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C1q/TNF-Related Protein 9 (CTRP9) attenuates hepatic steatosis via the autophagy-mediated inhibition of endoplasmic reticulum stress

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ABSTRACT

C1q/TNF-Related Protein (CTRP) 9, the closest paralog of adiponectin, has been reported to protect against diet-induced obesity and non-alcoholic fatty liver disease (NAFLD). However, the underlying mechanism has not been fully elucidated. We explored the protective effect of CTRP9 against hepatic steatosis and apoptosis, and identified the mechanisms through autophagy and endoplasmic reticulum (ER) stress using *in vitro* and *in vivo* experiments. Treating HepG2 cells with human recombinant CTRP9 significantly ameliorated palmitate- or tunicamycin-induced dysregulation of lipid metabolism, caspase 3 activity and chromatin condensation, which lead to reduction of hepatic triglyceride (TG) accumulation. CTRP9 treatment induced autophagy markers including LC3 conversion, P62 degradation, Beclin1 and ATG7 through AMPK phosphorylation in human primary hepatocytes. Furthermore, CTRP9 decreased palmitate- or tunicamycin-induced ER stress markers, such as eIF2α, CHOP and IRE-1, in HepG2 cells. Compound C, an AMPK inhibitor, and 3 methyladenine (3 MA), an autophagy inhibitor, canceled the effects of CTRP9 on ER stress, apoptosis and hepatic steatosis. In the livers of HFD-fed mice, adenovirus-mediated CTRP9 overexpression significantly induced AMPK phosphorylation and autophagy, whereas suppressed ER stress markers. In addition, both SREBP1-mediated lipogenic gene expression and apoptosis were significantly attenuated, which result in improvement in hepatic steatosis by overexpression of CTRP9. These results demonstrate that CTRP9 alleviates hepatic steatosis through relief of ER stress via the AMPK-mediated induction of autophagy.

1. Introduction

Non-alcoholic fatty liver disease (NAFLD) is the most common cause of liver disease in developed countries (Argo et al., 2009, Browning et al., 2004) and is closely related to the components of metabolic syndrome such as obesity, dyslipidemia and hyperglycemia (Tonjes et al., 2010). C1q/TNF-Related Proteins (CTRPs) are adipose tissue-derived proteins that were identified based on sequence homology with the globular domain of adiponectin (Wong et al., 2004). CTRPs have diverse metabolic functions in various organ systems including adipose tissue, liver, skeletal muscle, hypothalamus and the heart (Seldin et al., 2013). CTRP9 is the closest known paralog of adiponectin and shares multiple common structural and biochemical features with adiponectin, including its domain structure, adipose-selective expression, endocrine function, formation of higher-order oligomeric complexes and post-translational modifications (Peterson et al., 2013). CTRP9 is also thought to serve as an adipokine that facilitates communication among adipose tissue, skeletal muscle and liver (Peterson et al., 2013). Compared to the metabolic profile of control mice, CTRP9 transgenic mice have significantly improved fasting insulin and glucose levels, resistance to weight gain and attenuation of hepatic steatosis (Peterson et al., 2013). In contrast, CTRP9 knockout mice exhibit higher body weight, increased insulin resistance and hepatic steatosis (Wei et al., 2014). One clinical study found that serum CTRP9 concentration is inversely correlated with age, blood pressure, and fasting glucose and positively correlated with serum adiponectin (Hwang et al., 2013). Taken together, the

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prior literature suggests that CTRP9 may play an important role in the development and progression of NAFLD, as well as in lipid and glucose metabolism.

Ozcan et al. found that obese animal models have elevated levels of endoplasmic reticulum (ER) stress markers in liver (Ozcan et al., 2004, 2006). These animals also have increased expression of chemical chaperones, increased liver injury markers and higher prevalence of hepatic steatosis (Ozcan et al., 2004, 2006). The process of autophagy involves sequestration of cytoplasmic materials and intracellular organelles into a membrane-bounded vacuole called an autophagosome. Lysosomes fuse with the autophagosome, resulting in the consequent degradation of cytoplasmic materials and organelles and thus decreased biosynthetic burden on the ER (Klionsky, 2005). However, the effects of CTRP9 on hepatocytes through the mechanism regarding ER stress and autophagy has not been reported. Since AMP-activated protein kinase (AMPK) has been reported to sense cellular energy status to maintain energy homeostasis, it can be a potential candidate in autophagy regulator (Hardie, 2007). Furthermore, there are some evidences to support a role of AMPK in autophagy regulation in response to various cellular stresses (Herrero-Martin et al., 2009, Matsui et al., 2007, Vingtdeux et al., 2010). Therefore, this study focuses on the AMPK-autophagy-dependent pathway to relieve ER stress and ameliorate NAFLD.

In the present study, we investigated the effects of CTRP9 on (1) palmitate- or tunicamycin-induced impairment of lipid metabolism and apoptosis; (2) AMPK phosphorylation and induction of autophagy; (3) the impact of AMPK-autophagy pathway on ER stress, triglyceride (TG) accumulation and apoptosis in HepG2 and primary human hepatocytes; and (4) the effects of CTRP9 overexpression on AMPK phosphorylation, autophagy and ER stress, which are associated with steatosis in liver of high fat diet (HFD)-fed mice.

2. Materials and methods

2.1. Cell cultures, reagents, and antibodies

The human hepatoma HepG2 cell line (ATCC, Manassas, VA) and human primary hepatocytes (ZenBio, Research Triangle Park, NC) were cultured in Dulbecco's modified eagle medium (DMEM) (Invitrogen, Carlsbad, CA) or hepatocyte maintenance medium (ZenBio) supplemented with 10% fetal bovine serum (Invitrogen), 100 units/ml penicillin, and 100 μg/ml streptomycin (Invitrogen). Cells were incubated in a humidified atmosphere with 5% CO_2 at 37 °C. Human recombinant CTRP9 made in *E. Coli* was purchased from Aviscera Bioscience (Aviscera Bioscience, CA) (Kambara et al., 2012). Distilled water was used to dissolve 3-methyladenine (3 MA) (Sigma). Tunicamycin (Sigma) and compound C (Sigma) were dissolved in dimethyl sulfoxide (DMSO) and were added to the culture medium. Sodium palmitate (Sigma) was conjugated to 2% BSA (fatty acid free; Sigma) dissolved in the DMEM. Chloroquine (Sigma) was dissolved in distilled water. The final concentration of DMSO was <0.1% and did not affect cell viability. In all experiments, palmitate-BSA was applied for 24 h, while 2% BSA was used as the control. Anti-phospho AMPK, anti-AMPK, anti-P62, anti-phospho eIF2α, anti-total eIF2α, anti-total IRE-1, anti-CHOP, anti-Caspase 3, and total anti-PERK were purchased from Cell Signaling (Beverly, MA). Anti-SREBP1, anti-FAS, anti-SCD1, anti-GRP78, anti-HSP47, anti-HSP70, anti-Calnexin, anti-phospho PERK, anti-CTRP9, and beta-actin were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-LC3 was purchased from Novus Biologicals (Littleton, CO). Anti-phospho IRE-1 was purchased from Abcam (Cambridge, MA). All *in vitro* experiments were performed three or four times.

2.2. Experimental animals, feeding regimens, and viral introduction

This study was approved by the institutional animal review board (Institutional Animal Care and Use Committee) of Korea University, Seoul, Korea. Eight-week-old male C57BL/6J (B6) mice were purchased from Central Lab. Animal (Seoul, Korea) and housed in groups of seven in standard clear polycarbonate cages and allowed to acclimatize to their surrounds. The control group and two experimental groups were fed a normal fat diet (ND) (2.85 kcal/g−12.6% kcal from fat, 26.7% from protein, and 60.7% from carbohydrate, Brogaarden, Gentofte, Denmark) and a high fat diet (HFD), respectively, for eight weeks. The HFD contained 60% kcal from fat, 20% from protein, and 20% from carbohydrates (Research Diets, New Brunswick, NJ). Mice were randomly assigned to vehicle (Veh; empty adenovirus) injected ND-fed mice, vehicle injected HFD-fed mice, and CTRP9-overexpression adenovirus-injected HFD-fed mice. All groups had access to drinking water *ad libitum* during the eight weeks. Customized adenoviruses expressing mouse CTRP9 and empty were purchased from Koma Biotech (Seoul, Korea). Tail vein injections were performed once weekly for eight weeks in order to infect the mice with the indicated viruses using modified adenovirus infection protocols (Li et al., 2004, Saimura et al., 2002). For intraperitoneal glucose tolerance test (IPGTT), mice fasted for overnight (12 h) were given an intraperitoneal injection of glucose (2 g/kg b.w.; n = 7). After 3 days from IPGTT, blood samples were harvested from the tail vein before glucose challenge, as well as 30, 60, 90, and 120 min thereafter. For insulin tolerance test (ITT), mice fasted for 6 h were given an intraperitoneal injection of human insulin (1 U/kg b.w.; n = 7). Blood samples were harvested from the tail vein, as well as 15, 30, 45, and 60 min thereafter. Serum glucose levels were measured using Accu-Chek III glucose analyzer. Serum aspartate aminotransferase (AST) levels were measured using a AST ELISA kit (Cusabio, Wuhan, China) according to the manufacturer's instructions. After an 8-week study period, mice of all groups were sacrificed under anesthesia after fasting for overnight (12 h). Serum and tissue samples were harvested and stored at −80 °C prior to use. All efforts were made to minimize animal suffering and to reduce the number of animals used.

2.3. RNA isolation and quantitative real-time PCR

The gene expression of each cDNA sample was analyzed with quantitative real-time PCR using the fluorescent TaqMan 5′-nuclease assay on an Applied Biosystems 7000 sequence detection system. TaqMan real-time PCR was performed using 2× TaqMan Master Mix and 20× premade TaqMan gene expression assays (Applied Biosystems, Foster City, CA). Forty-five cycles of PCR were conducted under the following conditions: 95 °C for 10 min, 95 °C for 15 s, and 60 °C for 1 min. The levels of mRNA expression of human CPT1 (Hs03046298_s1; Applied Biosystems), ACO (Hs00158095_m1; Applied Biosystems), and LFBP (Hs00155026_m1; Applied Biosystems) were normalized to that of human beta-actin (Hs00969077_m1; Applied Biosystems). Furthermore, forward 5′TGCTGAGTCCGCAGCAGGTG3′ and reverse 5′GCTGGCAGGCTCTGGGGAAG3′ primers were used to detect the spliced XBP1 mRNA.

2.4. Immunoblotting analysis

HepG2 cells and human primary hepatocytes were harvested and extracted with lysis buffer (PRO-PREP™; Intron Biotechnology, Seoul, Korea) for 60 min at 4 °C. Protein samples (30 μg) were subjected to 10% SDS-PAGE, transferred to a nitrocellulose

membrane (Amersham Biosciences, Westborough, MA), and probed with a primary antibody. A secondary antibody conjugated with horseradish peroxidase (Amersham Biosciences) was then applied. The samples were detected with chemiluminescence kits (Amersham Biosciences).

2.5. Histological analysis

HepG2 cells and human primary hepatocytes were stained using the Oil Red-O method in order to measure the cellular accumulation of neutral lipid droplets. After fixation with 10% formalin for 30 min, the cells were stained with Oil Red-O solution for 30 min at 37 °C. In order to quantify the Oil Red-O content, isopropanol was added to each sample. The mixtures were shaken at room temperature for 5 min. Finally, 100 μl isopropanol-extracted samples were analyzed on a spectrophotometer at 510 nm.

2.6. Triglyceride measurement

Total lipids were extracted using a 2:1 chloroform:methanol (2:1, vol/vol) mixture. The organic layer was dried and dissolved in 60% methanol. The extracted triglycerides (TG) were measured using a colorimetric TG assay kit as instructed by the manufacturer (Biovision, Milpitas, CA).

2.7. Cell viability assay

Cell viability was measured using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (Sigma) assay, which is based on the MTT conversion into formazan crystals by mitochondrial dehydrogenases. In brief, HepG2 cells were plated at a density of 2×10^4 cells/well in a 96-well plate. Twenty microliters of 5 mg/ml MTT was added to each well. After a 5 h incubation at 37 °C, the MTT solution was removed, and the formazan was solubilized in 200 μl DMSO. A microplate reader was used to measure the absorbance at 490 nm.

2.8. Nuclear staining with Hoechst 33342

Cells were cultured in 6-well plates. After each different treatment, the cells were washed with PBS and fixed with 4% formalin for 5 min. In order to stain the nuclei, Hoechst 33342 (10 μg/ml) was applied to the plates for 20 min in a dark room. The stained cells were washed twice with PBS and observed under a fluorescence microscope. Apoptotic cells were determined by direct visualization and measurement of average fluorescence intensity. They were identified based on a nucleus containing brightly stained, condensed chromatin.

2.9. Caspase 3 activity assay

The caspase 3 activity assay was performed using a Caspase 3 Colorimetric Assay Kit™ (Abcam, Cambridge, MA) according to the manufacturer's instructions.

2.10. Autophagolysosome staining

Cells were fixed with 3% PFA for 10 min at 37 °C. They were then incubated for 10 min with 50 μM monodansylcadaverine (MDC) (Sigma), an autofluorescent compound that selectively stains autophagic vacuoles at 37 °C.

2.11. Statistical analysis

All analyses were performed using the SPSS/PC statistical program (version 12.0 for Windows; SPSS, Inc., Chicago, IL). Results are presented as fold-change from the control value (mean ± SEM). All of the experiments were conducted at least three times. Student's *t*-test or two-way ANOVA were used for the statistical analysis.

3. Results

3.1. CTRP9 ameliorates palmitate- or tunicamycin-induced dysregulation of lipid metabolism, resulting in improved TG accumulation in HepG2 cells

SREBPs are transcription factors for genes related to cholesterol or lipid metabolism (Horton, 2002). Palmitate and tunicamycin have been reported to induce the expression of SREBP, which subsequently increases the hepatic biosynthesis of TG, which lead to the development of hepatic steatosis (Wang et al., 2009, Werstuck et al., 2001). There was significant TG accumulation in HepG2 cells exposed to 200 μM palmitate or 1 μg/ml tunicamycin for 24 h which are optimized conditions for evaluation of lipid metabolism. In contrast, treatment of HepG2 cells with CTRP9 significantly suppressed palmitate- or tunicamycin-induced TG accumulation in a dose-dependent manner (Fig. 1A and D). CTRP9 treatment also significantly blocked palmitate- or tunicamycin-induced expression of SREBP1, FAS and SCD1 in a dose-dependent manner (Fig. 1B and E). Furthermore, CTRP9 treatment reversed the palmitate-induced suppression of CPT1 and ACO, which are associated with fatty acid oxidation. However, the mRNA expression of LFBP, which is involved in the intracellular transport of long-chain fatty acids in the liver, was not affected by CTRP9 treatment (Fig. 1C and F). Furthermore, treatment of human primary hepatocytes with CTRP9 showed similar patterns of data of HepG2 cells (Fig. 2).

3.2. CTRP9 inhibits the palmitate- or tunicamycin-induced apoptosis and restores cell viability in HepG2 cells

Palmitate has been reported to induce ER stress and apoptosis in hepatocytes (Cao et al., 2012). First, we treated HepG2 cells with 500 μM palmitate or 20 μg/ml tunicamycin for 12 h which significantly decreased cell viability. Furthermore, treatment of HepG2 cells with human recombinant CTRP9 significantly restored cell viability by inhibiting the palmitate- or tunicamycin-induced pro-apoptotic mechanisms (Fig. 3A and D). To verify the protective effect of CTRP9 on apoptosis, we measured cellular caspase 3 activity and chromatin condensation. Treatment of HepG2 cells with CTRP9 ameliorated palmitate- or tunicamycin-induced caspase 3 activity and chromatin condensation (Fig. 3B, C, E, and F). CTRP9 also ameliorated palmitate or tunicamycin-induced cell viability impairment and caspase 3 activity in human primary hepatocytes (Fig. 3G–J).

3.3. CTRP9 induces autophagy through AMPK activation in HepG2 cells and primary human hepatocytes

AMPK activation has been reported to attenuate ER stress in various cell types (Gu et al., 2012, Salvado et al., 2013, Wang et al., 2011). Autophagy helps the cell to adapt to stress through clearance of misfolded proteins and damaged organelles (Ogata et al., 2006). Therefore, activation of autophagy may improve the ER stress-mediated development of hepatic steatosis. Bachar-Wikstrom et al. demonstrated that rapamycin-induced autophagy prevents β-cell apoptosis (Bachar-Wikstrom et al., 2013). Furthermore, it has been reported that AMPK triggers autophagy through an mTOR-dependent (Meley et al., 2006) or −independent (Kim et al., 2011)

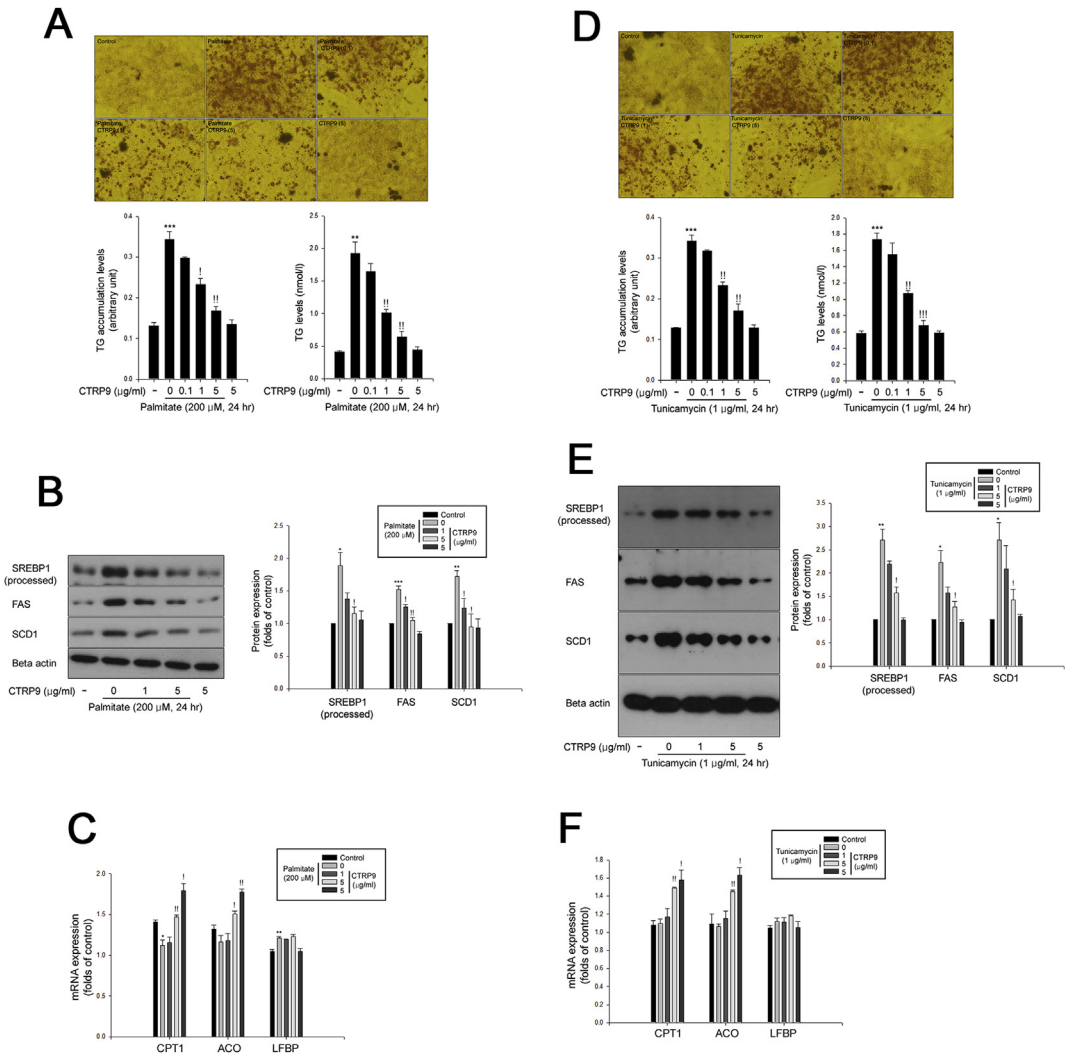

Fig. 1. C1q/TNF-Related Protein (CTRP) 9 suppresses TG accumulation via modulation of lipogenic genes in HepG2 cells. (A), (D) Oil Red-O staining of HepG2 cells treated with palmitate (200 μM, 24 h) or tunicamycin (1 μg/ml, 24 h), and CTRP9 (0–5 μg/ml, 24 h). (B), (E) Western blot analysis of processed SREBP1, FAS and SCD1 protein expression levels. (C), (F) Quantitative RT-PCR analysis of CPT1, ACO and L-FABP mRNA expression levels. Data are presented as the mean ± SEM of three separate experiments. $^{***}P < 0.001$, $^{**}P < 0.01$ and $^{*}P < 0.05$ compared to the control. $^{!!}P < 0.01$ and $^{!}P < 0.05$ compared to the palmitate treatment.

pathway. Therefore, we hypothesized that CTRP9 may stimulate autophagy through its interactions with AMPK. Through this mechanism, autophagy may play a critical role in the protective effects of CTRP9 against ER stress. As shown in Fig. 4A, CTRP9 treatment dose-dependently inhibited palmitate-induced ER stress marker expression including those of eIF2α, CHOP and IRE-1 (Fig. 4A). In addition, CTRP9 treatment of HepG2 cells stimulated autophagic markers in a dose-dependent manner. Compound C, a specific AMPK inhibitor abrogated the effect of CTRP9 on autophagic markers and MDC staining representing formation of autophagic vacuoles (Fig. 4B–D). Furthermore, in agreement with data from HepG2 cells, CTRP9 also augmented various autophagic markers including LC3 conversion, P62 degradation, Beclin1 and ATG7 through AMPK-dependent pathway in human primary hepatocytes (Fig. 4E and F). Next, we performed to examine whether CTRP9 facilitated autophagic flux. We compared LC3II accumulation by CTRP9 alone or in combination with chloroquine. Chloroquine neutralizes the lysosomal pH and interrupts autophagosomal degradation, and thereby blocks autophagic flux (Klionsky et al., 2012). CTRP9 in combination with chloroquine showed elevated levels of LC3II compared with either CTRP9 or chloroquine alone (Fig. 4G), indicating activation of autophagic flux is augmented by CTRP9.

3.4. CTRP9 alleviates palmitate-induced ER stress, apoptosis and hepatic steatosis through AMPK-mediated autophagy

Next, we evaluated the role of AMPK-mediated autophagy in the effects of CTRP9 on ER stress, TG accumulation and apoptosis. Suppression of AMPK activity using compound C and autophagy inhibition using 3 MA markedly reversed the effects of CTRP9 on palmitate-induced ER stress markers, such as eIF2α, CHOP, IRE-1 and XBP-1 (Fig. 5A–D). Furthermore, treatment of HepG2 cells with compound C or 3 MA canceled the suppressive effects of CTRP9 on palmitate-induced TG accumulation and apoptosis (Fig. 5E–J). Treatment of primary hepatocytes with CTRP9 also prevented palmitate-induced TG accumulation and apoptosis through an AMPK-autophagy-mediated pathway (Supplement Fig. 1). These results suggest that the effects of CTRP9 on ER stress-induced hepatic cellular dysfunction are mediated by an AMPK-induced autophagy-dependent pathway.

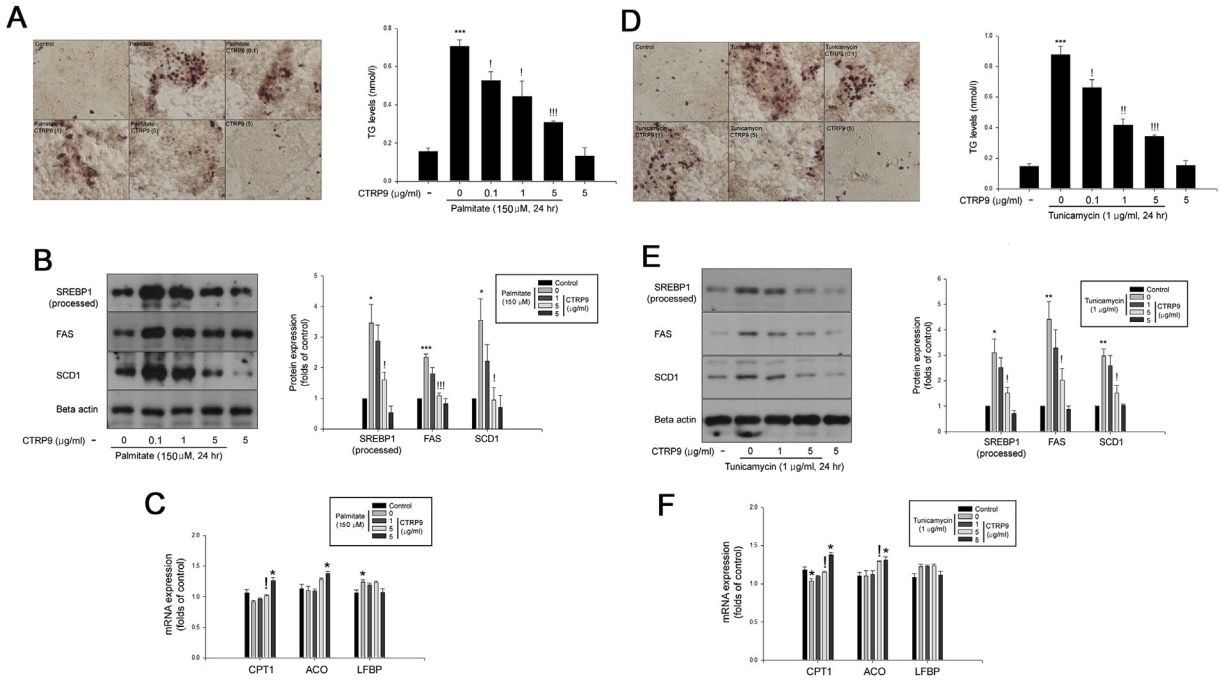

Fig. 2. CTRP9 prevents TG accumulation via modulation of lipogenic genes in primary hepatocytes. (A), (D) Oil Red-O staining of primary hepatocytes treated with palmitate (150 μM, 24 h) or tunicamycin (1 μg/ml, 24 h), and CTRP9 (0–5 μg/ml, 24 h). (B), (E) Western blot analysis of processed SREBP1, FAS and SCD1 protein expression levels. (C), (F) Quantitative RT-PCR analysis of CPT1, ACO and L-FABP mRNA expression levels. Data are presented as the mean ± SEM of three separate experiments. $^{***}P < 0.001$, $^{**}P < 0.01$ and $^{*}P < 0.05$ compared to the control. $^{!!!}P < 0.01$, $^{!!}P < 0.01$, and $^{!}P < 0.05$ compared to the palmitate treatment.

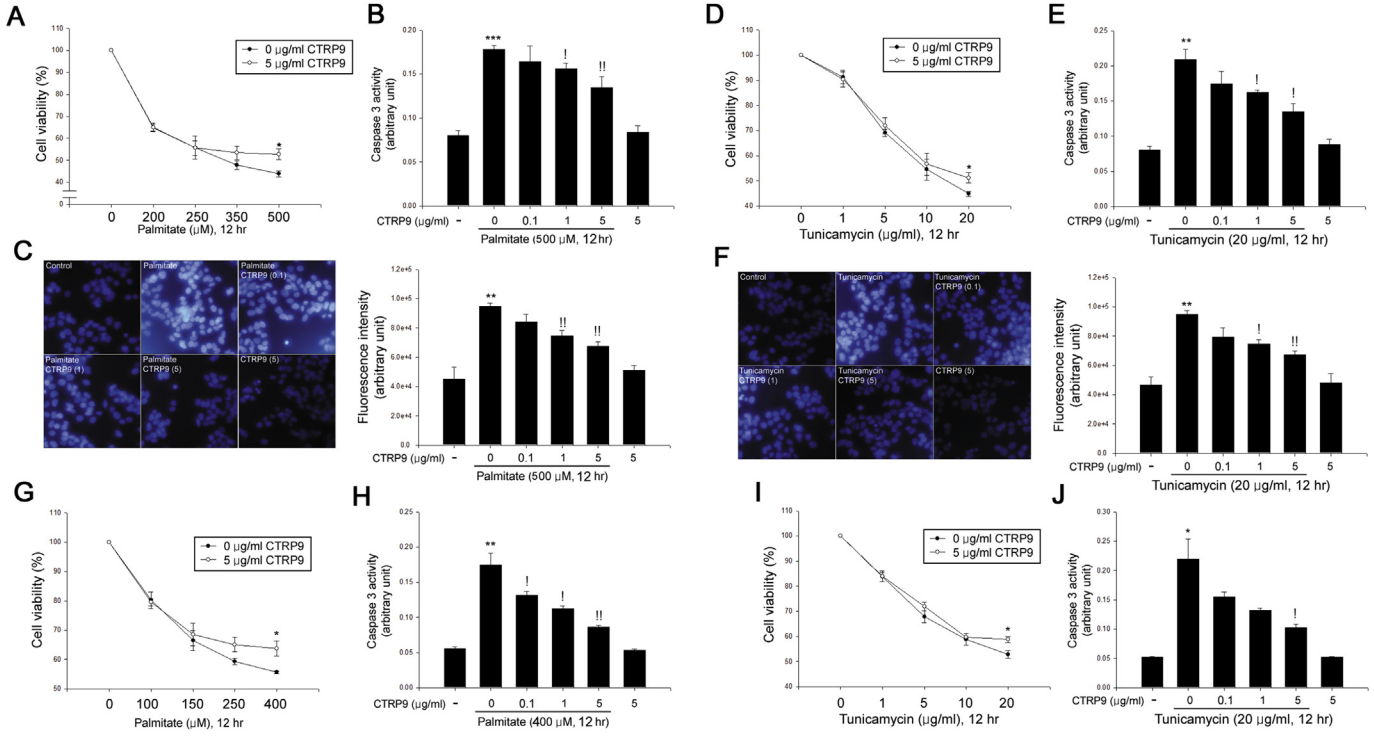

Fig. 3. CTRP9 suppresses palmitate- or tunicamycin-induced apoptosis in hepatocytes. (A), (D) Cell viability assay in HepG2 cells treated with palmitate (0–500 μM, 12 h) or tunicamycin (0–20 μg/ml, 12 h), and CTRP9 (0–5 μg/ml, 12 h) was determined using the MTT assay. (B), (E) Measurement of caspase 3 activity with caspase 3 activity assay. (C), (F) Chromatin condensation levels were determined after Hoechst staining. (G), (I) Cell viability assay in primary hepatocytes treated with palmitate (0–400 μM, 12 h) or tunicamycin (0–20 μg/ml, 12 h), and CTRP9 (0–5 μg/ml, 12 h) was determined using the MTT assay. (H), (J) Measurement of caspase 3 activity with caspase 3 activity assay in primary hepatocytes. Data are presented as the mean ± SEM of three separate experiments. $^{***}P < 0.001$, $^{**}P < 0.01$, and $^{*}P < 0.05$ compared to the control. $^{!!}P < 0.01$ and $^{!}P < 0.05$ compared to the palmitate or tunicamycin treatment.

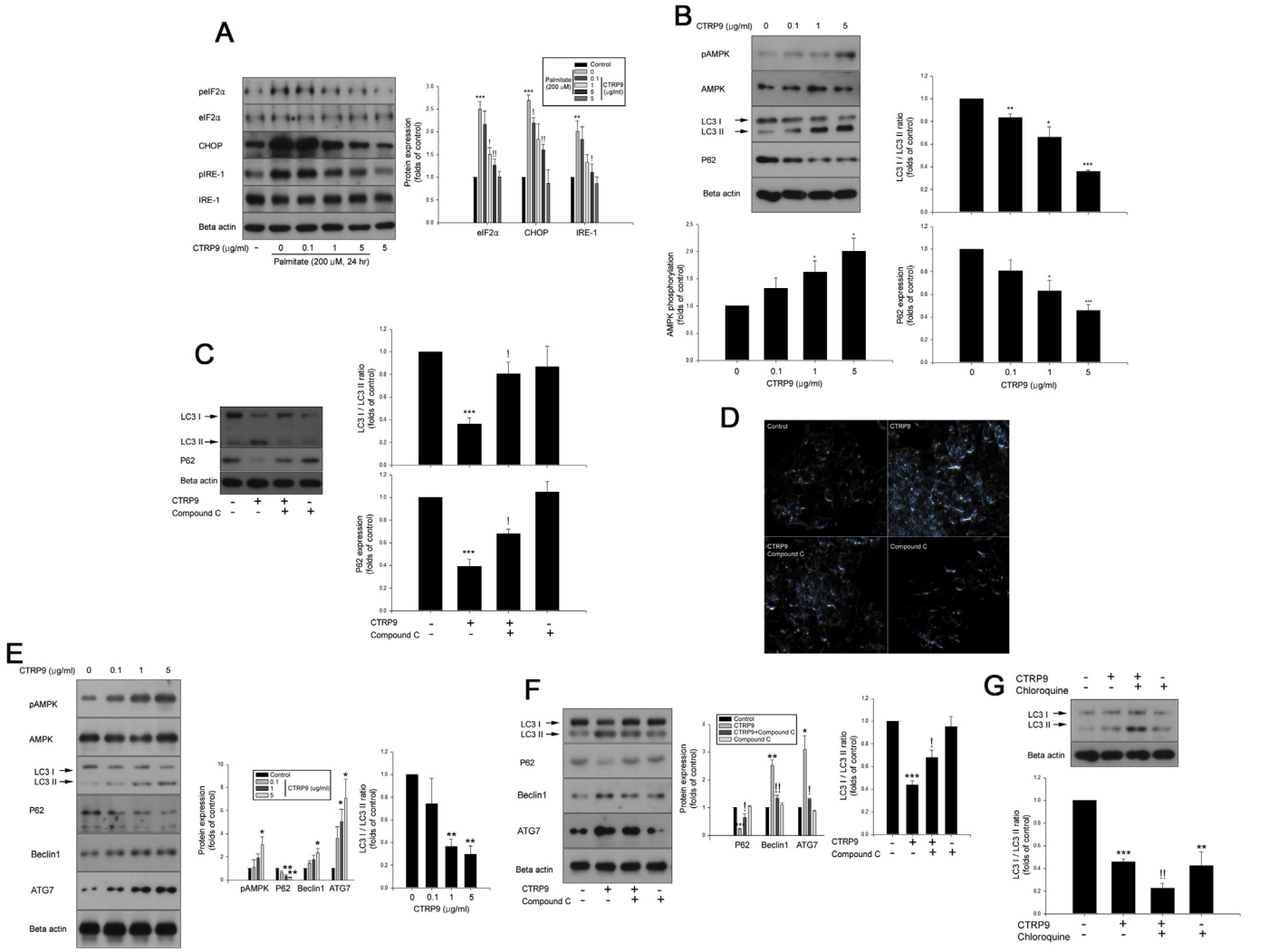

Fig. 4. CTRP9 ameliorates palmitate-induced ER stress through AMPK-mediated autophagy in HepG2 cells and primary human hepatocytes. (A) Various ER stress marker protein expression levels were determined by Western blot analysis in HepG2 cells treated with palmitate (200 μM, 24 h) and CTRP9 (5 μg/ml, 24 h). (B), (C) Western blot analysis of AMPK, LC3, and P62 protein expression levels in HepG2 cells treated with CTRP9 and compound C (20 μM, 16 h). (D) MDC staining of HepG2 cells treated with CTRP9 and compound C. (E), (F) Various autophagic marker protein expression levels were determined by Western blot analysis in human primary hepatocytes. (G) Western blot analysis of LC3 expression levels in HepG2 cells treated with CTRP9 and chloroquine (10 μM, 24 h). Data are presented as the mean ± SEM of three separate experiments. $^{***}P < 0.001$, $^{**}P < 0.01$, and $^{*}P < 0.05$ compared to the control. $^{!!}P < 0.01$ and $^{!}P < 0.05$ compared to the palmitate or CTRP9 treatment.

3.5. CTRP9 does not affect the expression of various chaperones in the ER

To attenuate excessive protein loading, cells induce the unfolded protein response (UPR), which leads to transient suppression of protein translation, degradation of misfolded proteins, and an increase in molecular chaperone expression. Induction of chaperone transcription augments the ER capacity of protein folding and degradation, consequently alleviating ER stress (Ni et al., 2007). Interestingly, we found that treating HepG2 cells with CTRP9 did not influence chaperone expression in the ER or in the cytoplasm (Supplement Fig. 2). This result suggests that CTRP9 treatment does not decrease ER stress through a chaperone-mediated mechanism.

3.6. CTRP9 overexpression prevents weight gain and improves glucose tolerance and insulin sensitivity in HFD-fed mice

Overexpression of human adiponectin in transgenic mice leads to significantly less weight gain than in normal mice (Otabe et al., 2007). Similarly, adenovirus-mediated adiponectin overexpression in Wistar rats improves glucose tolerance and insulin tolerance (Satoh et al., 2005). Given these findings, we examined the *in vivo* functional effect of adenovirus-mediated CTRP9 overexpression on weight gain. In agreement with a previous report, overexpression of CTRP9 induced modest weight loss (Fig. 6A) (Peterson et al., 2013) but did not influence food intake and calorie intake (Fig. 6B). As previously observed, CTRP9 also improved glucose tolerance and insulin sensitivity in HFD-fed mice (Peterson et al., 2013) (Fig. 6C and D).

3.7. CTRP9 modulates AMPK-mediated autophagy and alleviates hepatic ER stress in HFD-fed mice

In order to supplement and validate the results of our *in vitro* experiments, we examined the effects of CTRP9 overexpression on AMPK phosphorylation, autophagy and ER stress in HFD-fed mice. Adenovirus-mediated overexpression of CTRP9 was verified. The

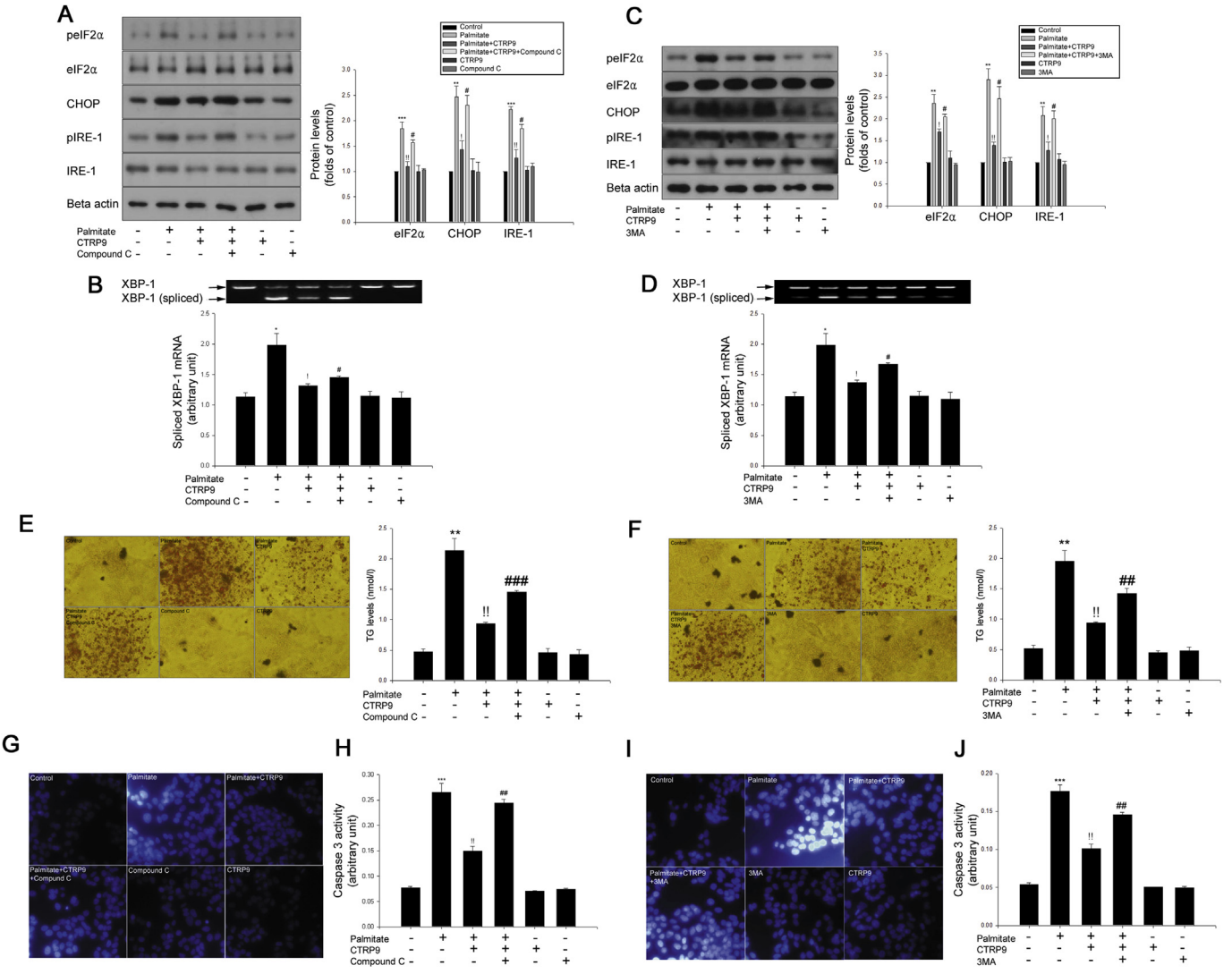

Fig. 5. CTRP9 attenuates palmitate-induced TG accumulation and apoptosis through AMPK-mediated autophagy. (A), (C) Various ER stress marker protein expression levels were determined by Western blot analysis in HepG2 cells treated with palmitate (200 μM, 24 h), CTRP9 (5 μg/ml, 24 h), compound C (20 μM, 16 h), or 3 MA (1 mM, 24 h). (B), (D) Spliced XBP-1 mRNA expression levels were determined by semi-quantitative RT-PCR and quantitative RT-PCR. (E), (F) Oil Red-O staining and TG quantification of HepG2 cells treated with palmitate, CTRP9, and compound C or 3 MA (G), (I) Hoechst staining of HepG2 cells treated with palmitate, CTRP9, and compound C or 3 MA (H), (J) Caspase 3 activity assay of HepG2 cells treated with palmitate, CTRP9, and compound C or 3 MA. Data are presented as the mean ± SEM of three separate experiments. $^{***}P < 0.001$, $^{**}P < 0.01$, and $^{*}P < 0.05$ compared to the control. $^{!!!}P < 0.001$, $^{!!}P < 0.01$, and $^{!}P < 0.05$ compared to the palmitate treatment. $^{\#\#\#}P < 0.001$, $^{\#\#}P < 0.01$, and $^{\#}P < 0.05$ compared to the palmitate plus CTRP9 treatment.

increased expression of CTRP9 induced both AMPK phosphorylation and autophagy, as demonstrated by LC3 conversion and P62 degradation. CTRP9 expression consequently decreased ER stress markers, such as eIF2α, CHOP and IRE-1, in the livers of HFD-fed mice (Fig. 7A and B).

3.8. CTRP9 improves hepatic steatosis in HFD-fed mice by attenuating SREBP1-mediated lipogenesis and apoptosis

We evaluated the effects of CTRP9 overexpression on liver histology using H&E and Oil red-O staining. CTRP9 suppressed HFD-induced TG accumulation (Fig. 7C) and the SREBP1-mediated gene expression patterns that are associated with lipogenesis in liver of HFD-fed mice (Fig. 7D). CTRP9 overexpression decreased HFD-induced hepatic apoptosis in mice (Fig. 7E and F). Finally, AST levels which represents liver injury were ameliorated by CTRP9 (Fig. 7G).

4. Discussion

Various adipokines from adipose tissue, including adiponectin, play an important role in systemic regulation of glucose and lipid metabolism. Therefore, adipokines have been studied extensively in the effort to discover novel drug candidates or targets for treatment of metabolic disorders. Wong et al. first identified CTRP9, which is a novel serum glycoprotein secreted by adipose tissue. CTRP9 can trimerize with adiponectin, activate AMPK and Akt signaling in cultured myotubes and lower serum glucose level in *ob/ob* mice (Wong et al., 2009). Interestingly, the circulating level of CTRP9 is significantly reduced in diet-induced obese (DIO) mouse models (Peterson et al., 2013). CTRP9 transgenic mice that are fed a high-fat diet showed improved systemic insulin sensitivity and protection against hepatic steatosis (Peterson et al., 2013). Recently, Wei et al. demonstrated that the targeted deletion of CTRP9 in mice increases food intake, decreases insulin sensitivity and aggravates

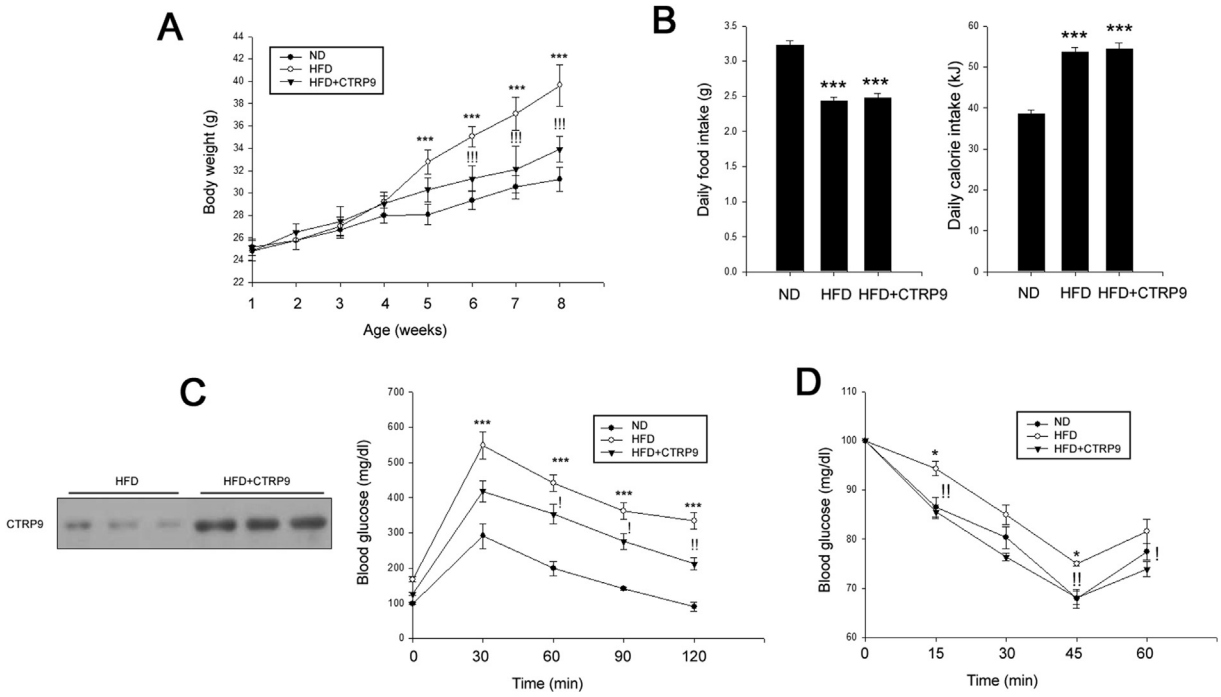

Fig. 6. Adenovirus-mediated overexpression of CTRP9 improves HFD-induced insulin resistance. (A) Measurement of body weight and (B) daily food intake in mice (C) CTRP9 overexpression was verified by Western blot analysis in mice serum (30 μl). Intraperitoneal glucose tolerance test (IPGTT) and (D) insulin tolerance test (ITT) in mice (n = 7 animals per treatment group). $^{***}P < 0.001$ and $^{**}P < 0.01$ compared to ND. $^{!!!}P < 0.001$, $^{!!}P < 0.01$, and $^{!}P < 0.05$ compared to HFD. Means ± SEMs were calculated from data from seven animals.

hepatic steatosis (Papackova et al., 2012). CTRP9 is a relatively small protein, the gene of which is located on human chromosome 13q12.12. Cai et al. found that insulin sensitivity phenotypes of Mexican Americans are closely correlated with one region of chromosome 13q (between markers D13S787 and D13S252) that includes the CTRP9 gene (Cai et al., 2004). Thus, they suggested that the CTRP9 gene and its genetic alteration may be predictable markers for diabetes in Mexican Americans (Wong et al., 2009). In human subjects, serum CTRP9 has been found to be inversely associated with visceral fat and the presence of metabolic syndrome (Hwang et al., 2013).

Although the underlying mechanisms of NAFLD have not been fully defined, various reports have suggested that ER stress plays a pivotal role in both the development of hepatic steatosis and progression to nonalcoholic steatohepatitis (NASH). The disruption of ER homeostasis leads to hepatic lipid accumulation and apoptosis during altered lipid metabolism (Werstuck et al., 2001). A previous clinical study has reported that NAFLD patients have increased expression of ER stress markers in the liver and other organs (Das et al., 2008). Persistent ER stress results in the accumulation of unfolded proteins, which can subsequently induce apoptosis in hepatocytes (Pfaffenbach et al., 2010). These data indicate that over-nutrition or obesity is sufficient to disrupt ER homeostasis and increase the amount of circulating saturated free fatty acids. Recent studies have reported that ER stress-induced UPR is associated with the regulation of lipogenesis and hepatic steatosis. Sha et al. have demonstrated strong binding affinity between XBP-1 and the promoter regions of various lipogenic genes in mouse embryonic fibroblasts and 3T3-L1 cells (Sha et al., 2009). The phosphorylation of eIF2α induces various target genes including ATF4. ATF4$^{-/+}$ mice are resistant to diet-induced obesity and hepatic steatosis (Seo et al., 2009). Therefore, a successful therapeutic approach to treating NAFLD may be discovered through the exploration of the mechanisms that disrupt ER homeostasis and regulate hepatic ER stress. This study is the first to demonstrate that both *in vitro* and *in vivo* CTRP9 treatment improves hepatic steatosis through the relief of ER stress.

Autophagy is an important intracellular catabolic process that allows cell components such as damaged organelles and unfolded proteins to be degraded by lysosomes. Previous literature has suggested an important link between autophagy and NAFLD. It has been reported that the impairment of autophagic flux is detected in the livers of patients and murine models with NAFLD (Gonzalez-Rodriguez et al., 2014). Autophagy enhancement has a protective effect against hepatic steatosis and insulin sensitivity (Klionsky et al., 2012). The hepatic overexpression of Atg7 was shown to improve fatty liver and insulin resistance in HFD-fed mice and *ob/ob* mice (Yang et al., 2010). Therefore, activation of hepatic autophagy may act as a therapeutic approach to NAFLD. Since autophagy eliminates misfolded and unfolded proteins, its impairment could result in the accumulation of such proteins and the induction of ER stress (Herrero-Martin et al., 2009). Previous studies have shown that stimulation of autophagy relieves ER stress. Bachar-Wikstrom et al. reported that rapamycin reduced ER stress through autophagy activation, thereby decreasing β-cell apoptosis and the subsequent development of diabetes in mice (Bachar-Wikstrom et al., 2013). Autophagy is thought to occur downstream to AMPK activation by resveratrol and metformin (Shi et al., 2012, Wu et al., 2011). AMPK activation directly phosphorylates ULK1 and Beclin-1 and inhibits mTORC1; this pathway leads to the induction of autophagy (Kim et al., 2014). We explored the effects of CTRP9 on ER stress-induced apoptosis and TG accumulation with regard to the AMPK-autophagy pathway. These are the first data demonstrating that CTRP9 induces autophagy and attenuates the palmitate- or tunicamycin-induced ER stress markers. Ultimately, CTRP9 decreases the development of hepatic steatosis and apoptosis. Both compound C and 3 MA significantly blocked the suppressive effects of CTRP9 on ER stress, TG accumulation and

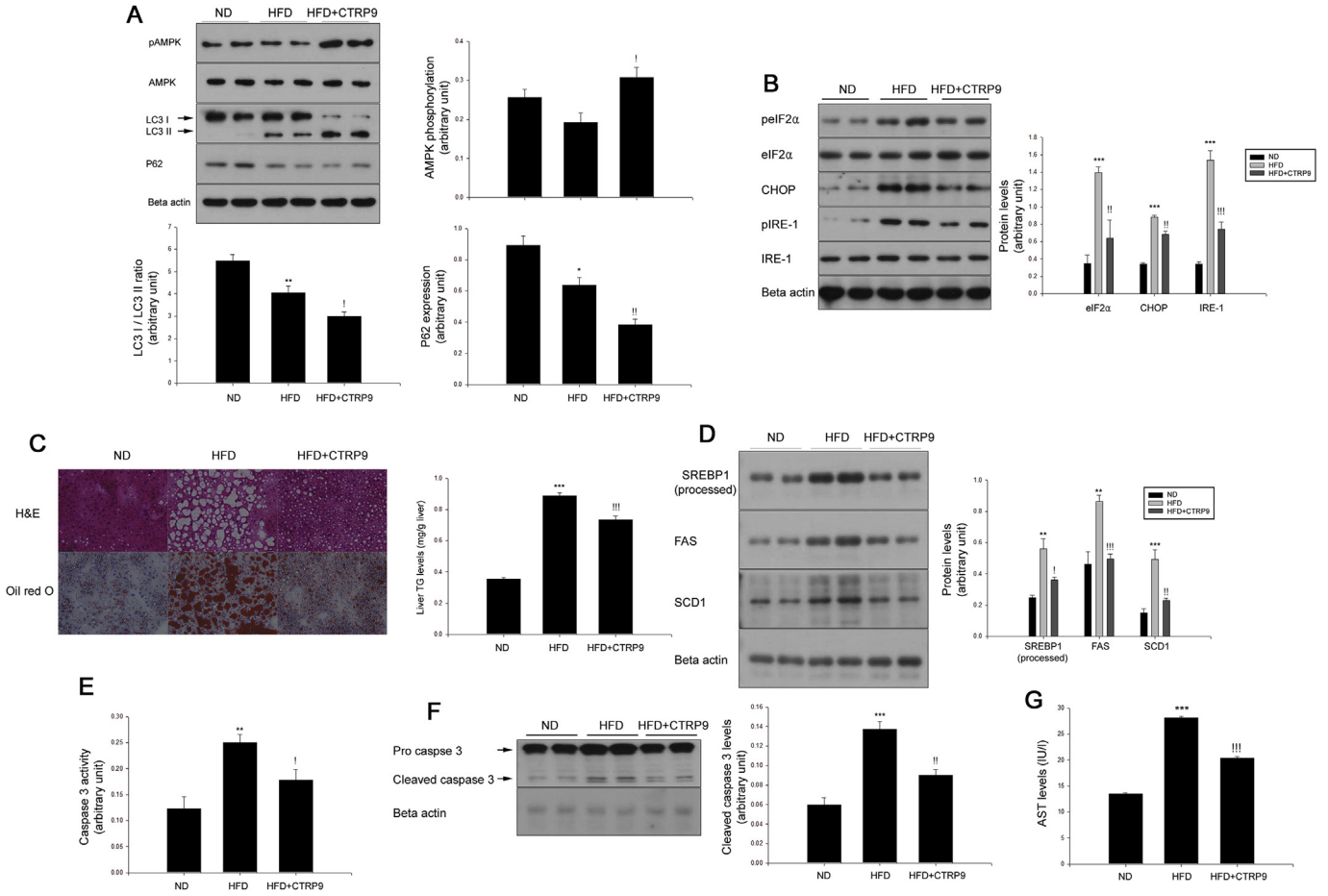

Fig. 7. Adenovirus-mediated overexpression of CTRP9 augments AMPK phosphorylation and autophagy and suppresses HFD-induced ER stress, thereby alleviating HFD-induced hepatic steatosis and apoptosis in liver of mice. (A) AMPK, LC3, and P62 expression levels were determined by Western blot analysis in liver of mice. (B) Various ER stress marker protein expression levels were determined by Western blot analysis in liver of mice. (C) H&E and Oil Red-O staining of liver sections. Hepatic TG accumulation levels were measured by TG extraction kit. (D) Western blot analysis of processed SREBP1, FAS, and SCD1 protein expression levels in liver of mice. (E) Caspase 3 activity was measured using the caspase 3 activity assay in liver of mice. (F) Western blot analysis of cleaved caspase 3 expression levels in liver of mice. (G) Serum AST levels were measured (n = 7 animals per treatment group). $^{***}P < 0.001$, $^{**}P < 0.01$, and $^{*}P < 0.05$ compared to ND. $^{!!!}P < 0.001$, $^{!!}P < 0.01$, and $^{!}P < 0.05$ compared to HFD. Means ± SEMs were calculated from data from seven animals.

apoptosis in hepatocytes. These results support the emerging concept that autophagy and apoptosis are antagonistic processes (Mei et al., 2011). Regardless, these results suggest that CTRP9 treatment is a promising strategy for NAFLD treatment based on induction of AMPK-mediated autophagy.

As a survival mechanism, the UPR signal pathways decrease protein synthesis, including that of various chaperone genes (Schroder et al., 2005). Therefore, we evaluated whether CTRP9 modulated chaperone expression to alleviate ER stress. However, CTRP9 treatment had no influence on chaperone expressions in HepG2 cells.

In conclusion, CTRP9-induced autophagy suppresses palmitate-induced ER stress markers and attenuates hepatic TG accumulation and apoptosis. The data presented here provide evidence that relief of ER stress through AMPK-mediated activation of autophagy by CTRP9 may be a novel therapeutic strategy for the treatment of NAFLD.

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Duality of interest

No conflicts of interest relevant to this article were reported.

Author contributions

TWJ, HCH, KMC: conception, design, experiment, analysis, interpretation of data, drafting of the manuscript, final approval given. HJH, HJY: experiment, analysis, and interpretation of data given. SHB: critical revision of article and final approval given.

Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.mce.2015.09.027.

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