepatocytes.

(A-B) Berberine stimulates the conversion of LC3-I to LC3-II in HepG2 cells (A) and primary mouse hepatocytes (B). (C) The redistribution of GFP-LC3 to form puncta is enhanced by berberine in HeLa cells. Cells were transfected with expressing plasmid encoding GFP-LC3 fusion protein, followed by treatment without or with 10 μM berberine for 10 h. (D-E) Berberine decreases lipid accumulation in HepG2 cells exposed to palmitate, as reflected by triglyceride levels determined by a colorimetric enzymatic assay (D) and Oil Red O staining (E). The data are presented as the mean±SEM, n=6. *p<0.05, versus control; #p<0.05, versus palmitate.
Fig. 4. Berberine induces autophagy to inhibit lipid accumulation through SIRT1 in hepatocytes.

(A) Effects of SIRT1 deficiency on LC3 mouse embryo fibroblasts (MEFs). MEFs were isolated from 8-week-old floxed SIRT1 mice containing the deleted SIRT1 exon4, and then infected with adenoviral Cre for 72 h. (B) SIRT1 deficiency diminishes berberine-stimulated conversion of LC3-I to LC3-II in primary hepatocytes. Primary hepatocytes were isolated from 8-week-old WT or SIRT1 LKO mice and treated as indicated. (C-D) The lipid lowering effects of berberine is abolished by a pharmacological SIRT1 inhibitor EX527 in HepG2 cells treated with palmitate, as determined by triglyceride levels (C) and Oil Red O staining (D). The data are presented as the mean±SEM, n=6. *p<0.05, versus control; #p<0.05, versus palmitate.
Fig. 5. Berberine stimulates SIRT1 deacetylation activity and induces autophagy in an Atg5-dependent manner.

(A-B) Berberine induces deacetylation of PGC-1α (A) or Atg5 (B). HEK293 cells stably expressing mouse FLAG-tagged PGC-1α (St-FLAG-PGC-1α), human FLAG-tagged Atg5 (St-FLAG-Atg5) or empty vector were treated with 10 μM berberine or 5 μM EX527 for 10 or 6 hours respectively, followed by immunoprecipitation and immunoblots with the indicated antibodies. (C) The mRNA amounts of Atg5 are decreased by Atg5 knockdown in HepG2 cells. The data are normalized to β-actin for unwanted sources of variation, and presented as the mean±SEM, n=5. *p<0.05, versus control siRNA. (D) Berberine-stimulated conversion of LC3-I to LC3-II is ablated by Atg5 knockdown in HepG2 cells. Cells were transfected with Atg5 and control siRNAs for 24 h, and incubated in serum-free DMEM containing 5.5 mM glucose overnight, followed by treatment without or with 10 μM berberine for 10 h. (E) Berberine’s lipid lowering effects are abrogated by the autophagy inhibitor chloroquine in HepG2 cells treated with palmitate as reflected by Oil Red O staining.
Fig.6. SIRT1 is required for berberine to potentiate fasting-induced autophagy in mouse livers.

Male C57BL/6 mice at eight weeks of age, SIRT1 LKO or WT mice at sixteen weeks of age were treated without or with berberine (5 mg/kg/day) or vehicle (PBS) by intraperitoneal injection once-daily for four weeks, and then subjected to feeding (Fed), or fasting for 24 hours (Fasted). (A) The conversion of LC3-I to LC3-II was increased by fasting in mice. Band intensity of LC3-II was quantified by densitometry and normalized to the levels of LC3-I for unwanted sources of variation, and presented as the mean±SEM, n=5. *p<0.05, versus Fed mice. (B) Berberine stimulates the conversion of LC3-I to LC3-II and reduces the p62 in mice in response to fasting, n=5. (C) Hepatic steatosis was assessed by Oil Red O staining (scale bars: 50 μm). (D) SIRT1 deficiency abrogated berberine-induced conversion of LC3-I to LC3-II and the reduction of p62 in the liver of fasted mice, n=6. (E-F) Electron micrographs (E) and the average numbers of autophagic vacuoles (F) of the liver tissue were measured.
Images 1-4 showing autophagic vacuoles (AVs) are high magnifications of the “white box” area indicated in the images of berberine-treated WT or SIRT1 LKO mice in response to fasting. Arrows denote autophagic vacuoles. N, nucleus; LD, lipid droplet. The average numbers of AVs were quantified from a randomly selected pool of 5 fields under each condition. The data are presented as the mean±SEM, n=5. *p<0.05, versus WT and vehicle; #p<0.05, versus WT and BBR. (G) The mRNA levels of BECN1 and p62 were measured by real-time PCR. The data are normalized to β-actin for unwanted sources of variation, and presented as the mean±SEM, n=5.
Fig. 7. Administration of BBR induces production and secretion of FGF21 and promotes whole-body energy expenditure in mice.

Male C57BL/6 mice at eight weeks of age were treated without or with BBR (5 mg/kg/day) or vehicle (PBS) via intraperitoneal injection for 4 weeks, and then subjected to feeding (Fed),
fasting for 24 hours (Fasted), or refeeding for 6 hours after a 24-hour fast (Refed). (A) Administration of berberine is sufficient to promote hepatic gene expression and circulating levels of FGF21 in mice. The mRNA levels are normalized to β-actin for unwanted sources of variation, n=5. (B) Circulating ketone bodies levels were robustly induced by treatment with berberine. The data are presented as the mean±SEM, n=5. *p<0.05, versus fed and vehicle; #p<0.05, versus fasted and vehicle. (C) SIRT1 deficiency abolishes berberine induced expression of FGF21 in mouse primary hepatocytes. The mRNA levels are normalized to β-actin for unwanted sources of variation, and presented as the mean±SEM, n=6. *p<0.05, versus WT and vehicle; #p<0.05, versus WT and BBR. (D) Effects of SIRT1 inhibitor EX527 on FGF21 secretion in hepatocytes. Media FGF21 levels were measured in HepG2 cells treated with berberine (10 μM) and EX527 (5 μM). The data are presented as the mean±SEM, n=5. *p<0.05, versus vehicle; #p<0.05, versus vehicle and BBR. (E) Food intake was measured during the fed state. (F) The rate of VO₂, energy expenditure, VCO₂, and Respiratory quotient (RQ) were measured by comprehensive metabolic monitoring in mice over a 24-hour period with food and over a 24-hour fast, and normalized to lean body mass. (G) Administration of berberine increases the transcription of brown-like genes in WAT and BAT in mice. The mRNA levels are normalized to β-actin for unwanted sources of variation, and presented as the mean±SEM, n=8. *p<0.05, versus vehicle. (H) The proposed model for SIRT1 in mediating berberine’s effects on improving hepatic steatosis and obesity in diet-induced insulin-resistant mice. Administration of berberine stimulates hepatic SIRT1, which in turns increases autophagic function to regulate intracellular lipid stores, and improves fatty liver disease. Activation of berberine-SIRT1-FGF21 axis may represent a new approach to treat obesity by promoting white fat browning and stimulating energy expenditure.