

Metformin alleviates hepatosteatosi  
through Sirt1-mediated autophagy  
induction

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# Metformin alleviates hepatosteatosis through Sirt1-mediated autophagy induction

Directed by Professor Bong Soo Cha

The Doctoral Dissertation  
submitted to the Department of Medical Science,  
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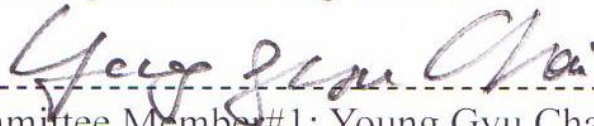
Young Mi Song

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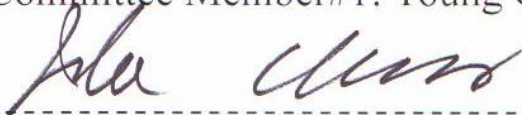
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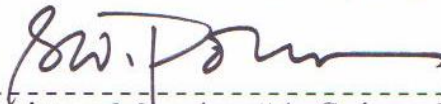
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I remain yours truly

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## **Abstract**

# **Metformin alleviates hepatosteatosis through Sirt1-mediated autophagy induction**

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(Directed by Professor **Bong Soo Cha**)

Metformin activates both PRKA and SIRT1. Furthermore, autophagy is induced by either the PRKA-MTOR-ULK1 or SIRT1-FOXO signaling pathways. We aimed to elucidate the mechanism by which metformin alleviates hepatosteatosis by examining the molecular interplay between SIRT1, PRKA, and autophagy. ob/ob mice were divided into 3 groups: one with ad libitum feeding of a standard chow diet, one with 300 mg/kg intraperitoneal metformin injections, and one with 3 g/day caloric

restriction (CR) for a period of 4 weeks. Primary hepatocytes or HepG2 cells were treated with oleic acid (OA) plus high glucose in the absence or presence of metformin. Both CR and metformin significantly improved body weight and glucose homeostasis, along with hepatic steatosis, in *ob/ob* mice. Furthermore, CR and metformin both upregulated SIRT1 expression and also stimulated autophagy induction and flux *in vivo*. Metformin also prevented OA with high glucose-induced suppression of both SIRT1 expression and SIRT1-dependent activation of autophagy machinery, thereby alleviating intracellular lipid accumulation *in vitro*. Interestingly, metformin treatment upregulated SIRT1 expression and activated PRKA even after siRNA-mediated knockdown of *PRKAA1/2* and *SIRT1*, respectively. Taken together, these results suggest that metformin alleviates hepatic steatosis through PRKA-independent, SIRT1-mediated effects on the autophagy machinery.

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Key words: autophagy, hepatoseatosis, metformin, PRKA (protein kinase, AMP-activated), SIRT1 (sirtuin class of histone/protein deacetylases)

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**I. INTRODUCTION**

Efforts for treating non-alcoholic fatty liver disease (NAFLD) have traditionally focused on burning excess energy and unloading caloric burdens, on either the individual or cellular level. To achieve the aims of increasing energy

expenditure and mimicking caloric restriction (CR) in the treatment of metabolic disease, a thorough understanding of the molecular machinery involved in these processes is required. Understanding how this molecular machinery is dysregulated or dysfunctional in NAFLD is also crucial. Although metformin is the first-line anti-diabetic drug, particularly for overweight and obese people and those with normal kidney function, the idea that metformin exerts pleiotropic effects via novel mechanisms of action is now gaining popularity.<sup>1,2,3</sup> In this regard, the mechanism by which metformin alleviates hepatosteatosis and the effects of metformin on autophagy are two current areas of debate and investigation.

Autophagy is a conserved cellular process that degrades damaged organelles, unfolded proteins and intracellular pathogens by fusion with lysosomes for maintenance of cellular homeostasis.<sup>4,5</sup> Three types of autophagy have been known: macroautophagy, chaperone-mediated autophagy (CMA) and microautophagy.<sup>6,7</sup> Macroautophagy (hereafter referred to as autophagy) involves the isolation of a portion of cytosol by a double membrane structure termed an autophagosome. The autophagosome fuses with lysosome resulting in the degradation of the intra-cellular components.<sup>6</sup> In CMA, soluble proteins containing a specific KFERQ motif in their amino acids sequence are bound to a chaperone protein for translocation to the lysosome, where binding to the lysosome-associated membrane protein type 2A (LAMP-2A) receptor results in protein internalization and degradation.<sup>7,8</sup> The third form of autophagy, microautophagy, involves the sequestration of intracellular components within lysosomes by an invagination of the lysosomal membrane. However, these forms of autophagy are little known about the regulation or function.

As previously mentioned, there are two general functions of autophagy. First,

autophagy can maintain energy homeostasis by generating a source of metabolic substrate under limited nutrients. Second, autophagy can also remove damaged cellular constituents such as mitochondria and long-lived protein. These functions of autophagy sustain cell survival under stress condition.<sup>9-11</sup> Recent studies have suggested that novel function for autophagy is active in the regulation of cellular homeostasis such as lipid metabolism and insulin sensitivity.<sup>12,13</sup>

Liu HY et al. and Yang L et al. have demonstrated that autophagy is impaired in the liver in insulin resistant state.<sup>13,14</sup> In addition, autophagy machinery has been found to be associated with lipid metabolism through demonstrating colocalization of lipid with autophagosomes and lysosomes assessed by fluorescence microscopy, lipid in autophagic vacuoles by electron microscopy, and an association of the autophagosome-associated protein microtubule-associated protein light chain 3 (LC3) with lipid droplets by immunogold staining. Furthermore, an inhibition of autophagy by either genetic knockdown using siRNA against autophagy gene *atg5* or pharmacological inhibition with 3-methyladenine (3MA) in hepatocytes which was exposed to free fatty acid or methionine and choline-deficient medium, significantly increased intracellular TG content.

*In vivo* study of mouse livers, hepatocyte-specific *atg7*, essential autophagy gene, knockout led to inhibition of autophagy and consequently increased levels of hepatic TGs and cholesterol.<sup>12</sup>

Taken together, these results suggested autophagy contributed to hepatic lipid stores.<sup>15</sup> Subsequently, autophagy inhibition has been reported to induce hepatosteatosis in alcohol-induced hepatic injury models as well.<sup>16</sup> Therefore, autophagy is a central mechanism for the lipid droplet breakdown of lipid droplet by lipophagy.<sup>12,17</sup>



The traditional pathways of low nutrition-induced autophagy are either through PRKA (protein kinase , AMP-activated)-MTOR-ULK1 mediated signaling, activated by an increase in the AMP/ATP ratio, or the sirtuin class of histone/protein deacetylases (SIRT1)-FOXO pathway, activated by increased NAD<sup>+</sup>.<sup>18,19</sup>

Under starvation conditions, active PRKA, which are activated by increase in AMP/ATP ratio, positively regulates the activity of the TSC1-TSC2 complex by phosphorylating the TSC2 protein (resulting in the opposite activity to the AKT-1 phosphorylation of TSC2), which then turns off the RHEB G-protein and reduces MTOR activity, central regulator in autophagy.<sup>20,21</sup> MTOR is present in two complexes: MTORC1 and MTORC2 (TORC, target of rapamycin complex). Inactivation of MTOR allows the dissociation of the MTORC1 complex, leading to ULK1 activation, phosphorylation of ATG13 and FIP200, and consequently the induction of autophagy. The MTORC2 complex is less rapamycin-sensitive.<sup>22,23</sup>

And CR also stimulates autophagy through the activation of the NAD<sup>+</sup>-dependent deacetylases, called sirtuins. SIRT1 can directly deacetylate several essential autophagic modulators, such as ATG5, ATG7 and ATG8, thereby inducing autophagy.<sup>24</sup> SIRT1-mediated deacetylation of FOXO1 induces expression of Rab7 which in turn stimulate autophagy.<sup>25,26</sup>

Metformin has been reported to mimic the benefits associated with caloric restriction.<sup>27,28</sup> In addition, metformin is well-established to activate both PRKA and the NAD<sup>+</sup>-dependent deacetylase SIRT1.<sup>29</sup> Based on these reports, we aimed to elucidate the mechanism by which metformin alleviates NAFLD by investigating the molecular interplay between PRKA, SIRT1, and autophagy.

## **II. MATERIALS AND METHODS**

### **1. Animal procedure**

Eight-week-old ob/ob mice (C57bl/6j background, Jackson Laboratory, 000632) and C57bl/6j control mice (Jackson Laboratory, Bar Harbor, ME, USA) were used in this study. ob/ob mice were separated into 3 groups and subjected to the following treatment regimens: 1) ad libitum feeding of chow diets (Dyets Inc., Bethlehem, PA, USA) and vehicle injection of phosphate-buffered saline (PBS, pH 7.8; GIBCO, Carlsbad, CA, USA, n=8); 2) ad libitum feeding of chow diets and intraperitoneal injections of 300 mg/kg metformin (Merck KGaA, Darmstadt, Germany, n=8); and 3) CR group (3 g/day, n=8). Mice were maintained at  $60 \pm 5$  % relative humidity and  $22 \pm 2$  °C, with a 12-h light/dark cycle. Mouse weight and food intake were measured daily over a 4-week course of treatment. All experimental procedures performed in this study followed ethical guidelines for animal studies, and were approved by the Institutional Animal Care and Use Committee of Yonsei University College of Medicine (IACUC No. 2011-0302-1).

### **2. Leupeptin assay for measuring autophagic flux**

Mice were intraperitoneally injected with sterile PBS or PBS containing 25 mg/kg leupeptin hemisulfate (Sigma, St. Louis, MO, USA). After injection, mice were returned to their cages and provided water without any food for 5 h.<sup>30</sup>

### **3. Metabolic and biochemical analyses**

For IPGTTs, 3-week-old mice were fasted overnight and given an

intraperitoneal injection of glucose (2 mg/g body weight; Sigma, St. Louis, MO, USA). Blood was collected from the tail vein, and glucose concentrations were measured at 0, 15, 30, 60, 90, and 120 min using a handheld glucose meter (SD Code Free, Standard Diagnostics, Inc., Gyeonggido, Korea). After sacrificing the mice at 4 weeks, blood was obtained by cardiac puncture, and mouse livers were removed and weighed. Serum levels of GOT1/AST (Asan Pharm Inc., Seoul, Korea), GPT/ALT (Asan Pharm Inc., Seoul, Korea), and T-CHO were quantified using a commercial kit (Asan Pharm Inc., Seoul, Korea).

#### **4. Histological analysis**

After each animal was sacrificed, fresh tissues were immediately frozen and subsequently placed in prelabeled base molds filled with embedding medium to ensure optimal cutting temperature. Frozen sections (7  $\mu$ M) were stained with ORO (Sigma, St. Louis, MO, USA); paraffin-embedded sections were stained with H&E (Dako, Carpinteria, CA, USA). To evaluate hepatic steatosis, morphometric analysis was performed on 2 randomly selected fields (at 40 $\times$  magnification) from a section of each animal using an Olympus IX71 microscope equipped with an Olympus DP70 camera (Olympus Optical Company, Tokyo, Japan). Immunohistochemical analysis for the expression of SIRT1 was conducted as previously described.<sup>31</sup> After epitope retrieval by heat in citrate, deparaffinized sections were incubated with anti-SIRT1 antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) for 1 h, rinsed with Tris-buffered saline (Sigma, St. Louis, MO, USA) containing 0.1% Tween-20 (Sigma, St. Louis, MO, USA), and incubated with a secondary antibody (Vector Laboratories, Inc., Burlingame, CA, USA). After washing in PBS, sections were detected with the

horseradish peroxidase–conjugated anti-biotin labeling solution (Vector Laboratories, Inc., Burlingame, CA, USA). Counterstaining was then performed before examination under a light microscope. The sections were mounted and dehydrated permanently in nonaqueous mounting medium. The slides were photographed at 20× and 40× magnification) using an Olympus IX71 microscope equipped with an Olympus DP70 camera.

## 5. Real-Time PCR

Total RNA was isolated using TRIZOL reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions and subjected to reverse transcription followed by quantitative real-time PCRs using SuperScript III First-strand kit (Invitrogen, Carlsbad, CA, USA). cDNA from liver from ob/ob mice was used for real-time PCR with SYBR Green (Takara, Shiga, Japan) and run on an StepOnePlus™ (Life technologies, Carlsbad, CA, USA) to detect relative mRNA levels. The sequences of the primers used for mouse samples were: *Acadm*, forward primer 5'-TGA CGG AGC AGC CAA TGA-3' and reverse primer 5'-TCG TCA CCC TTC TTC TCT GCT T-3'; *Sirt1*, forward primer 5'-ACC TCC CAG ACC CTC AAG-3' and reverse primer 5'- TTC CTT CCT TAT CTG ACA AAG C-3'; *Sqstm1*, forward primer 5'- GCT GCC CTA TAC CCA CAT CT-3' and reverse primer 5'- CGC CTT CAT CCG AGA AAC-3'; *Actb*, forward primer 5'- TGG CGC TTT TGA CTC AGG AT -3' and reverse primer 5'- GGG ATG TTT GCT CCA ACC AA -3'. The sequences of the primers used for HepG2 cells were: *SQSTM1*, forward primer 5'- CAG GGC TGA AGG AAG CTG C-3' and reverse primer 5'-AAG CCC ATG GAC AGC ATC TG-3'; *ACTb*, forward primer 5'- TCA TGA AGT GTG ACG TTG ACA TCC GT -3'

and reverse primer 5'- CCT AGA AGC ATT TGC GGT GCA CGA TG -3'. Quantitative analyses were performed using the  $\Delta\Delta$ cycle threshold method and StepOne Software version 2.2.2.

## **6. Isolation of primary hepatocyte and cell culture**

Primary mouse hepatocytes were prepared according to the two-step perfusion method as previously described<sup>32</sup> with minor modifications. Livers were digested with Hanks' balanced salt solution containing 30  $\mu$ g/mL of Liberase Blendzyme 3 (Roche Diagnostics, Indianapolis, IN, USA), 5 mM CaCl<sub>2</sub> (Sigma, St. Louis, MO, USA), 0.1 mg/mL DNaseI (Roche Diagnostics, Indianapolis, IN, USA), and 100 mM HEPES (Sigma, St. Louis, MO, USA) for 5 min. After centrifugation, pellets were resuspended in growth medium consisting of DMEM (Welgene, Daegu, Korea) supplemented with 2.7 mM D-glucose (Sigma, St. Louis, MO, USA) and 10% fetal bovine serum and subsequently incubated at 37 °C in humidified air containing 5% CO<sub>2</sub>. After determining cell viability via trypan blue exclusion testing, hepatocytes were seeded on collagen-coated 6-well plates (SPL, Gyeonggido, Korea;  $5 \times 10^5$ /well) and incubated for 24 h prior to all experiments. HepG2 cells were maintained in Dulbecco's modified Eagle's medium (Welgene, Daegu, Korea) supplemented with 10% fetal bovine serum and antibiotics. For pharmacological studies, cells were incubated with either control (5.5 mM) or high-glucose (30 mM) medium in the presence of 1 mM oleic acid (Sigma, St. Louis, MO, USA) after pretreatment with 0.5 mM metformin for 2 h.

## **7. Quantification of lipids by oil red o staining and triglyceride assay**

Lipid droplets were initially visualized and subsequently quantified by ORO staining. Primary mouse hepatocytes and HepG2 cells were exposed to 2 mM and 1 mM OA, respectively, in the presence of 30 mM glucose after pretreatment with or without 0.5 mM metformin, followed by fixation with 10% formalin for 1 h. Fixed cells were then stained in prewarmed ORO solution (Sigma, St. Louis, MO, USA) for 1 h in a 60 °C water bath, and red-stained lipid droplets were subsequently observed with a light microscope. To quantify lipid accumulation, ORO was eluted with 100% isopropanol and the optical densities of the eluates were measured using a spectrophotometer at 520 nm. TG levels in liver tissue, primary mouse hepatocytes, and HepG2 cells were measured with the Triglycerides Quantification Kit (Biovision, Mountain view, CA, USA) according to the manufacturer's instructions.

## **8. Plasmid transfection and sirt1 activity assay**

The plasmid construct driving overexpression of SIRT1 was kindly provided by Professor Hyun-seok Kim (Ewha Womans University, Seoul, Republic of Korea). Transient transfections of plasmid DNA were performed using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. To measure cellular SIRT1 activity, nuclear proteins were extracted from mouse livers, primary hepatocytes, and HepG2 cells using a Nuclear/Cytosol Fractionation Kit (Pierce Biotechnology, Rockford, IL, USA). SIRT1 activity was quantified with a SIRT1 Fluorometric Assay Kit (Sigma, St. Louis, MO, USA) according to the manufacturer's protocol. Fluorescence intensities were measured with a microplate fluorometer (excitation wavelength = 360 nm, emission wavelength = 450 nm).

## **9. RNA interference**

Primary hepatocytes and HepG2 cells were transfected with siRNAs targeting *ATG5* (Sigma, St. Louis, MO, USA), *SIRT1* (Sigma, St. Louis, MO, USA), *PRKAA1/2* (Santa Cruz Biotechnology, Santa Cruz, CA, USA), or a control siRNA (Sigma, St. Louis, MO, USA) using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) by following the manufacturer's instructions. Cells were incubated for 16 h with a transfection mixture containing a final siRNA concentration of 100 pM, and then supplemented with fresh medium.

## **10. Immunoblotting and antibodies**

Mouse livers, primary hepatocytes and HepG2 cells were lysed in PRO-PREPTM protein extraction solution (iNtRON Biotechnology, 17081), and the protein contents of the resulting lysates were measured with the Bradford assay (Bio-Rad, Hercules, CA, USA). Equal amounts of proteins were resolved by SDS-PAGE and electroblotted onto a nitrocellulose membrane (Bio-Rad, Hercules, CA, USA). Membranes were subsequently probed as indicated with the following primary antibodies: LC3B (Cell Signaling Technology, Danvers, MA, USA), PRKA (Cell Signaling Technology, Danvers, MA, USA), pPRKA (Cell Signaling Technology, Danvers, MA, USA), pACAC (Cell Signaling Technology, Danvers, MA, USA), SIRT1 (Santa Cruz Biotechnology, Santa Cruz, CA, USA) and GAPDH (Santa Cruz Biotechnology, Santa Cruz, CA, USA).

## **11. Detection of autophagy**

To monitor autophagy, levels of conversion of the autophagy marker protein

MAP1LC3/LC3 (microtubule-associated protein 1 light chain 3)-I to LC3-II were measured by immunoblotting. Detection of autophagosomes was also performed by transfecting HepG2 cells with a plasmid driving the expression of GFP-LC3 (ORIGINE Technologies, Inc., Rockville, MD, USA). Transfected cells were fixed with 4% paraformaldehyde and then observed with a confocal microscope. Cells were classified as having either a predominantly diffuse GFP stain, or as having numerous punctate structures representing autophagosomes. To assay autophagic flux, LC3 processing either in the presence or absence of 50  $\mu$ M of the lysosomal alkalinizing agent, CQ (Sigma, St. Louis, MO, USA), was assayed by immunoblotting. For detection of autophagosomes using electron microscopy, liver tissues of ob/ob mice were fixed with 2% glutaraldehyde-2% paraformaldehyde buffered with 0.1 M phosphate buffer (pH 7.2) overnight at 4 °C, post-fixed with 1% osmium tetroxide in a 0.1 M sodium cacodylate buffer (pH 7.4) for 1 h at room temperature, and were dehydrated with a graded series of ethanol as previously described.<sup>8</sup> The dehydrated tissues were then embedded in Epon, poly/Bed812 Embedding kit/DMP-30 (Polysciences, Warrington, PA, USA). Sectioning for electron microscopy examination was accomplished with an ultramicrotome (Leica, Wetzlar, Germany), and electron microscopy was performed with a JEM-1011 transmission electron microscope (JEOL, Peabody, MA, USA). Autophagic vacuoles were counted from at least 20 random cells, averaged and expressed as the number of autophagic vacuoles per cell.

## **12. Statistical analysis**

Statistical analysis was performed using PRISM (GraphPad Software Inc., San

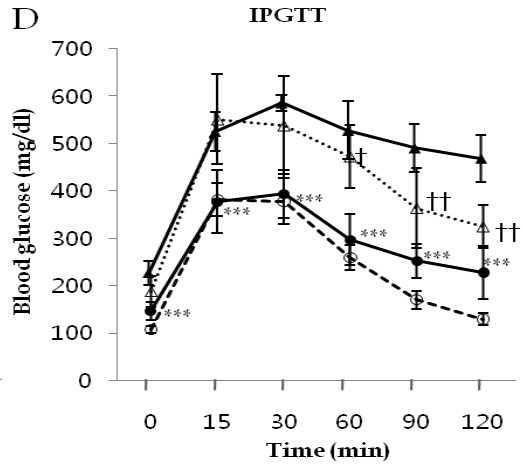
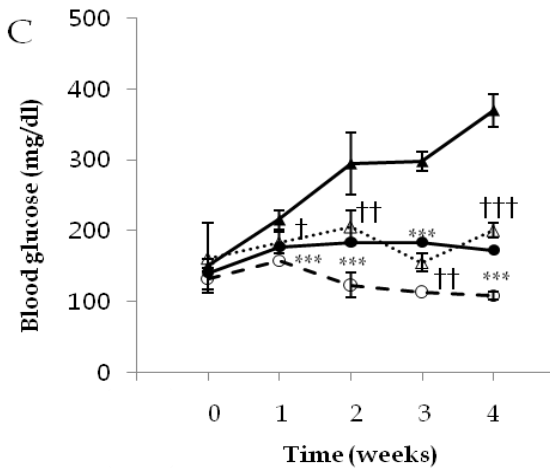
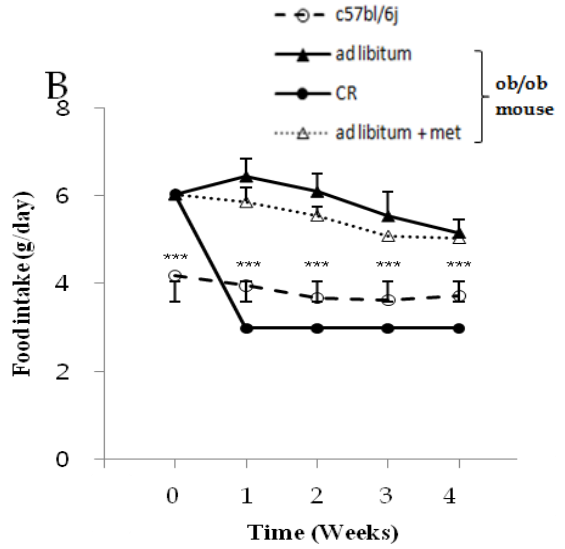
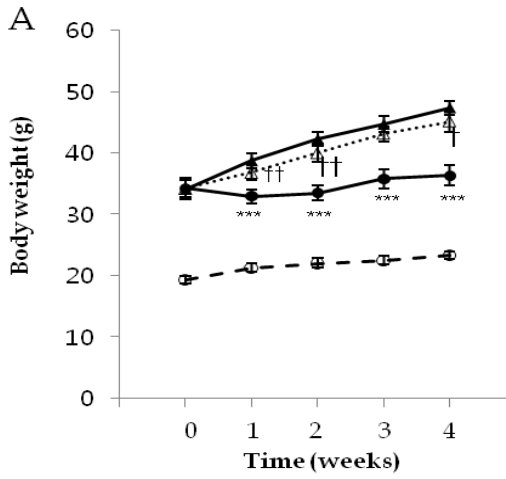


diego, CA, USA). Results are expressed as means  $\pm$  SE, and statistical significance was calculated using Student's t-test. Alternatively, for comparisons involving more than 2 groups, one-way analysis of variance (ANOVA) with a post hoc Bonferroni multiple comparison test was used to assess the differences between the groups. Differences in liver weights among 4 mice groups were compared by using analysis of covariance (ANCOVA) with adjustment for body weights. Statistical significance was defined as the conventional p value of  $<0.05$ .

### III. RESULTS

#### 1. Metformin and caloric restriction decrease body weight and blood glucose in ob/ob mice

To confirm the reported beneficial metabolic phenotypes of metformin treatment and CR *in vivo*, body weight and gluco-metabolic parameters were assessed in C57bl/6j control and ob/ob mice through weekly fasted blood glucose measurements and intraperitoneal glucose tolerance tests (IPGTTs). The ob/ob mouse, an animal model of obesity and type 2 diabetes, eats excessively and expresses mutation in the gene encoding leptin, which is important in the control of appetite. Despite increased levels of insulin, ob/ob mice develop high blood glucose.<sup>33</sup> Whole body weights of ob/ob mice treated with 3 g/day CR ( $p < 0.001$  at 1, 2, 3, and 4 weeks) and 300 mg/kg metformin ( $p < 0.01$  at 1 and 2 weeks,  $p < 0.05$  at 4 weeks) were significantly lower than those fed ad libitum (**Fig. 1A**). Except for ob/ob mice treated with CR (all  $p < 0.001$ ), food intakes were not different among ob/ob mice (**Fig. 1B**). As shown in **Fig. 1C**, blood glucose levels in the fasting condition were significantly decreased throughout the entire experiment by metformin treatment ( $p < 0.05$  at 1 week;  $p < 0.01$  at 2, 3 weeks;  $p < 0.001$  at 4 weeks) and CR treatment ( $p < 0.001$ ) compared with ad libitum-fed mice, whose blood glucose levels ranged from 300 to 400 mg/dL at 2 weeks post-ad libitum. To investigate the effect of metformin treatment and CR on glucose tolerance, we performed 2 g/kg body weight IPGTTs at 3 weeks (**Fig. 1D**). Compared to ad libitum-fed mice, tolerances to glucose loading were significantly improved in CR-treated mice at all-time points tested ( $p < 0.001$ ) and in metformin-treated mice at 60, 90, and 120 min ( $p < 0.05$ ,  $p < 0.01$ , and  $p < 0.01$ , respectively).



ob/ob

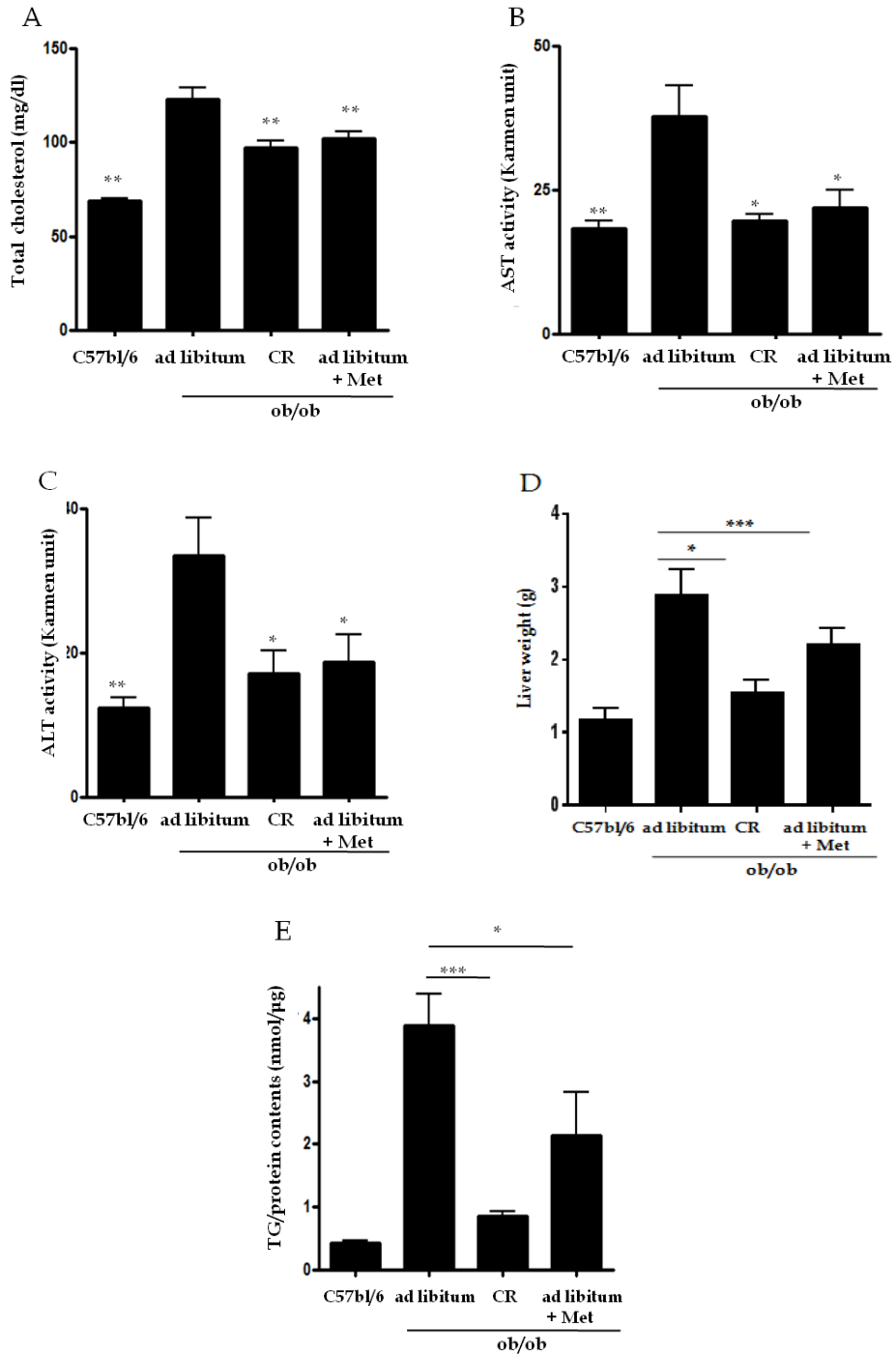
| Parameter                     | C57bl/6j     | ad libitum    | CR            | ad libitum + met |
|-------------------------------|--------------|---------------|---------------|------------------|
| Body weight (g)               | 23.4 (0.23)  | 47.11 (0.35)  | 36.36 (0.47)  | 45.31 (0.49)     |
| Food intake (g/day)           | 3.67 (0.03)  | 3 (0)         | 4.98 (0.13)   | 5.15 (0.12)      |
| Fasting blood glucose (mg/dl) | 108.3 (2.49) | 369.5 (23.78) | 176.8 (5.34)  | 200.3 (4.04)     |
| IPGTT (mg/dl)                 | 130.1 (4.86) | 473.1 (45.20) | 289.7 (21.07) | 325.1 (19.09)    |

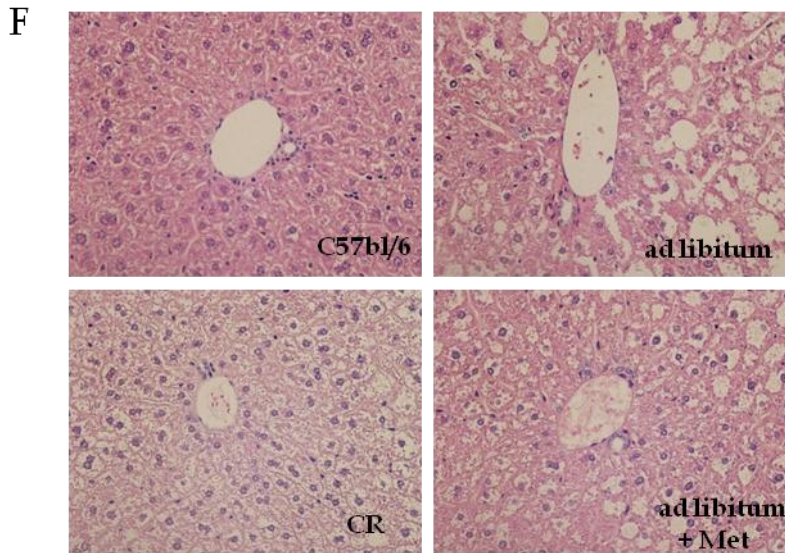
**Figure 1. Metabolic effects of metformin and caloric restriction on ob/ob mice.**

(A) The effects of metformin and CR on body weight, (B) food intake, and (C) blood glucose in the 8-week-old C57bl/6j control (○) and ob/ob mice were evaluated. The latter mice were classified into 3 groups: ad libitum feeding of chow diets (▲), 300 mg/kg intraperitoneal metformin (Δ), and 3 g/day CR (●). (A) During a 4-week treatment course of either metformin or CR, body weight significantly decreased compared to ad libitum-fed ob/ob mice. (B) Due to the protocol of this study, food intake was only reduced in calorie-restricted mice. (C) Blood glucose levels were significantly decreased throughout the entire 4 weeks in ob/ob mice treated either with metformin or CR, compared to ad libitum-fed ob/ob mice. (D) At 3 weeks, glucose tolerance tests were performed after an intraperitoneal injection of glucose (2 g/g body weight). Blood was collected from the tail vein, and glucose concentrations were measured at 0, 15, 30, 60, 90, and 120 min. CR significantly improved glucose tolerance throughout the IPGTTs, but metformin only significantly improved glucose tolerance at 60, 90, and 120 min. Values displayed are means ± SEM of 8 mice per group. \*\*\* p < 0.001, CR vs. ad libitum-fed ob/ob mice; †p < 0.05, ††p < 0.01, and †††p < 0.001, metformin vs. ad libitum-fed ob/ob mice.

## 2. Metformin and caloric restriction alleviate liver dysfunction and hepatic lipid accumulation in ob/ob mice

To examine whether metformin and CR alleviated hepatic fat accumulation *in vivo*, serum levels for total cholesterol (T-CHO), GOT1/AST (glutamic-oxaloacetic transaminase 1, soluble), and GPT/ALT, [glutamic-pyruvate transaminase (alanine aminotransferase)], as well as triglyceride (TG) contents and hepatic histology were evaluated in C57bl/6j control and ob/ob mice. Serum levels of T-CHO were significantly reduced in both the CR- ( $97.3 \pm 10.4$ ,  $p < 0.01$ ) and metformin-treated groups ( $101.9 \pm 8.9$ ,  $p < 0.01$ ) compared to the ad libitum-fed group ( $122.8 \pm 15.6$  mg/dL, **Fig. 2A**). The elevated GOT1 and GPT serum levels of the ad libitum-fed group ( $37.74 \pm 5.49$  and  $33.53 \pm 5.15$ , respectively) were significantly lowered by treatment with CR ( $19.54 \pm 1.28$  and  $17.09 \pm 3.26$ , both  $p < 0.05$ ) and metformin ( $21.95 \pm 3.07$  and  $18.74 \pm 3.82$ , both  $p < 0.05$ , respectively; **Fig. 2B and C**). As shown in Fig. 2D, liver weights adjusted by body weights in the CR- ( $1.53 \pm 0.15$  g,  $p < 0.05$ ) and metformin-treated groups ( $2.20 \pm 0.16$  g,  $p < 0.001$ ) were significantly lower than those in the ad libitum-fed group ( $2.88 \pm 0.35$  g). Compared to the ad libitum-fed mice ( $3.89 \pm 0.49$  TG/protein nm/ $\mu$ g), CR- ( $0.85 \pm 0.06$ ,  $p < 0.001$ ) and metformin-treated mice ( $2.13 \pm 0.70$ ,  $p < 0.05$ ) showed a significant decrease in hepatic TG contents as assessed by a Quantification Colorimetric Kit (**Fig. 2E**). Liver sections were also stained with hematoxylin and eosin (H&E,  $\times 40$ ) to visualize lipid accumulation under all treatment conditions. Extensive macrovesicular steatosis surrounding the perisinusoidal areas was present in ad libitum-fed ob/ob mice. However, visible decreases in the accumulation of intracellular lipid droplets were observed in ob/ob mice upon treatment with both metformin and CR (**Fig. 2F**).





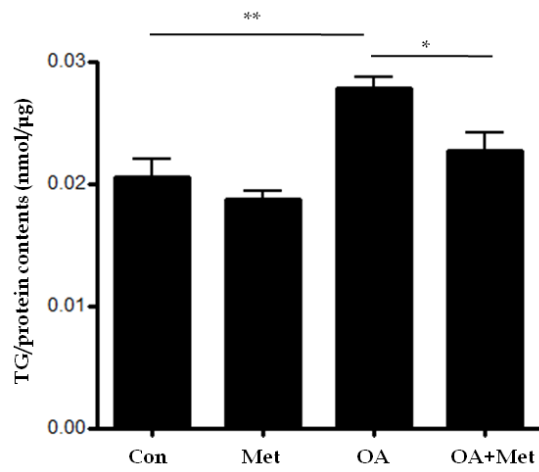
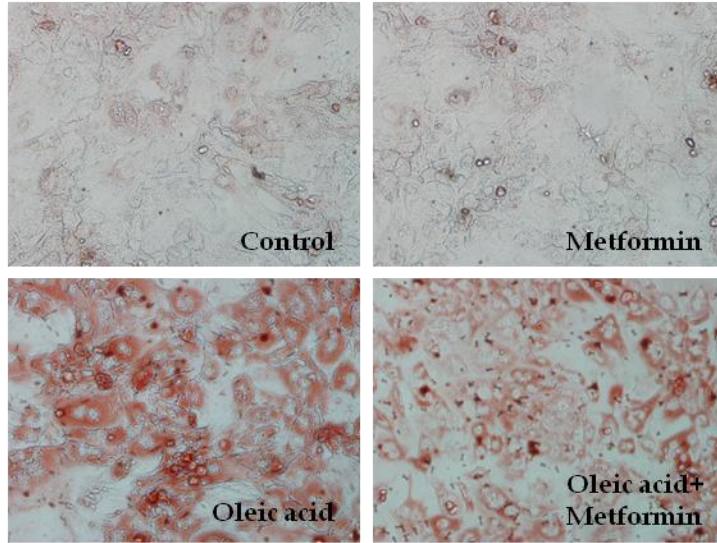
**Figure 2. Hepatic effects of metformin and caloric restriction on ob/ob mice.** (A-C) Serum chemistry values, (D-F) as well as TG contents and hepatic histology, were evaluated after 4 weeks of metformin administration or CR. (A-C) Levels of T-CHO, as well as GOT1 and GPT activity, were significantly decreased in ob/ob mice treated with metformin or CR compared to ad libitum-fed ob/ob mice. Mice treated with either metformin or CR also showed a significant decrease both in (D) liver weights adjusted by body weights and also in (E) hepatic fat accumulation compared to ad libitum-fed ob/ob mice. (F) Staining of liver sections with H&E (x 40) revealed extensive macrovesicular steatosis around the perisinusoidal area in ad libitum-fed ob/ob mice. Values displayed are means  $\pm$  SEM of 8 mice per group. Asterisks ( $*p < 0.05$ ,  $**p < 0.01$ ,  $***p < 0.001$ ) indicate significant differences compared to ad libitum-fed ob/ob mice.

### **3. Metformin reduces oleic acid-induced lipid accumulation in hepatocytes *in vitro***

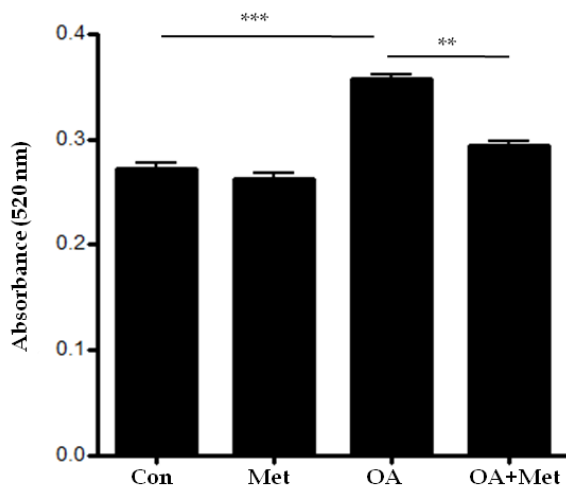
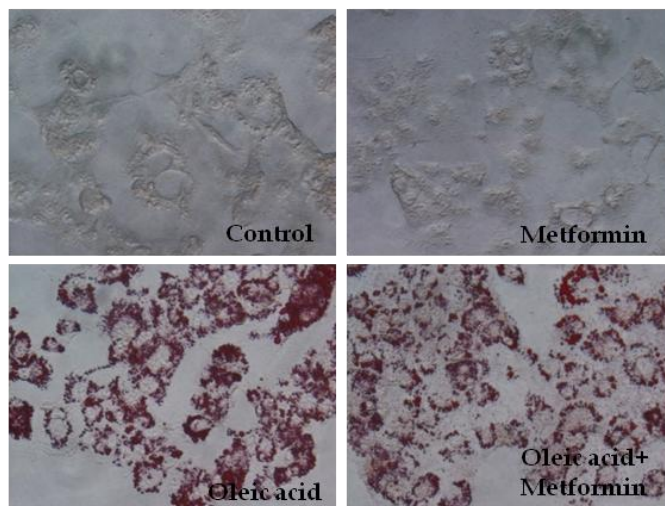
To confirm the lipid clearance effects of metformin on primary mouse hepatocytes *in vitro*, we stained lipid droplets with Oil Red O (ORO) and measured TG with Triglycerides Quantification Kit (**Fig. 3A**); furthermore, we also quantified TGs in HepG2 cells via spectrophotometry (**Fig. 3B**). As shown in Fig. 3, treatment with 2 mM OA for primary hepatocytes ( $0.021 \pm 0.004$  vs.  $0.028 \pm 0.002$ ,  $p < 0.01$ ) and 1 mM OA for HepG2 cells ( $0.27 \pm 0.01$  vs.  $0.36 \pm 0.005$ ,  $p < 0.001$ ) significantly increased intracellular lipid accumulation in the presence of 30 mM glucose for 8 h. However, pretreatment with 0.5 mM metformin significantly attenuated OA-induced lipid accumulation in both primary mouse hepatocytes ( $0.028 \pm 0.002$  vs.  $0.022 \pm 0.004$ ,  $p < 0.05$ ) and HepG2 cells ( $0.36 \pm 0.005$  vs.  $0.29 \pm 0.01$ ,  $p < 0.01$ ).



A



B



**Figure 3. Metformin reduces oleic acid-induced lipid accumulation in hepatocytes *in vitro*.** Treatment with OA (2.0 mM for primary hepatocytes, 1.0 mM for HepG2 cells) in combination with 30 mM glucose for 8 h significantly increased

intracellular lipid accumulation. In contrast, pretreatment with 0.5 mM metformin for 2 h before OA exposure significantly reduced OA-induced lipid accumulation in both primary (A) hepatocytes and (B) HepG2 cells, as assessed by visualization and quantification of ORO staining (x 40). Values displayed are means  $\pm$  SEM of 8 independent experiments. Asterisks (\*  $p < 0.05$ , \*\*  $p < 0.01$ , \*\*\*  $p < 0.001$ ) indicate significant differences.

#### **4. SIRT1 expression and induction of autophagy are suppressed in type 2 diabetic ob/ob mice, but are ameliorated by metformin**

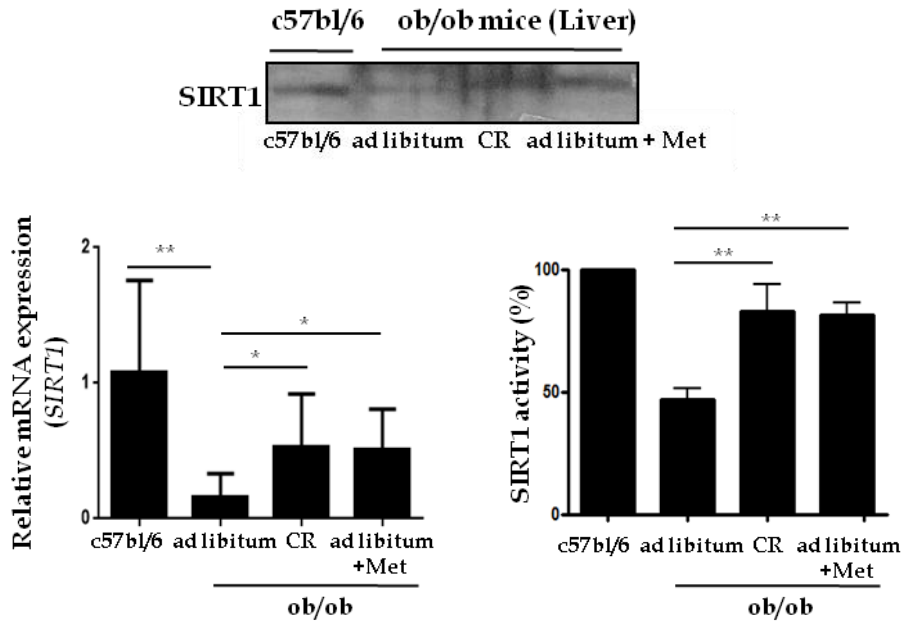
SIRT1 regulates autophagy by deacetylating autophagy machinery, including the autophagic players ATG5, ATG7, and LC3. To test the hypothesis that hepatic SIRT1 expression and autophagy induction are suppressed in type 2 diabetes, and that restoration of SIRT1 expression and autophagy induction can be achieved by treatment with metformin and CR in the ob/ob mouse model of type 2 diabetes, SIRT1 and autophagy induction were evaluated in this model. Compared to C57bl/6j mice, downregulated hepatic expression of SIRT1 protein as well as mRNA and SIRT1 activity were observed in C57bl/6j background ob/ob mice, while SIRT1 expression and activity were restored in ob/ob mice treated with a 4-week course of CR or metformin (**Fig. 4A**). With respect to autophagy, LC3-I to LC3-II conversion were significantly increased, while expression of SQSTM1 was decreased in ob/ob mice treated with a 4-week course of CR or metformin compared to those fed ad libitum (**Fig. 4B**).

To verify autophagic flux under the *in vivo* condition, leupeptin was administered into each mouse before sacrifice and the rate of LC3-II accumulation after leupeptin injection was calculated from immunoblots (**Fig. 4C**). Consistent with *in vitro* findings, this leupeptin-based assay demonstrated that leupeptin treatment significantly increased the amount of LC3-II accumulation (designated as LC3 net flux) in ob/ob mice treated with CR or metformin compared to ad libitum-fed mice.

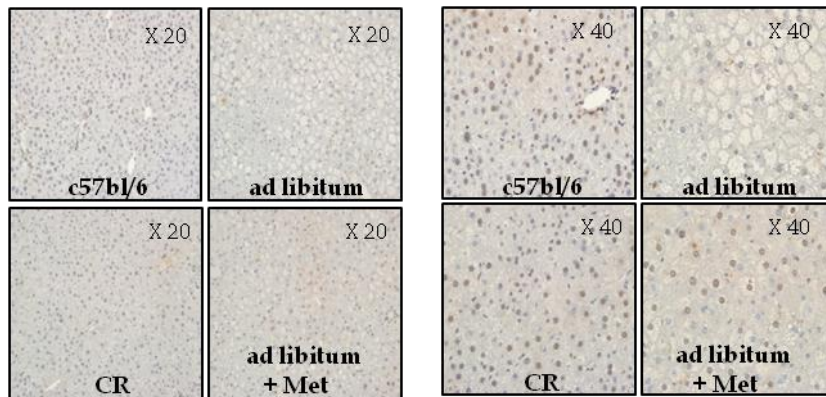
Furthermore, autophagosomes and autolysosomes were determined in the liver tissue of each group using an electron microscope to support the evidence of autophagy induction (**Fig. 4D**). Hepatocytes in ob/ob mice treated with a 4-week course of CR or

metformin had a significantly increased number of autophagic vacuoles compared with those fed ad libitum.

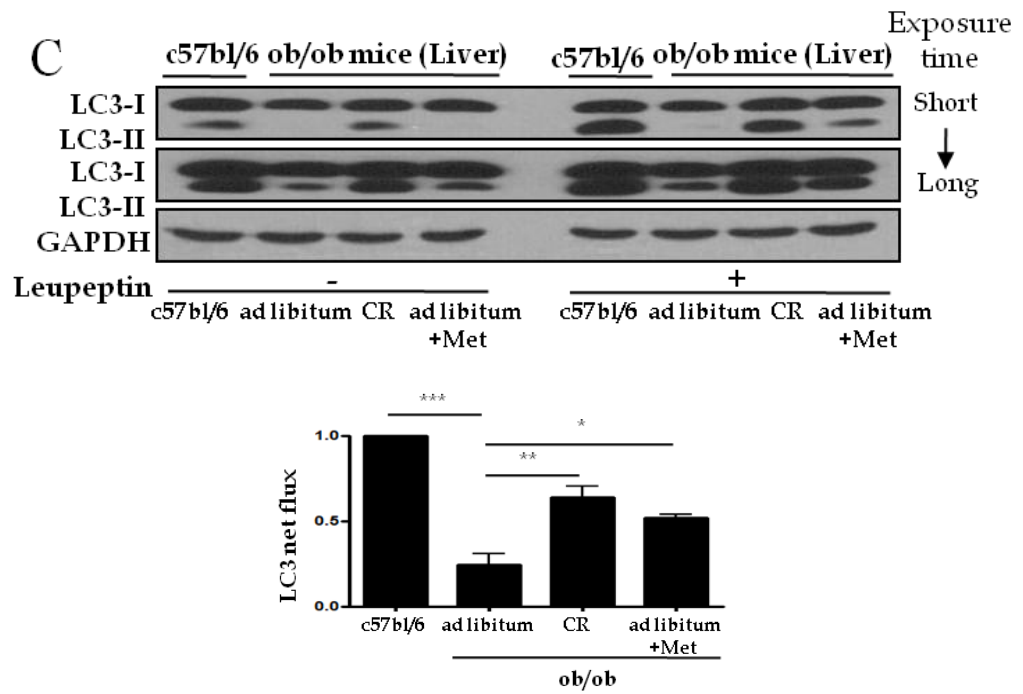
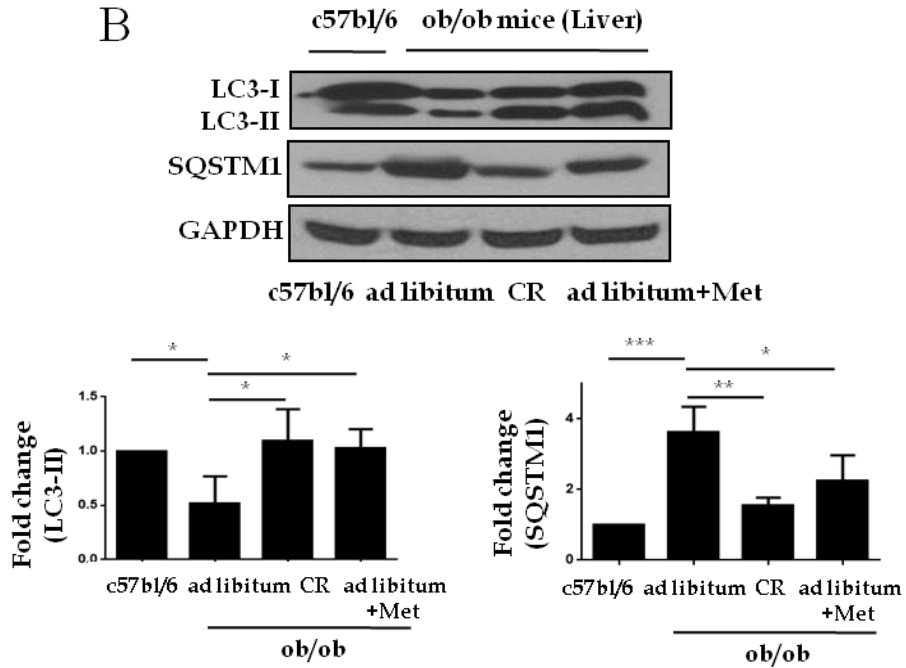
**A**

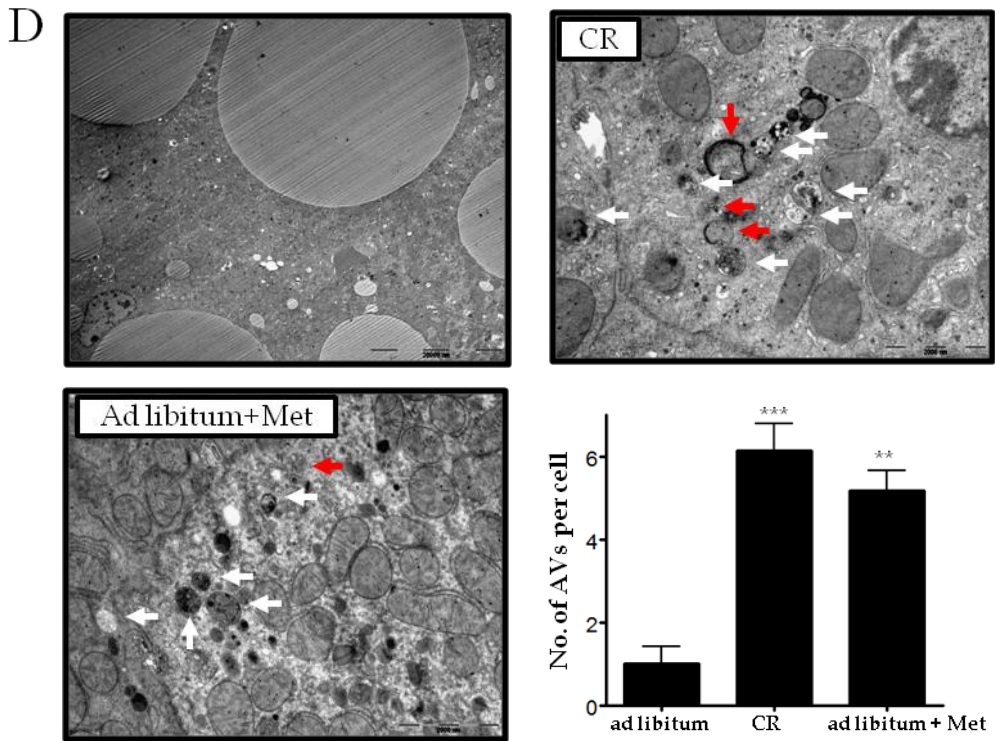


**Immunohistochemistry - SIRT1**



**B**





**Figure 4. SIRT1 expression and autophagy induction are decreased in ob/ob mice.**

(A) Immunoblots, SIRT1 activity assay and real time PCR and immunohistochemistry for SIRT1 were performed in mice liver tissues. Hepatic expression and activity of SIRT1 and induction of autophagy were significantly decreased in ad libitum-fed ob/ob mice, compared to C57bl/6j control mice. Furthermore, a 4-week treatment course of either metformin or CR significantly restored the expression and activity of SIRT1 and induction of autophagy in ob/ob mice. (B) Expression of LC3 and SQSTM1 was shown as a densitometric graph of the optical density-based data of immunoblots. (C) LC3 immunoblots were conducted in liver tissues from 4



experimental groups to analyze autophagy flux in vivo using a leupeptin-based assay. Autophagy flux was expressed as the subtraction of the amount of LC3-II in the absence of leupeptin from the amount of LC3-II in the presence of leupeptin for each of the conditions, (C, right graph) which is defined as “LC3 net flux” and graphically displayed. (D) Autophagosomes (red arrows) and autolysosomes (white arrows) were shown in the electron microscopy images from ad libitum-fed ob/ob mice and ob/ob mice treated with CR or metformin. Scale bars are indicated. (D, right graph) The numbers of autophagic vacuoles (autophagosomes and autolysosomes) per cell (n=20) were counted and graphically displayed. Values displayed are means  $\pm$  SEM of at least 5 independent experiments. Asterisks ( $* p < 0.05$ ,  $** p < 0.01$  and  $*** p < 0.001$ ) indicate significant differences.

## **5. Oleic acid suppresses hepatic expression of SIRT1 and autophagy induction, but metformin restores SIRT1 expression and activates the autophagy machinery**

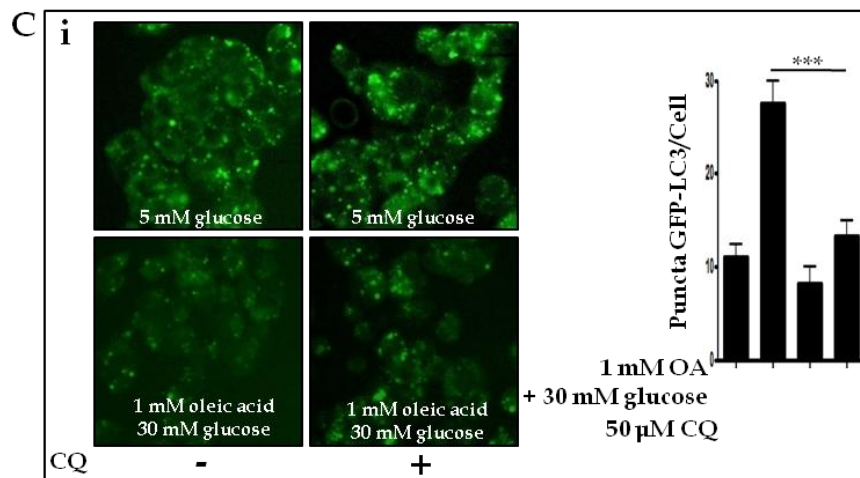
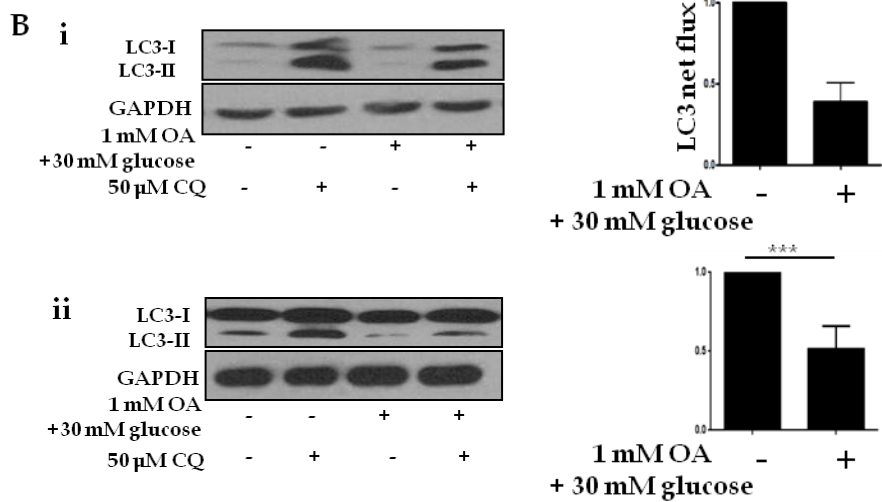
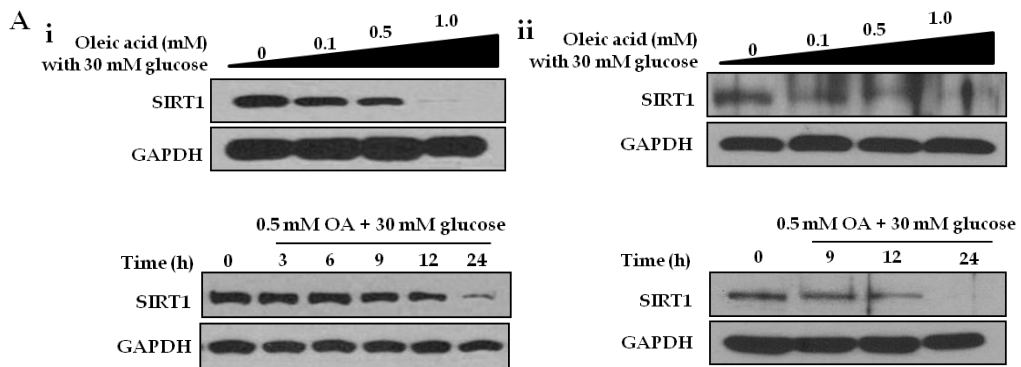
To test the possible mechanisms by which a dysfunctional autophagic pathway results in lipid accumulation, and functional autophagy mediates lipid clearance, the integrity of the autophagy machinery was assessed on several levels. First, we demonstrated OA-induced suppression of SIRT1 in both a dose- (0, 0.1, 0.5, 1.0 mM) and time- (0, 3, 6, 9, 12, 24 h) dependent manner in both HepG2 cells and primary hepatocytes (**Fig. 5Ai and Aii**). Second, we employed multiple techniques to demonstrate OA-induced dysfunctional autophagy *in vitro*. Dysfunctional autophagy was first validated with autophagic flux assays; as shown in Fig. 5B, 1.0 mM OA in the presence of 30 mM glucose suppressed conversion of LC3-I to LC3-II (designated as LC3 net flux), compared to the control group. Next, we employed fluorescence confocal microscopy to visualize autophagosome formation. HepG2 cells and primary hepatocytes ectopically expressing GFP-LC3 exhibited a decrease in the number of punctate GFP-LC3 structures upon exposure to 1.0 mM OA, either in the presence or absence of 50  $\mu$ M chloroquine (CQ; **Fig. 5Ci and Cii**).

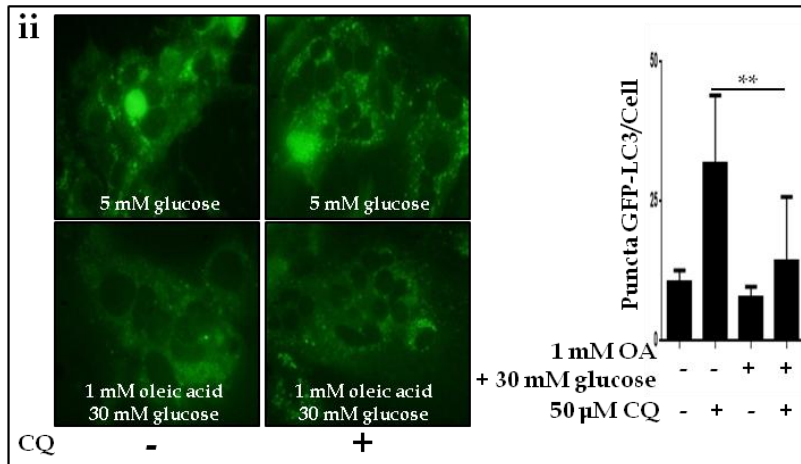
Then, we examined metformin-induced upregulation of SIRT1 expression and autophagy induction *in vitro*. As shown in Fig. 6A, HepG2 cells and primary hepatocytes exposed to different concentrations of metformin (0, 0.1, 0.5, 1.0 mM) showed significantly increased activity and expression of SIRT1, in a dose-dependent manner. Metformin also upregulated the expression of phosphorylated PRKA (pPRKA) and pACAC (phosphorylated acetyl-CoA carboxylase) in a dose-dependent manner (**Fig. 6A**). Consistent with these findings *in vitro*, both metformin and CR

significantly induced mRNA expression of the gene encoding ACADM (acyl-CoA dehydrogenase, C-4 to C-12 straight chain), one of the key enzyme in the  $\beta$ -oxidation pathway<sup>34</sup> in liver tissues compared with those fed ad libitum (**Fig. 6B**). In autophagy flux assays, increased conversion of LC3-I to LC3-II and reduction of SQSTM1 expression were observed in both metformin-treated HepG2 cells and primary hepatocytes (**Fig. 6C**). This increase in LC3 net flux and decrease in SQSTM1 expression occurred in a dose-dependent manner. In addition, electron microscopy analysis showed that the numbers of autophagosomes and autolysosomes were significantly increased after metformin treatment in hepatocytes (**Fig. 6D**).

Furthermore, pretreatment with 0.5 mM metformin prevented OA-induced suppression of SIRT1 expression and LC3-II conversion (**Fig. 7A**). An autophagic flux assay using CQ showed that the conversion of LC3-I to LC3-II was blocked in OA-treated hepatocytes, but was restored after metformin treatment. In addition, increased expression of SQSTM1 by OA was significantly reduced after metformin pretreatment. To test the hypothesis that metformin reduces lipid accumulation by inducing autophagy, we inhibited the induction of autophagy with either siRNA directed against *ATG5*, or a pharmacological inhibitor of autophagy, 3-methyladenine (3MA). Intracellular lipid accumulation in HepG2 cells and primary mouse hepatocytes was analyzed by using a spectrophotometer at 520 nm and a colorimetric quantification kit, respectively (**Fig. 7B-D**). While 1.0 mM OA significantly increased the lipid contents of HepG2 cells ( $0.17 \pm 0.003$  vs.  $0.37 \pm 0.01$ ,  $p < 0.001$ ), 0.5 mM metformin significantly decreased intracellular fat loading ( $0.37 \pm 0.01$  vs.  $0.34 \pm 0.01$ ,  $p < 0.05$ ). However, after siRNA-mediated knockdown of *ATG5*, metformin no longer attenuated OA-induced lipid accumulation ( $0.39 \pm 0.01$  vs.  $0.37 \pm 0.003$ ,  $p = 0.15$ ; **Fig.**

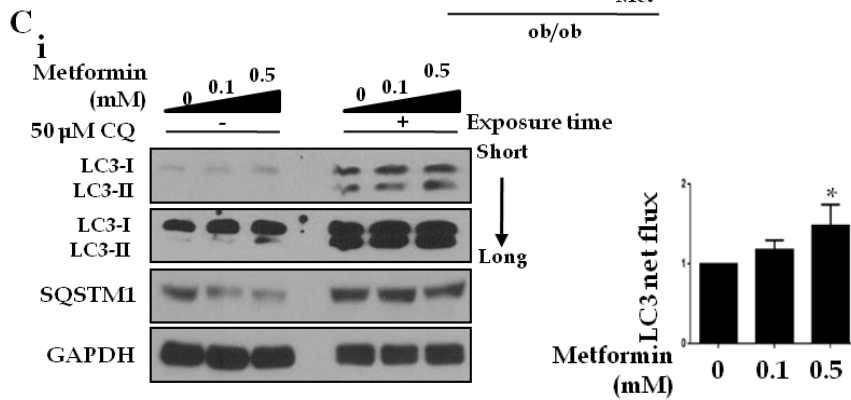
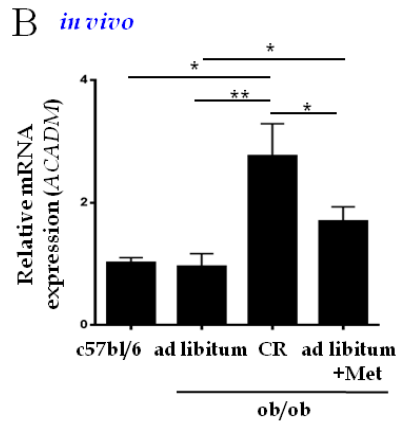
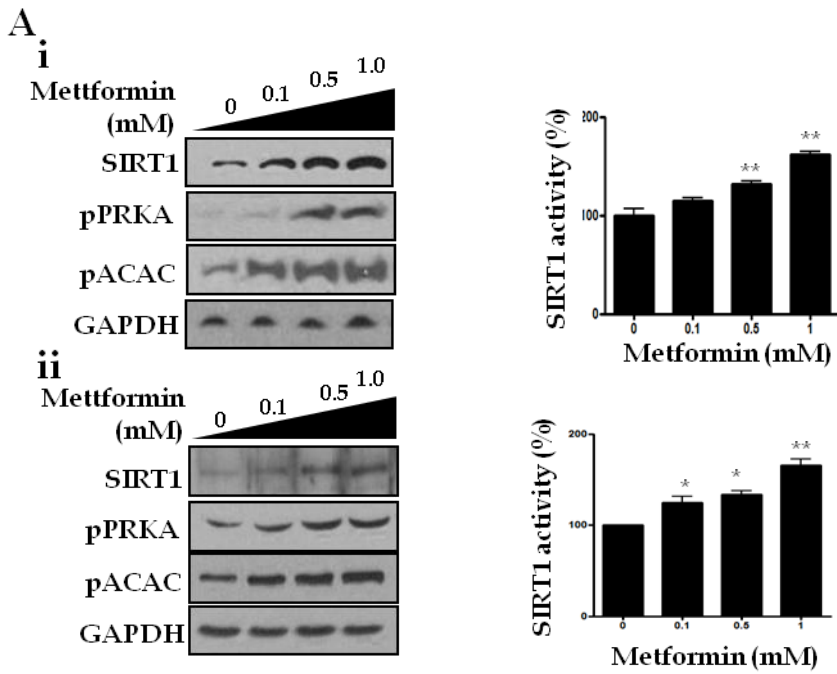
**7B**). Similarly, metformin was also unable to attenuate OA-induced lipid accumulation in the presence of 5 mM 3MA in both HepG2 cells ( $0.11 \pm 0.003$  vs.  $0.12 \pm 0.002$ ,  $p=0.08$ ; **Fig. 7C**) and primary mouse hepatocytes ( $0.029 \pm 0.001$  vs.  $0.03 \pm 0.001$ ,  $p=0.61$ ; **Fig. 7D**).



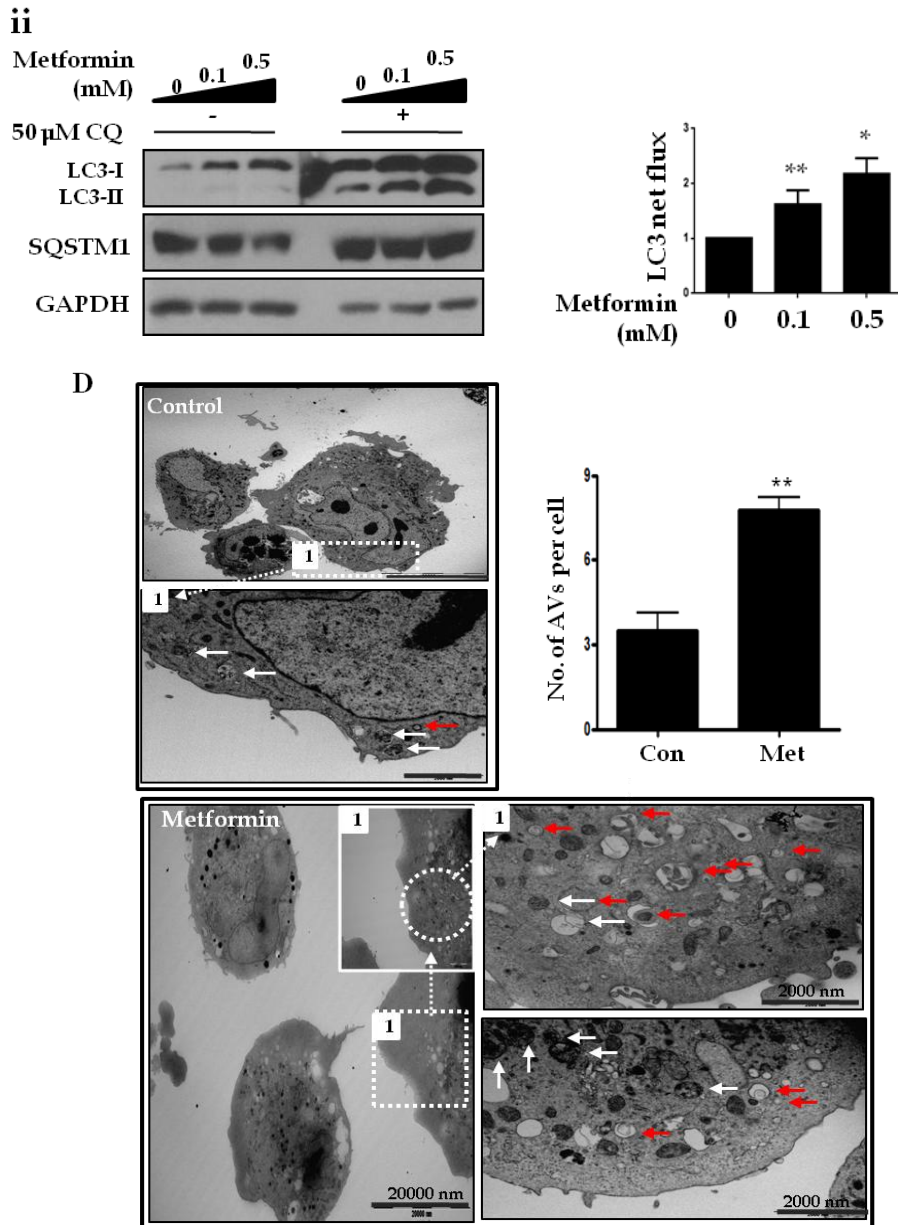


**Figure 5. Oleic acid downregulates hepatic expression of SIRT1 and suppresses autophagy machinery.** (Ai and Aii designated as HepG2 cells and primary hepatocytes, respectively) HepG2 cells and primary hepatocytes exposed to OA in combination with 30 mM glucose exhibited decreased levels of SIRT1, as assessed by immunoblots, in a dose- (0, 0.1, 0.5 and 1.0 mM OA) and time- (0, 3, 6, 9, 12, and 24 h) dependent manner. (Bi and Bii) Furthermore, treatment with the lysosomal inhibitor CQ (50  $\mu$ M) in combination with 1.0 mM OA and 30 mM glucose decreased LC3-II conversion, whereas CQ alone upregulated LC3-II levels in LC3 immunoblot flux assays. Autophagy flux was expressed as the subtraction of the amount of LC3-II in the absence of CQ from the amount of LC3-II in the presence of CQ for each of the conditions, which is defined as “LC3 net flux” and graphically displayed. (Ci and Cii) Fluorescence confocal microscopy was used to monitor the vesicle formation step of autophagy and autophagosome-lysosome fusion in the absence or presence of 50  $\mu$ M

CQ. The number of GFP-LC3 puncta was quantified and plotted. Punctate patterns of the autophagic marker GFP-LC3 were decreased in response to autophagic inhibition by 1.0 mM OA. Asterisks (\*\*  $p < 0.01$  and \*\*\*  $p < 0.001$ ) indicate significant differences.

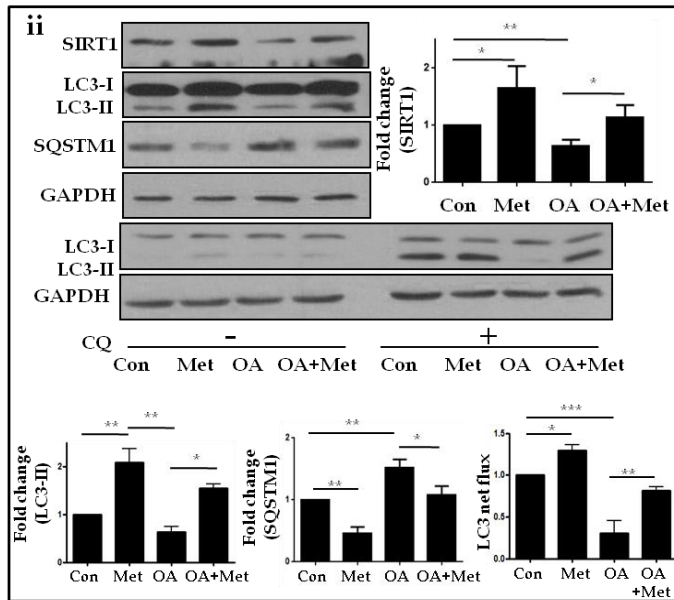
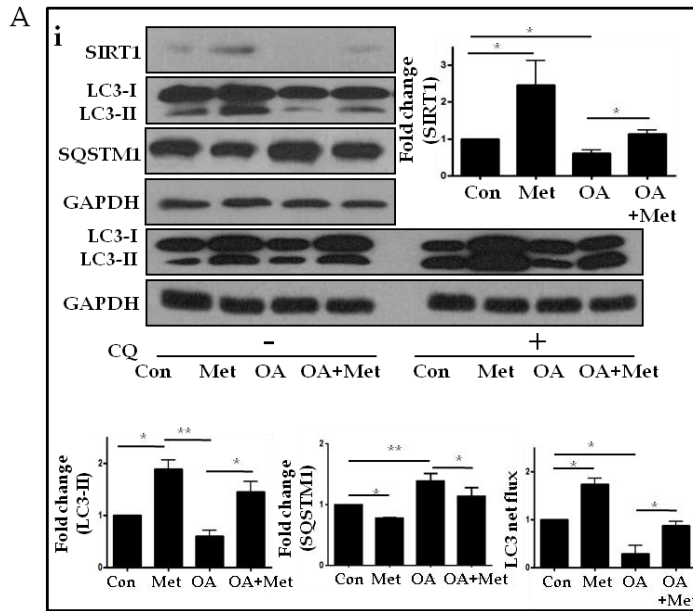


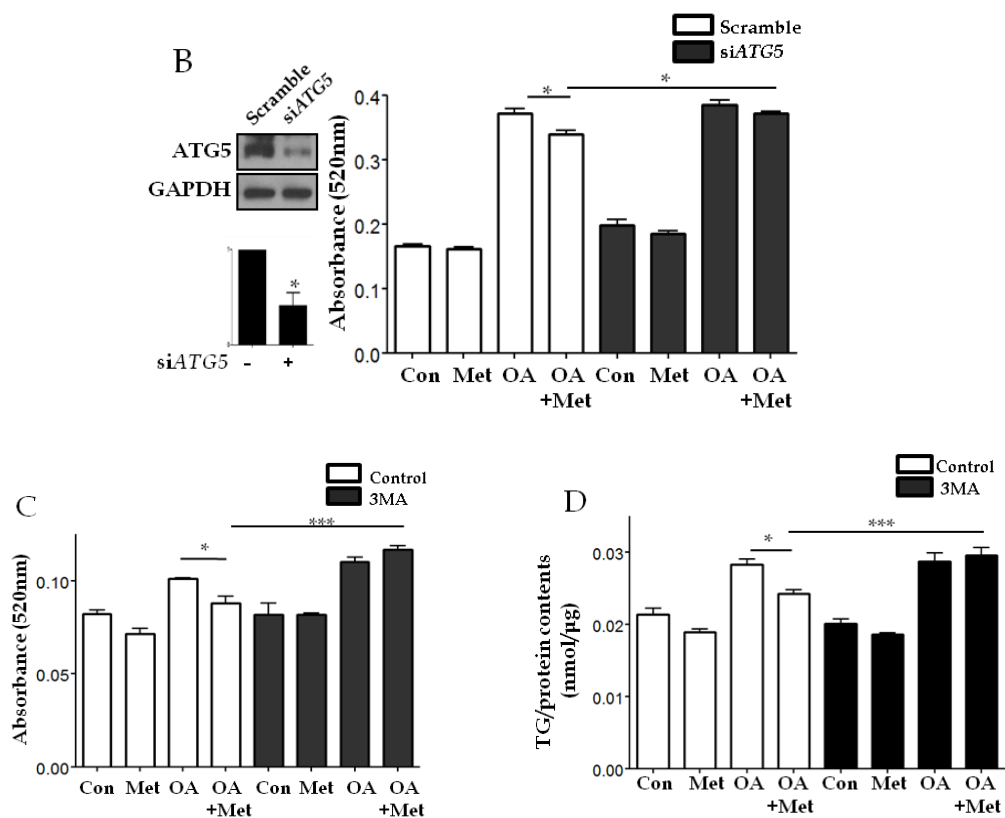




**Figure 6. Metformin upregulates SIRT1 and stimulates induction of autophagy.** Metformin upregulated the expression and activity of SIRT1, phosphorylated PRKA

(pPRKA), and phosphorylated ACAC (pACAC) in a dose-dependent manner, as shown by immunoblots from (Ai) HepG2 cells and (Aii) primary hepatocytes. Asterisks (\* $p < 0.05$  and \*\* $p < 0.01$ ) indicate significant differences compared to the no-metformin control. (B) Real-time RT-PCR of *Acadm* (acyl-CoA dehydrogenase, C-4 to C-12 straight chain) expression in mouse livers showed that CR and metformin treatment significantly increased the level of *Acadm*. (Ci and Cii) In LC3 flux assays, metformin increased LC3-II expression in a dose-dependent manner (0, 0.1, and 0.5 mM), and CQ (50  $\mu$ M) upregulated LC3-II conversion in HepG2 cells and primary hepatocytes, as assessed by immunoblots. Autophagy flux was expressed as the subtraction of the amount of LC3-II in the absence of CQ from the amount of LC3-II in the presence of CQ for each of the conditions, which is defined as “LC3 net flux” and graphically displayed. Expression of SQSTM1 was decreased in metformin-treated cells in a dose-dependent manner, while it increased after addition of CQ (50  $\mu$ M). (D) In transmission electron microscopy images, the number of autophagosomes (red arrows) and autolysosomes (white arrows) were significantly increased in primary hepatocytes treated with metformin compared with controls. Scale bars are indicated. (D, right graph) The numbers of autophagic vacuoles (autophagosomes and autolysosomes) per cell ( $n=20$ ) were counted and graphically displayed.



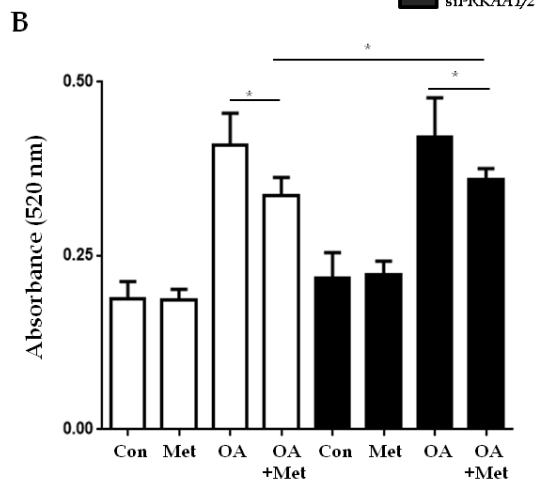
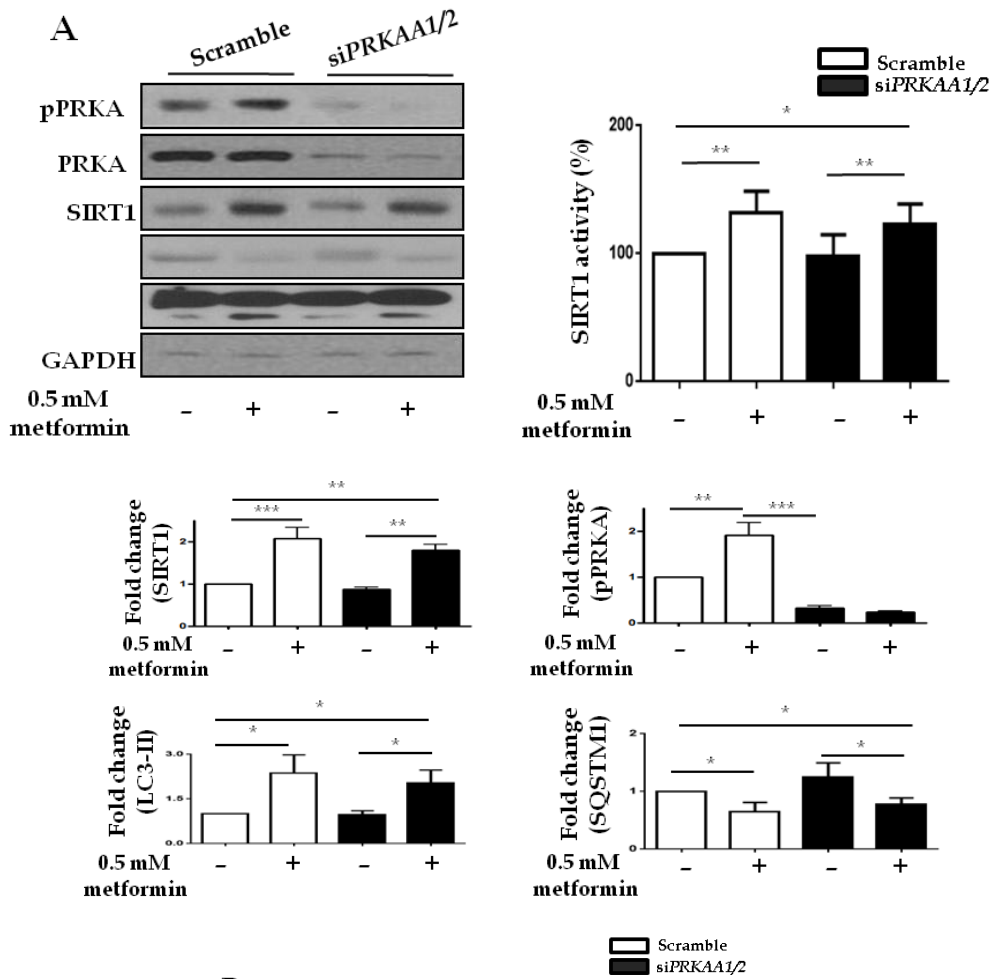


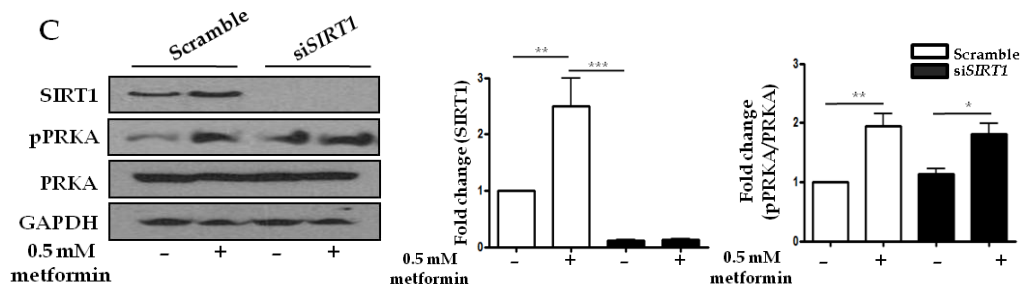
**Figure 7. Metformin reduced OA-induced lipids by autophagy induction.** (A) Metformin also inhibited OA-induced downregulation of SIRT1 expression and autophagy induction. (Ai) HepG2 cells and (Aii) primary hepatocytes exposed to 1.0 mM OA showed a significant decrease in SIRT1 expression and autophagy induction, whereas pretreatment with 0.5 mM metformin significantly reduced OA-induced autophagy dysfunction. Expression of indicated proteins was shown as a densitometric graph of the optical density-based data of immunoblots. Furthermore, LC3 immunoblot flux assays showed that treatment with the lysosomal inhibitor CQ (50  $\mu$ M) in combination with 1.0 mM OA alone decreased LC3-II conversion,

whereas treatment with 0.5 mM metformin in the presence of CQ increased LC3-II expression in both HepG2 cells and primary hepatocytes regardless of OA. Autophagy flux was expressed as the subtraction of the amount of LC3-II in the absence of CQ from the amount of LC3-II in the presence of CQ for each of the conditions, which is defined as “LC3 net flux” and graphically displayed. (B-D) Lipid accumulation was determined by fat quantification in the presence or absence of the autophagic inhibitor 3MA, and also after siRNA-mediated knockdown of *ATG5*. OA-treated primary mouse hepatocytes and HepG2 cells exhibited a significant increase in lipid accumulation. However, treatment with 0.5 mM metformin significantly decreased the OA-induced intracellular lipid accumulation. Furthermore, the lipid contents of both HepG2 cells transfected with (B) siRNA against *ATG5* and (C) HepG2 cells or (D) primary hepatocytes treated with 5 mM 3MA in the presence of metformin and OA were similar to those of cells treated with OA alone. Values displayed are means  $\pm$  SEM of 5 independent experiments. Asterisks (\*  $p < 0.05$ , \*\*  $p < 0.01$  and \*\*\*  $p < 0.001$ ) indicate significant differences.

## **6. Metformin activates SIRT1 expression through a PRKA-independent pathway**

To determine whether metformin induced SIRT1 expression through a PRKA-independent pathway, siRNAs directed against *PRKAA1/2* or *SIRT1* were transfected into HepG2 cells. Metformin upregulated SIRT1 activity as well as its expression, regardless of PRKA expression levels (**Fig. 8A**). With respect to autophagy, metformin significantly increased the level of LC3-II, while it decreased SQSTM1 expression in both PRKA-sufficient and -deficient conditions. In Fig. 8B, metformin treatment significantly reduced lipid accumulation in OA-treated HepG2 cells regardless of the presence of PRKA. Interestingly, the amount of lipid was much lower in OA and metformin co-treated cells transfected with control siRNA compared to those with *PRKAA1/2* siRNA ( $0.337 \pm 0.01$  vs.  $0.359 \pm 0.005$ ,  $p < 0.05$ ). After transfection with siRNA against *SIRT1*, *SIRT1* knockdown did not interfere with metformin-induced phosphorylation of PRKA (**Fig. 8C**).





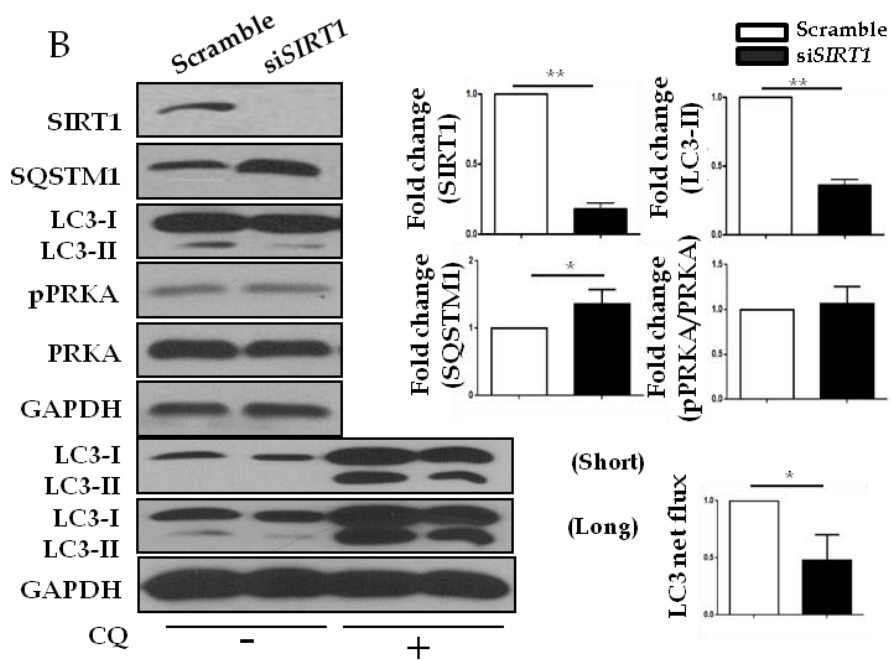
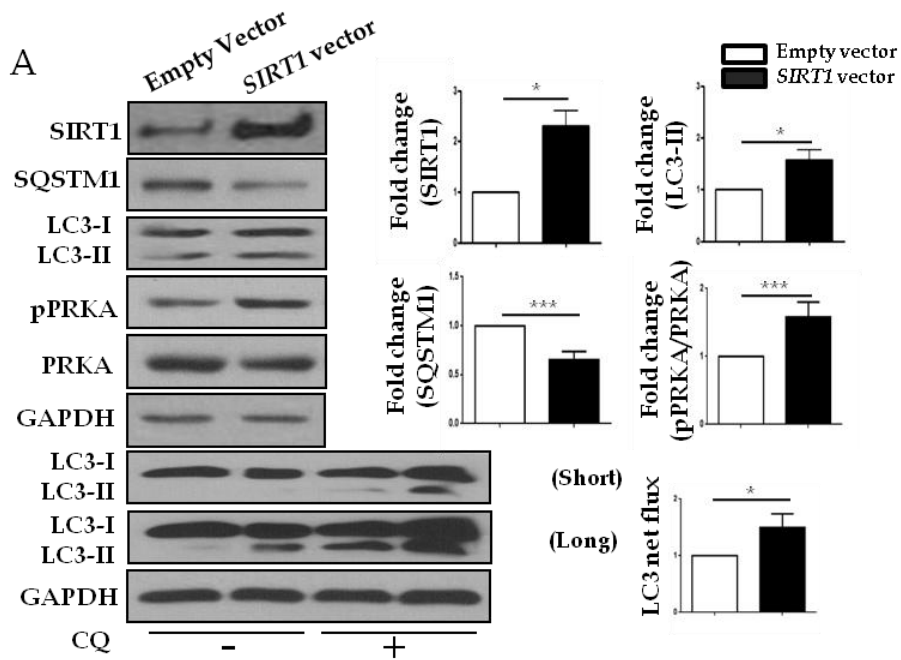
**Figure 8. Metformin upregulates SIRT1 expression through a PRKA-independent pathway.** (A) HepG2 cells were transfected with siRNA against *PRKAA1/2* or a scrambled siRNA control. Densitometric graphs of the optical density-based data of immunoblots showed that SIRT1 expression and activity were significantly upregulated after metformin treatment in HepG2 cells regardless of PRKA expression levels. Metformin also decreased SQSTM1 expression while it increased LC3-II levels in both control and *PRKAA1/2* siRNA-treated cells. (B) Oil Red O staining and spectrophotometer analysis of HepG2 cells transfected with either siRNA against *PRKAA1/2* or scrambled siRNA control demonstrated that metformin significantly decreased lipid accumulation in cells treated with OA. (C) HepG2 cells were then transfected with siRNA against *SIRT1*, and a scrambled siRNA control, showing that the ratio of pPRKA and PRKA expression was increased upon treatment with metformin regardless of SIRT1 levels. Values displayed are means  $\pm$  SEM of 3 to 6 independent experiments. Asterisks (\*  $p < 0.05$ , \*\*  $p < 0.01$  and \*\*\*  $p < 0.001$ ) indicate significant differences.

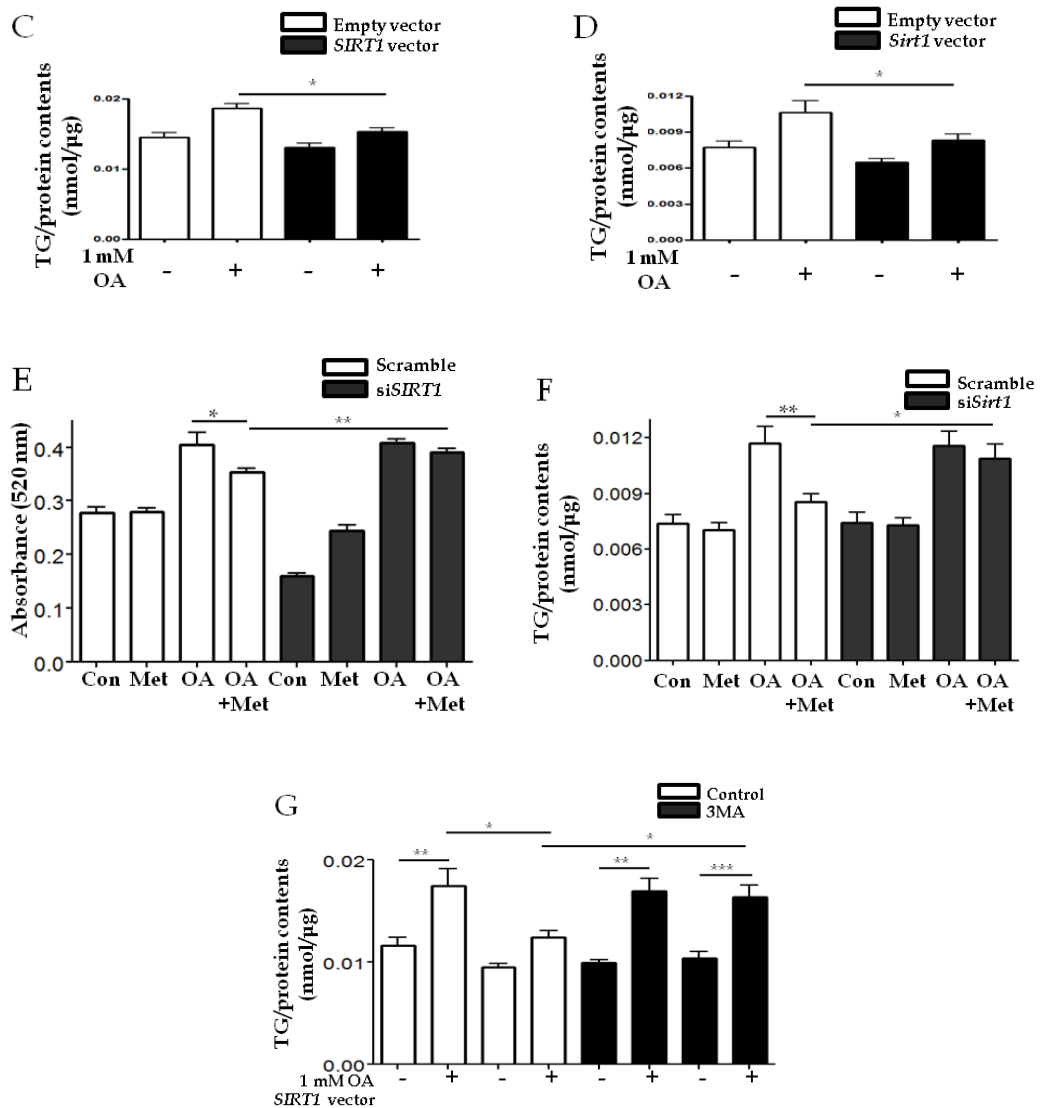


## **7. SIRT1 attenuates lipid accumulation in hepatocytes through SIRT1-mediated autophagy induction**

To confirm that SIRT1-mediated autophagy induction was responsible for hepatic lipid clearance, either a vector driving SIRT1 overexpression or siRNA directed against *SIRT1* were transfected into HepG2 cells. As shown in Fig. 9A and B, the SIRT1 vector increased SIRT1 expression, decreased SQSTM1 levels and promoted subsequent conversion of LC3-II, whereas *SIRT1* siRNA downregulated SIRT1 expression, increased SQSTM1 levels and decreased the conversion of LC3-II. OA-induced lipid accumulation was significantly alleviated by transfecting the SIRT1 vector into both HepG2 cells ( $0.019 \pm 0.0007$  vs.  $0.015 \pm 0.0005$ ,  $p < 0.05$ ; **Fig. 9C**) and primary hepatocytes ( $0.011 \pm 0.001$  vs.  $0.008 \pm 0.0005$ ,  $p < 0.05$ ; **Fig. 9D**). To elucidate whether hepatic lipid clearance required the SIRT1-mediated autophagy pathway, we disrupted this pathway through both siRNA knockdown of SIRT1 expression and the pharmacological inhibitor 3MA. Intracellular lipid accumulation in HepG2 cells and primary mouse hepatocytes were analyzed using a spectrophotometer at 520 nm and a colorimetric quantification kit, respectively. While 2 mM OA significantly increased the lipid contents in HepG2 cells ( $0.28 \pm 0.007$  vs.  $0.4 \pm 0.02$ ,  $p < 0.05$ ), 0.5 mM metformin significantly decreased this intracellular fat loading ( $0.4 \pm 0.02$  vs.  $0.35 \pm 0.008$ ,  $p < 0.05$ ). However, upon siRNA-mediated *SIRT1* knockdown, metformin was unable to attenuate OA-induced lipid accumulation ( $0.41 \pm 0.007$  vs.  $0.39 \pm 0.008$ ,  $p = 0.11$ ) in HepG2 cells (**Fig. 9E**). Similarly, after siRNA-mediated *SIRT1* knockdown in primary mouse hepatocytes, metformin also did not attenuate 1 mM OA-induced lipid accumulation ( $0.012 \pm 0.0008$  vs.  $0.01 \pm 0.0007$ ,  $p = 0.54$ , **Fig. 9F**). In the absence of 5 mM 3MA, 1 mM OA-induced lipid accumulation was significantly attenuated by

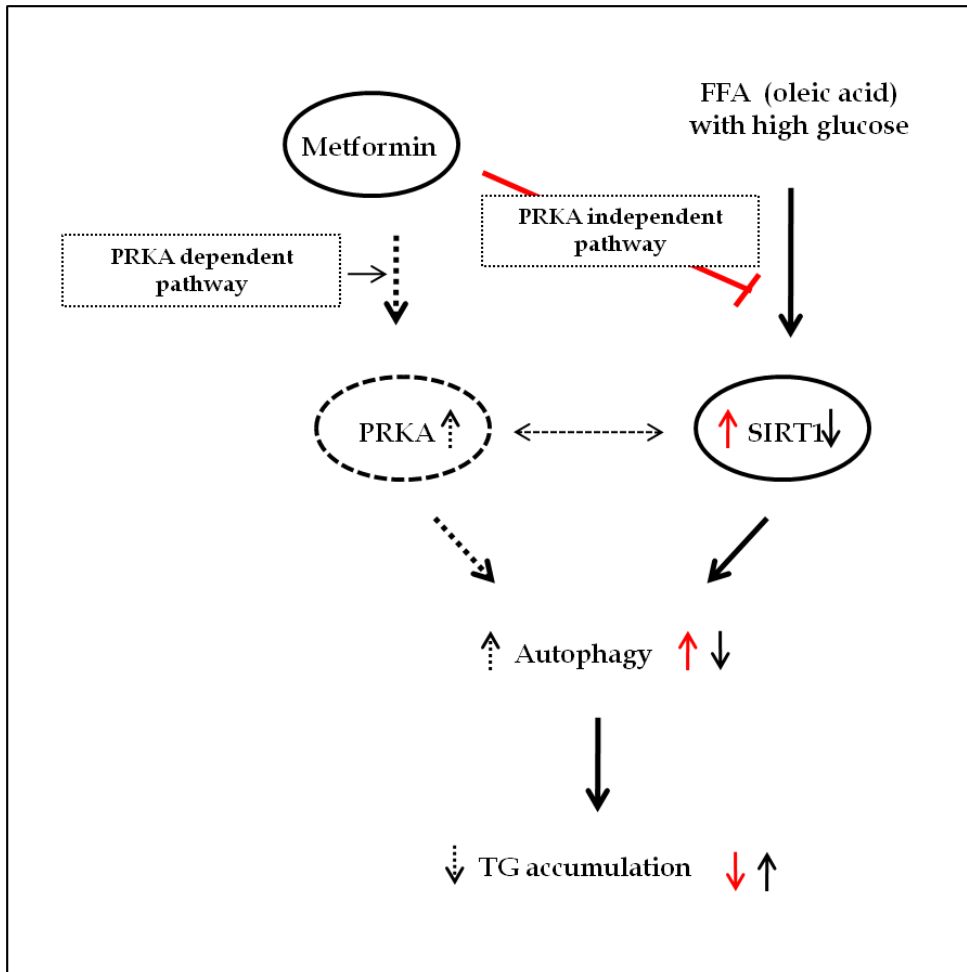
overexpression of SIRT1 ( $0.017 \pm 0.0017$  vs.  $0.012 \pm 0.0006$ ,  $p < 0.05$ ). However, in the presence of 5 mM 3MA, OA-induced lipid accumulation was not alleviated by SIRT1 overexpression ( $0.017 \pm 0.0013$  vs.  $0.016 \pm 0.0011$ ,  $p = 0.76$ , **Fig. 9G**).





**Figure 9. SIRT1 attenuates lipid accumulation in hepatocytes through SIRT1-mediated induction of autophagy.** (A) Increased protein levels of SIRT1 in HepG2 cells transfected with a *SIRT1* vector increased LC3-II conversion, while decreased expression of SQSTM1 was verified by immunoblots. Expression ratio of

pPRKA/PRKA was also increased in SIRT1 overexpression cells. (B) Furthermore, transfection with siRNA against *SIRT1* decreased both SIRT1 protein levels and also conversion of LC3-I to LC3-II. Expression of SQSTM1 was significantly increased, while pPRKA levels were not affected. To investigate whether SIRT1 could regulate lipid accumulation in hepatocytes, either a SIRT1 overexpression vector or siRNA directed against *SIRT1* were transfected into HepG2 cells and primary mouse hepatocytes. In (C) HepG2 cells and (D) primary hepatocytes, OA-induced increases in intracellular TG contents were significantly reduced by SIRT1 overexpression. In accordance with the data presented in Fig. 6D, OA-induced intracellular lipid accumulation was significantly alleviated by metformin treatment of control siRNA-treated (E) HepG2 cells and (F) primary hepatocytes. In contrast, reduction of lipid contents by metformin was negligible in SIRT1-knockdown cells. (G) Lipid accumulation was also examined in the presence or absence of the autophagic inhibitor 3MA. OA-treated HepG2 cells exhibited a significant increase in lipid accumulation. However, SIRT1 overexpression significantly decreased OA-induced intracellular lipid accumulation. Lipid contents of cells treated with 5 mM 3MA and transfected with *SIRT1* were similar to those of cells treated with OA alone. Values are displayed as means  $\pm$  SEM of 8 mice per group. Asterisks (\*  $p < 0.05$ , \*\*  $p < 0.01$ , \*\*\*  $p < 0.001$ ) indicate significant differences.



**Figure 10. Summary of working thesis of the study (See the text for details).**

## IV. DISCUSSION

Autophagy facilitates the removal of damaged proteins and aged organelles, thereby maintaining cellular homeostasis. Under conditions of nutrient deprivation, induction of autophagy provides a simple response for cells to reutilize their own constituents for energy.<sup>35,36</sup> From a translational point of view, nutritional deprivation through CR causes weight reduction on the individual level, and induces autophagy on the cellular level. Similar to CR, metformin causes weight reduction and induces autophagy through PRKA activation.<sup>27,28,37-40</sup> Metformin belongs to the biguanide class of drugs and inhibits complex I of the electron transport chain by sequentially lowering the cellular energy charge and then activating PRKA.<sup>41,42</sup> In this regard, lifestyle modification through CR and taking metformin are thought to induce autophagy; a promising approach for treating NAFLD.<sup>35,12,17,43,44</sup> Based on these reports, we used an animal model to implement CR as a lifestyle modification, and metformin as a drug therapy in both cellular and animal models.

The traditional role of PRKA is to act as a cellular stress and energy sensor, and its activity increases upon glucose deprivation.<sup>37</sup> It is also well established that PRKA can cooperate with the fuel/NAD<sup>+</sup>-dependent sirtuin class of histone/protein deacetylases (SIRTs). SIRT1 has been reported to mediate the beneficial metabolic effects of CR through deacetylation of transcription factors such as PPARG (peroxisome proliferator-activated receptor gamma), PPARA, PPARGC1A (peroxisome proliferator-activated receptor gamma, coactivator1 alpha) and FOXO (forkhead box O). These transcription factors are involved in diverse processes such as hepatic lipid metabolism, gluconeogenesis, mitochondrial biogenesis, and energy

expenditure.<sup>45-48</sup> Particularly, liver-specific *SIRT1* deletion induces hepatic steatosis and inflammation via impairment of PPARA signaling.<sup>48</sup> Conversely, both PRKA and SIRT1 are downregulated in the liver under stress conditions of nutrient excess, including a high-fat diet, obesity, and type 2 diabetes.<sup>45,15,49</sup> In addition, SIRT1-deficient mice exhibit a premature aging phenotype and develop fatty livers.<sup>48,50,51</sup> Based on these reports, we investigated the molecular interplay between SIRT1, PRKA, and autophagy in response to metformin.

The present study aimed to determine whether metformin alleviates hepatosteatosis through SIRT1-mediated autophagy induction, and whether this mechanism is PRKA-independent. This work had 3 main findings: First, SIRT1 expression and induction of autophagy are significantly downregulated both in the livers of ob/ob mice and also in OA- plus 30 mM glucose-treated hepatic cell lines. Second, both CR and metformin treatment caused weight reduction and alleviated hepatosteatosis and glucose homeostasis in ob/ob mice in association with increasing SIRT1 expression and induction of autophagy. Third, metformin treatment restored SIRT1 expression and, consequently, induction of autophagy, via a PRKA-independent pathway.

Regarding the effects of metformin on hepatosteatosis, definitive evidence that metformin is the treatment option of choice for fatty livers in humans is still lacking. Even taking into account the comorbidities of NAFLD with other diseases such as obesity, glucose intolerance and dyslipidemia, the primary cause of lipid accumulation in the liver is not yet clear. An increased supply of free fatty acids, *de novo* hepatogenic lipogenesis, decreased oxidation of free fatty acids, and secretion of hepatic very-low-density lipoprotein-triglycerides have all been implicated in the development of NAFLD.<sup>30,35,52</sup> The need to develop a new model of the molecular



pathways responsible for NAFLD has generated intense interest in autophagy as a pathway that could be manipulated for the management of NAFLD.<sup>12,35</sup> Here, we used the ob/ob mouse model, which mimics type 2 diabetes, to show that these mice exhibited increased body weight, an accumulation of hepatic fat, decreased hepatic levels of SIRT1, and suppressed autophagic machinery when fed ad libitum. However, metformin treatment of these mice caused significant weight reduction and alleviated hepatic lipid accumulation. *In vitro* experiments with both primary hepatocytes and HepG2 cells were performed with a monounsaturated fatty acid (OA) in the presence of high glucose, which minimizes inflammatory lipogenic effects. In these experiments, metformin decreased intracellular fat contents and activated the autophagy machinery. The activation of the autophagy machinery was demonstrated on several levels, including examining LC3 turnover in the absence or presence of CQ to measure the induction of autophagy, and using fluorescence microscopy to visualize autophagosome formation via GFP-LC3 localization. Most importantly, a leupeptin-based *in vivo* animal assay<sup>53</sup> was applied to assess the dynamics of autophagic flux under the *in vivo* condition, showing that both metformin and CR induced autophagic flux in the liver of ob/ob mice. However, considering previous reports showing age-related decline in autophagy function in livers,<sup>54-58</sup> it is quite possible that metformin may be less able to alleviate hepatic steatosis in elderly human subjects by activation of autophagy.

Regarding the mechanisms by which CR and metformin unload caloric burden, the role of PRKA as a key energy sensor for suppressing lipid accumulation and cell growth is well established.<sup>59-63</sup> In contrast, the MTOR (mechanistic target of rapamycin) signaling pathway has been proposed to have an opposing role to PRKA

with respect to protein synthesis, cell growth, and cell division.<sup>64-66</sup> Inhibition of MTOR induces autophagy, a process which plays a homeostatic role not only by breaking and removing cellular components, but also by decreasing cellular stores of TGs, as demonstrated in this study in hepatocytes.<sup>67,68</sup> Under low-nutrient conditions, increased PRKA activity controls the induction of autophagy by activating either the MTOR-ULK1 or the SIRT1-FOXO signaling pathway.<sup>18,19,25,69,70</sup> In addition, metformin activates both PRKA and SIRT1 through a PRKA-mediated increase in gene expression of NAMPT (nicotinamide phosphoribosyltransferase), the rate-limiting enzyme of the salvage pathway for NAD<sup>+</sup>.<sup>29</sup> In this study, we tested whether SIRT1 regulates the autophagic machinery, and whether this regulation required PRKA activation; to this end, we used siRNA to manipulate cellular levels of both *SIRT1* and *PRKAA1/2*. We observed that both SIRT1 and pPRKA were still upregulated upon treatment with metformin even under *PRKAA1/2* and *SIRT1* knockdown conditions, respectively. Taken together, we suggest that metformin induces *SIRT1* gene expression via a PRKA-independent pathway, thereby resulting in autophagy induction.

The present study has some limitations, which should be addressed by further research. Although it is well known that autophagic activation induces lipophagy, which leads to decreased cellular lipid content, we did not demonstrate direct evidence of lipophagy as a link between increased autophagic flux by metformin and reduced lipid accumulation in hepatocytes. Moreover, additional experiments with genetically engineered animal models such as liver-specific *Atg7*- or *Sirt1*-knockout mice should be conducted in the future to provide a more definitive mechanistic role of metformin.

Regarding autophagy-induced clearance of hepatic fat content, metformin was no longer able to alleviate lipid accumulation either in the presence of an inhibitor of autophagy, 3MA, or upon siRNA-mediated knockdown of *ATG5*, an important player in the elongation step of autophagosome formation. In addition, overexpression of SIRT1 stimulated induction of autophagy, thereby alleviating the increase in hepatic fat content normally caused by OA. Furthermore, the TG-lowering action of SIRT1 was also abolished by incubation with 3MA. Collectively, these data suggest that metformin-mediated alleviation of increased hepatic fat content involves the autophagy machinery, a finding that may have implications for the pathogenesis and treatment of hepatosteatosis.

## V. CONCLUSION

Taken together our results suggest that metformin apparently improved the hepatosteatosis. These beneficial effects are thought to be related with SIRT-mediated autophagy induction via a PRKA-independent pathway.

However, further studies with genetically engineered animal models such as liver-specific *Atg7*- or *Sirt1*-knockout mice are necessary to elucidate the definitive mechanistic role of metformin.

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Abstract (in Korean)

Metformin의 Sirt1-autophagy 활성화를 통한 지방간 개선

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자가포식작용 (Autophagy) 활성화 감소에 의한 외부 스트레스에 대한 방어 기전 억제는 불안정 세포 항상성을 의미한다. 억제된 자가포식작용의 활성화가 지방간을 호전 시킨다는 기존의 연구를 바탕으로 ‘자가포식작용 활성화가 지방간의 새로운 치료기전’으로 연구가 진행되고 있다. 임상적으로 칼로리 제한은



지방간을 호전 시키며, 이러한 기전으로 SIRT1 의 증가에 의한 자가포식작용 유도가 그 기전으로 제시되었다. 메트포민은 PARK, SIRT1 모두를 활성화 시킬 수 있으며, 앞서 언급했듯이 자가포식작용은 PRKA-MTOR-ULK1 또는 SIRT1-FOXO 를 통해 유도된다. 본 연구에서는 SIRT1, PRKA 와 자가포식작용의 상호작용 연구를 통하여 메트포민의 지방간 완화 메커니즘을 설명하고자 한다. 이를 위하여 ob/ob 쥐를 대조군 (자유식), 300 mg/kg 메트포민을 복강을 통해 주사한 군 (자유식), 식이 제한 군 (3 g/day)으로 나누어 4주 동안 유지하였다.

또한, 일차배양 간세포와 HepG2 세포는 올레산과 고농도 포도당을 처리한 그룹, 올레산과 고농도 포도당이 있는 세포에 메트포민을 함께 처리한 그룹으로 나누어 비교하였다.

식이 제한 군과 메트포민 처리한 군의 ob/ob 쥐의 몸무게, 포도당 항상성, 지방간이 향상 되었다. 또한, SIRT1과 자가포식작용이 증가하였다.

생체 외 실험에서 메트포민이 올레산과 고농도 포도당처리에 의한 SIRT1과 자가포식작용의 발현 억제를 막는 것을 확인하였다. 지방 축적 또한 메트포민에 의해 개선됨을 관찰 하였다.

마지막으로 siRNA를 통한 PRKAA1/2를 억제 시켰을 때도 메트포민에 의한 SIRT1의 발현은 유지 되었고, 또한 SIRT1이 억제

되었을때도 phospho-PRKA의 발현이 유지 되었다.

결론적으로 메트포민이 PRKA를 거치지 않고 SIRT1을 통해 자가포식작용을 유도하여 지방간을 완화 시킬 수 있음을 제시한다.

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핵심되는 말: 자가포식작용, 지방간, 메트포민, PRKA, SIRT1