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Basic Science

Adiponectin homolog novel osmotin protects obesity/diabetes-induced NAFLD by upregulating AdipoRs/PPARα signaling in *ob*/*ob* and *db*/*db* transgenic mouse models

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ABSTRACT

Background: In metabolic disorders, adiponectin and adiponectin receptors (AdipoR1/R2) signaling has a key role in improving nonalcoholic fatty liver disease (NAFLD) in obesity-associated diabetes.
Objective: To the best of our knowledge, here, we reported for the first time the underlying mechanistic therapeutic efficacy of the novel osmotin, a homolog of mammalian adiponectin, against NAFLD in leptin-deficient *ob*/*ob* and *db*/*db* mice.
Methods: The *ob*/*ob* and *db*/*db* mice were treated with osmotin at a dose of 5 μg/g three times a week for two weeks. To co-relate the *in vivo* results we used the human liver carcinoma HepG2 cells, subjected to knockdown with small siRNAs of AdipoR1/R2 and PPARα genes and treated with osmotin and palmitic acid (P.A.). MTT assay, Western blotting, immunohistofluorescence assays, and plasma biochemical analyses were applied.
Results: Osmotin stimulated AdipoR1/R2 and its downstream APPL1/PPAR-α/AMPK/SIRT1 pathways in *ob*/*ob* and *db*/*db* mice, and HepG2 cells exposed to P.A. Mechanistically, we confirmed that knockdown of AdipoR1/R2 and PPARα by their respective siRNAs abolished the osmotin activity in HepG2 cells exposed to P.A. Overall, the *in vivo* and *in vitro* results suggested that osmotin protected against NAFLD through activation of AdipoR1/R2 and its downstream APPL1/PPAR-α/AMPK/SIRT1 pathways as shown by the reduced body weight, blood glucose level and glycated hemoglobin, improved glucose tolerance, attenuated insulin resistance and hepatic glucogenesis, regulated serum lipid parameters, and increased fatty acid oxidation and mitochondrial functions.
Conclusion: Our findings strongly suggest that novel osmotin might be a potential novel therapeutic tool against obesity/diabetes-induced NAFLD and other metabolic disorders.

1. Introduction

Obesity-associated diabetes-induced nonalcoholic fatty liver disease (NAFLD) is a common cause of chronic liver diseases. NAFLD is representative of the increasing prevalence of metabolic syndrome [1]. NAFLD represents a spectrum of diseases ranging from simple steatosis to steatohepatitis through fibrosis and cirrhosis and is considered to represent the hepatic component of metabolic syndrome, which is related to insulin resistance and other metabolic risk factors such as diabetes mellitus, central abdominal obesity and dyslipidemia [2,3]. NAFLD has become an area of interest in research due to an increasing incidence of obesity-associated diabetes in both children and adults [4]. NAFLD is strongly associated with obesity, insulin resistance conditions such as diabetes mellitus and certain other features of metabolic syndrome such as elevated triglyceride (TG) and low-density lipoprotein (LDL) levels and reduced high-density lipoprotein (HDL) levels. NAFLD is reported to reduce insulin sensitivity in muscle, liver and adipose tissue [5]. Excessive lipids in pancreatic beta cells dysregulate

Abbreviations: AdipoRs, adiponectin receptors; AdipoR1, adiponectin receptor1; AdipoR2, adiponectin receptor2; AD, Alzheimer's disease; ALT, alanine aminotransferase; AMPK, AMP-activated protein kinase; AST, aspartate aminotransferase; BMI, body mass index; DMSO, dimethyl sulfoxide; FBS, fetal bovine serum; FOXO1, forkhead box protein O1; FFA, free fatty acid; G-6-P, glucose-6-phosphate; GTT, glucose tolerance test; HbA1C, glycated hemoglobin; HDL, high-density lipoprotein; I.P., intraperitoneally; IRS-1, insulin receptor substrate 1; LDL, low-density lipoprotein; MEM, modified Eagle's medium; MTT, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide; NAFLD, nonalcoholic fatty liver disease; PBS, phosphate-buffered saline; PR-5, pathogenesis-related protein family 5; PPAR-α, peroxisome proliferator-activated receptor-alpha; PGC-1α, proliferator-activated receptor-gamma coactivator 1 alpha; p-ACC, phosphorylated acetyl-CoA carboxylase; PEPCK, phosphoenol pyruvate carboxy kinase; siRNA, small interference RNA; TG, triglyceride; WT, wild-type.

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insulin secretion and alter the expression of peroxisome proliferator-activated receptor-alpha (PPAR-α) and glucokinase, ultimately leading to insulin resistance as a result of free fatty acid (FFA)-induced beta cell apoptosis [6]. Conditions such as obesity, diabetes mellitus and hyperlipidemia are the major players that promote NAFLD [4].

Normally, adiponectin levels are high in human serum; however, a decline in the adiponectin level has been observed in obese patients and those with hepatic steatosis [7]. Adiponectin reduces the storage of excess lipids in the liver and provides protection against inflammation and fibrosis due to its insulin-sensitizing property [8]. Established studies have reported that adiponectin and its receptors (AdipoR1/R2) have been associated with various metabolic disorders, such as diabetes, obesity, and cardiovascular and neurodegenerative diseases [9–14]. Moreover, AdipoR1/R2 signaling regulates substrate metabolism through activation of a couple of essential players in cellular energy management including 5, AMP-activated protein kinase (AMPK), sirtuins (SIRT1), and the nuclear receptor PPAR-γ [15,16]. The AdipoR1/R2 regulate age-associated metabolic disorder pathways such as lipid oxidation, glucose uptake and insulin signaling [17]. The osteoanabolic agent GTDF was found to bind to AdipoRs where it enhanced adiponectin signaling and improved the metabolic homeostasis system in rodent diabetes models [18]. Recently, Polyzos et al., reviewed the key role of adipokines in NAFLD and suggested that adipokines approach as noninvasive diagnostic markers of NAFLD and for the therapeutic purpose of NAFLD [19]. Adiponectin is a pleiotropic endogenous adipokine that displays anti-inflammatory and protective activities in various metabolic disorders such as obesity and diabetes [1,20]. Numerous ligands of AdipoRs have been reported, and among them, osmotin is the most accessible and interesting natural plant-derived novel tool and acts as a ligand of AdipoRs. Osmotin, previously known as an antimicrobial protein associated with the plant defense system and a member of the pathogenesis-related protein family 5 (PR-5), is reported to be a structural and functional homolog of the mammalian adiponectin protein [21,22]. Recently, osmotin was reported to possibly act as a homolog of the mammalian hormone adiponectin and induce similar effect in various *in vitro* and *in vivo* models [23]. Moreover, we recently reported that osmotin acts *via* AdipoRs and has neuroprotective properties against metabolic disorders and age-associated neurological disorders such as Alzheimer's disease (AD) [24–26]. Metabolic disorders and age-associated neurological disorders are closely related; AD is even referred to as type 3 diabetes [27]. Further recently a few exciting studies explored the protective effect of osmotin in HUVEC cells and 9Hc2 cells *via* activation of the AdipoRs [28,29]. Therefore, we hypothesized that osmotin might have beneficial therapeutic effects on the obesity- and diabetes-associated metabolic disorder NAFLD *via* regulating AdipoR1/R2 and its downstream APPL1/PPAR-α/AMPK/SIRT1 signaling in leptin-deficient *ob*/*ob* and *db*/*db* mice and that all of these changes in signaling were verified mechanistically in human liver carcinoma HepG2 cells exposed to palmitic acid *in vitro*.

Table 1
Primary antibodies information used in the Western blotting (WB) and immunofluorescence (IF) staining.

Antibody	Host	Application	Manufacturer	Catalog Number	Concentration
AdipoR2	Mouse	IF/WB		SC-514045	1;100/1:1000
APPL1	Rabbit	IF/WB		SC-67402	1;100/1:1000
SIRT1	Rabbit	WB		SC-15404	1:1000
p-AMPK	Rabbit	WB		SC-33524	1:1000
PGC-1α	Rabbit	WB		SC-13067	1:1000
PPARα	Rabbit	IF/WB		SC-9000	1:100/1:1000
p-FOXO1	Rabbit	IF/WB		SC-101681	1:100/1:1000
PEPCK	Rabbit	WB	Santa Cruz Biotechnology, USA	SC-32879	1:1000
p-IRS-1 Ser 636	Rabbit	WB		SC-33957	1:1000
IRS-1	Rabbit	WB		SC-559	1:1000
p-Akt	Rabbit	WB		SC-7985	1:1000
p-PI3K	Rabbit	WB		SC-2931	1:1000
β–Actin	Mouse	WB		SC-47778	1:2000
p-ACCβ Ser219/221	Rabbit	WB		SC-30446	1:2000
ACCβ	Mouse	WB		SC-337313	1:2000
G-6-P	Rabbit	WB/IF	ABCAM, USA	AB83690	1:1000/1:100
AdipoR1	Rabbit	WB/IF		AB126611	1:1000/1:100
Foxo1	Rabbit	WB		2880S	1:1000
AMPK	Rabbit	WB		2532S	1:1000
PI3K	Rabbit	WB	Cell Signaling, USA	4228S	1:1000
Akt	Rabbit	WB		9272S	1:1000

2. Materials and Methods

2.1. Osmotin Purification

Osmotin was purified from salt-adapted cultured *Nicotiana tabacum* cells. The detail methods for the osmotin isolation and purification are described in the supplementary information.

2.2. Animal Genotyping, Housing, Ethical Considerations, Grouping and Drug Treatment

The mice used, including wild-type (WT) 7-month-old male (C57BL/6J) mice with a body weight of (22 ± 1.5 g), 7-month-old male leptin-deficient obese (*ob/ob*) mice (B6.cg.Lepr. <Ob>/J) with a body weight of (49.6 ± 1.5 g), and diabetes (*db/db*) mice (B6.BKS (D) Lepr, db>/J) with a body weight of (47.4 ± 2.0 g), were purchased from Jackson's laboratory. All mice were housed in the soft wood bedding materials at a control temperature of (22 ± 2 °C), relative (55 ± 5%) humidity with a 12-h light and dark cycle. All mice were kept with restricted access to normal food and free access to fresh water. The maintenance and treatment of the mice were carried out in accordance with the Institutional Animal Care and Use Committee (IACUC) guidelines issued by the Division of Applied Life Science, Gyeongsang National University, South Korea. All efforts were made to minimize the suffering of animals. The experimental methods with mice were carried out in accordance with the approved guidelines (Approval ID: 125), and all experimental protocols were approved by the IACUC of the Division of Applied Life Science, Gyeongsang National University, South Korea. The mice were randomly divided into five groups ($n = 12$ for each group): Veh-treated WT mice

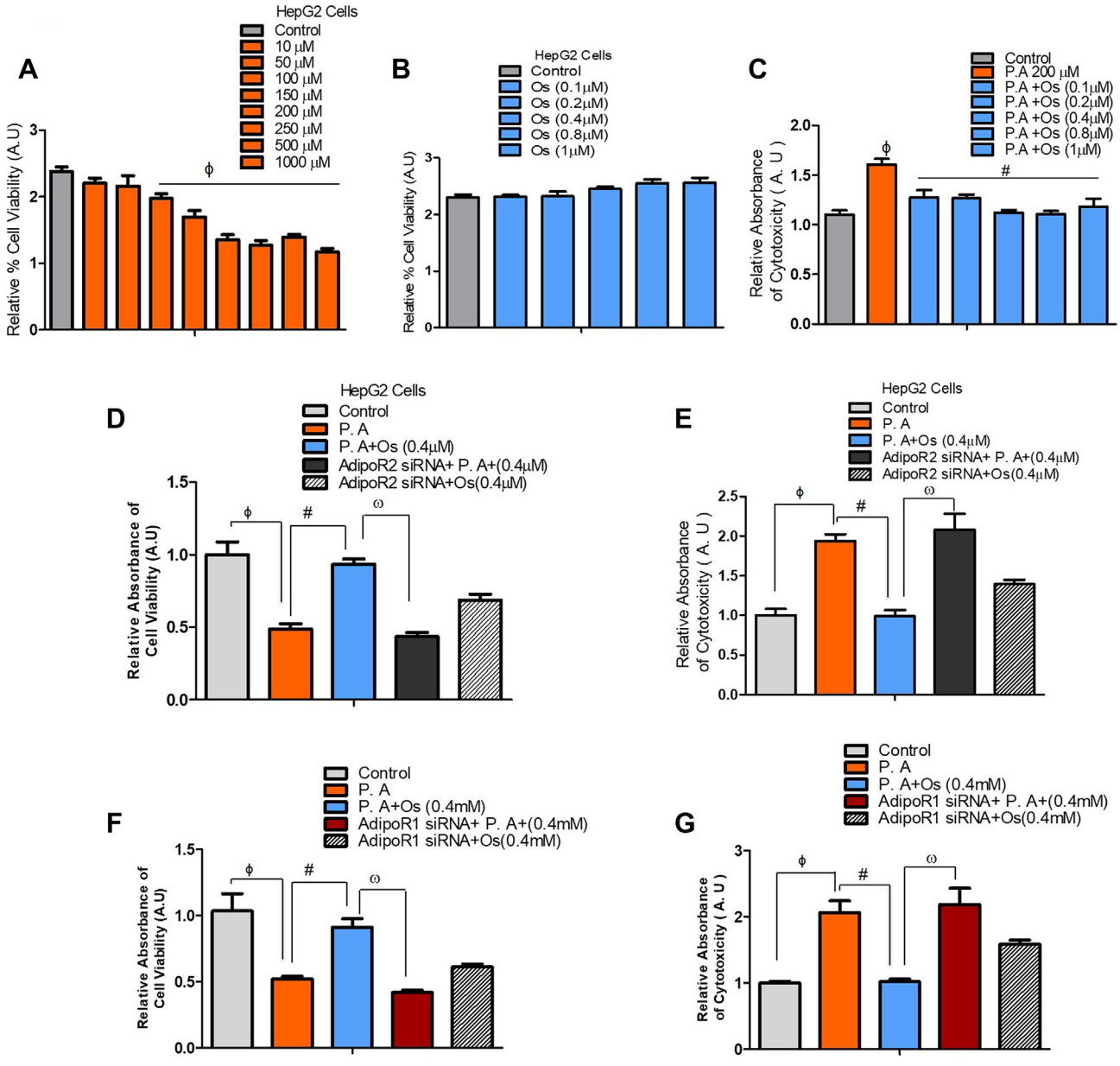


Fig. 1. Osmotin acted *via* AdipoR1/R2-dependent mechanism to reduce palmitic acid-induced toxicity *in vitro*. (A) MTT assay of HepG2 cells exposed to different concentrations of palmitic acid (10 μM–1000 μM) for 12 h. Cell viability was gradually reduced with increasing palmitic acid concentrations. (B) Histogram showing that osmotin at various concentrations (0.1, 0.2, 0.4, 0.8 and 1 μM) increased HepG2 cell viability. (C) Histogram illustrating that osmotin at five different concentrations (0.1, 0.2, 0.4, 0.8 and 1 μM) significantly decreased the cytotoxic effect of palmitic acid in HepG2 cells. (D–G) The relative cell viability and relative cytotoxicity of HepG2 cells subjected to AdipoR1/R2 siRNAs and treated with palmitic acid (200 μM) and osmotin (4 μM). $N = 3$ independent experiments. The data are expressed as the means ± SEM. Significance, $p < 0.05$. ϕ compared to control; # compared to palmitic acid. Palmitic acid = P.A. Osmotin = Os.

as a control for the *ob*/*ob* and *db*/*db* mice, Veh-treated *ob*/*ob* mice, osmotin-treated *ob*/*ob* mice, Veh-treated *db*/*db* mice, and osmotin-treated *db*/*db* mice. Osmotin was injected intraperitoneally (I.P.) at a dose of 5 μg/g of body weight three times a week for two weeks duration.

2.3. Analysis of Body Weight and Body Mass Index (BMI) of ob/ob and db/db Mice

The body weights and BMI were checked every other day after Veh and osmotin injection. BMI (weight divided by length squared) as well as the waist circumference and body length from tail to nose were regularly measured over the course of the last two weeks to evaluate the difference in the deposited fats between the Veh-treated *ob*/*ob* and *db*/*db* mice and osmotin-treated mice.

2.4. Glucose Tolerance Test (GTT)

The blood glucose level after 4 h of fasting was measured every other day with blood taken from the tail vein using a glucometer for two weeks. While mice were maintained on fasting conditions for 8 h, a glucose (20%) solution (2 mg/g of mice) was injected i.p., and blood samples were collected by pricking the tail vein at 0 (baseline),15, 30, 60, 90 and 120 min through a glucometer (OneTouch UltraMini; LifeScan, Milpitas, CA). The data were plotted as blood glucose concentrations over time.

2.5. Biochemical Analysis

For biochemical analysis, mice were deeply anesthetized, and blood samples were obtained by cardiac puncture of the right atrium. Blood serum was obtained by centrifugation (3000 rpm for 10 min) at room temperature and stored at −20 °C until it was sent to SCL Labs for biochemical evaluations to measure TC, TG, HDL, LDL, FFA, AST and ALT levels as well as HbA1C analysis.

2.6. Protein Collection and Western Blotting

Western blot analysis was performed according to the previously described method according our previous protocols (the details are described in the supplementary information) [24].

2.7. Antibodies

The details of the antibodies used in this study are described in Table 1.

2.8. Tissue Collection for Morphological Analysis

The liver tissue samples ($n = 5$/group) were prepared as we described previously according our previous protocols (the details are described in the supplementary information) [24].

2.9. Immunofluorescence Staining

Immunofluorescence staining was performed according to the previously described method according our previous protocols (the details are described in the supplementary information) [24,25].

2.10. Immunohistological Hematoxylin and Eosin (H&E) Staining

The details are described in the supplementary information.

2.11. HepG2 Cells Culture and Treatment

The human hepatocellular carcinoma (HepG2) cell line was cultured in modified Eagle's medium (MEM) supplemented with 10% (v/v) fetal bovine serum (FBS) (Gibco, CA, USA) and 1% penicillin-streptomycin antibiotics. The cells were kept at 37 °C in a humidified incubator with 5% CO_2 in a 95% humidified atmosphere. Palmitic acid was dissolved in DMSO at a concentration of 10 mM (stock solution) and then filtered. After reaching 70% confluence, the cells were treated with palmitic acid (200 μM), palmitic acid (200 μM) + osmotin (0.4 μM), or DMSO

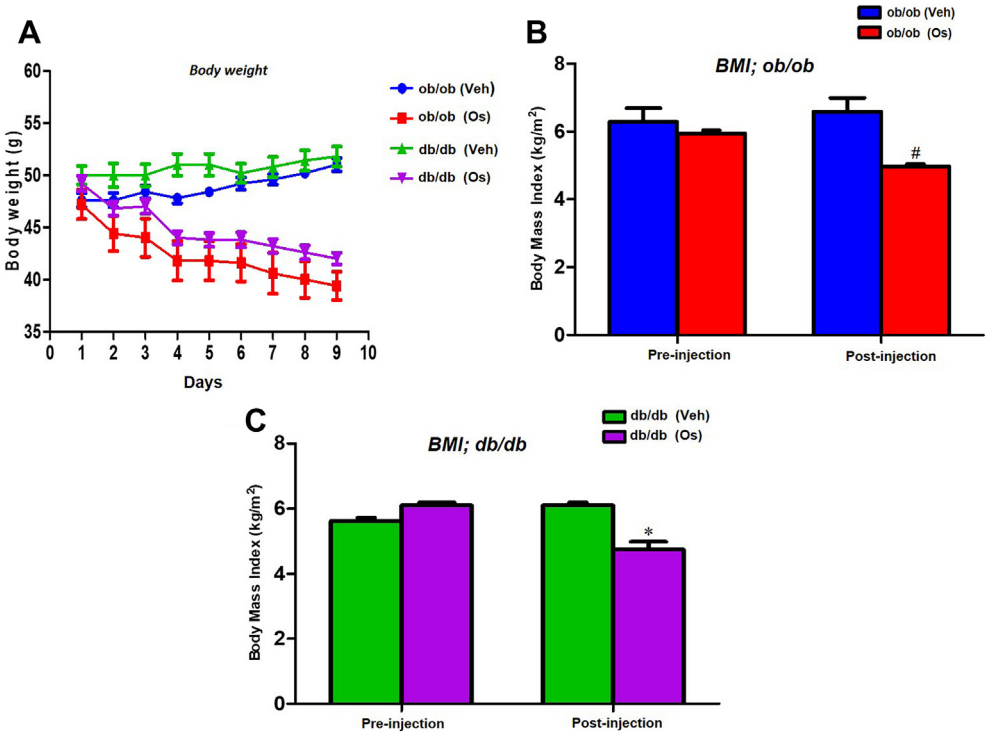


Fig. 2. Beneficial effect of osmotin on body weight and BMI of *ob*/*ob* and *db*/*db* mice. (A) Histogram represents the body weight of the mice. (B, C) Histogram represents the body mass index. The data collected are expressed as the mean ± SEM ($N = 12$ mice/group). Significance, $p < 0.05$. # compared to Veh-treated *ob*/*ob* mice, * compared to Veh-treated *db*/*db* mice.

(0.01%) (Control group) and incubated for 12 h. We proceeded accordingly to perform Western blotting and other *in vitro* experimental analyses.

2.12. HepG2 Cells Transfection and Gene Silencing by Small Interference RNA (siRNA)

HepG2 cells were seeded in 6-well culture plates at a density of 1×10^5 cells/ml. When the confluence reached 70–75%, the HepG2 cells were transfected with AdipoR1/R2-specific (h) (SC-60123/SC-60125) and PPAR-α-specific (h) (SC-36307) (Santa Cruz Biotechnology, Inc.) gene-silencing siRNA at a concentration of 10 μM per transfection for 72 h according to the manufacturer's protocol (Santa Cruz Biotechnology, Inc.) using Lipofectamine 3000 (Life Technologies). Negative siRNA (Ambion, USA) was used as a control. After 72 h of transfection, the cells were treated with palmitic acid (200 μM) and osmotin (0.4 μM) for 12 h, and the cell lysate was further processed for Western blotting.

2.13. MTT Assay

The colorimetric MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide) assay was used to measure the cell viability after treatment with different concentrations of palmitic acid and osmotin. The details are described in the supplementary information.

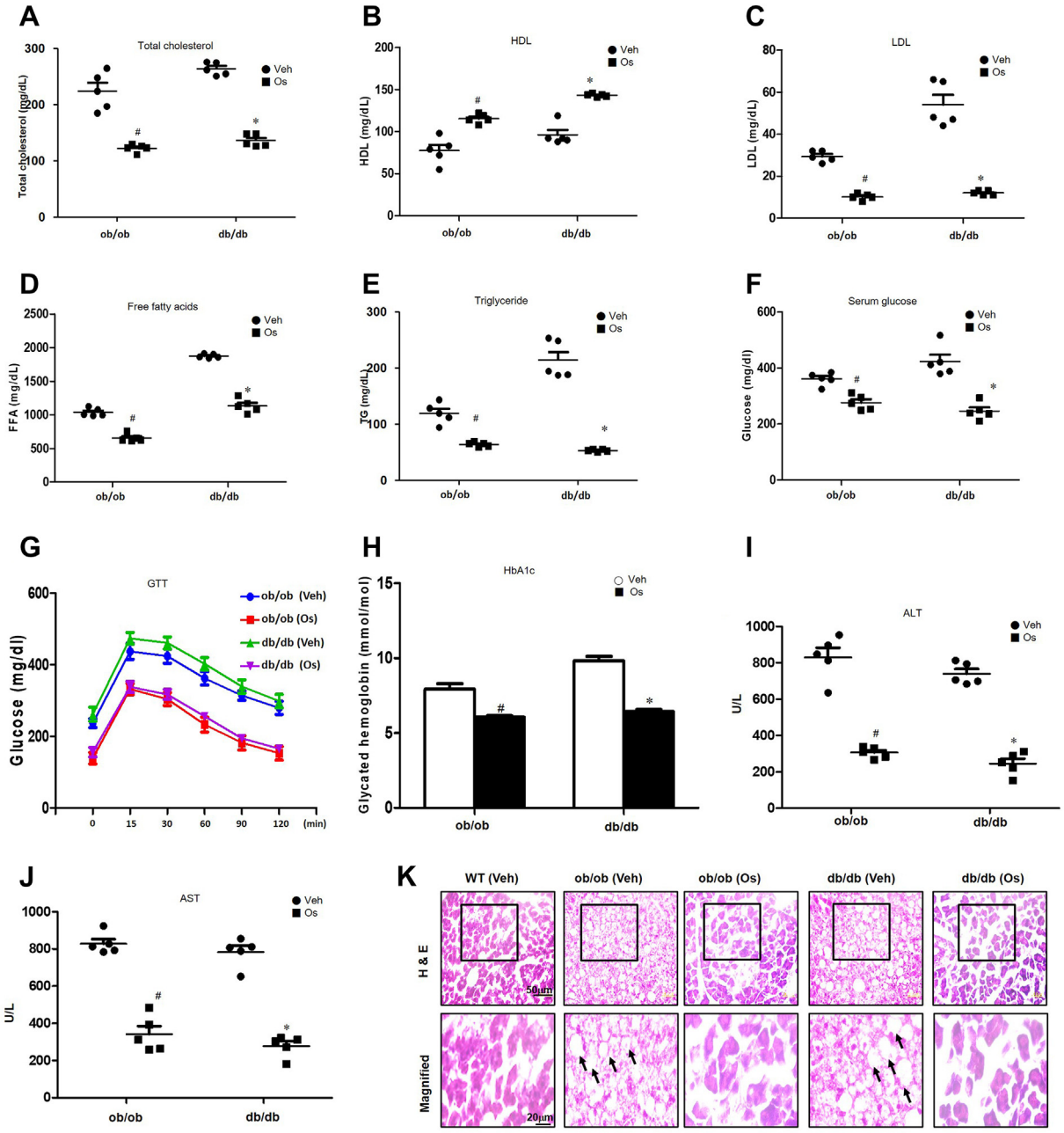
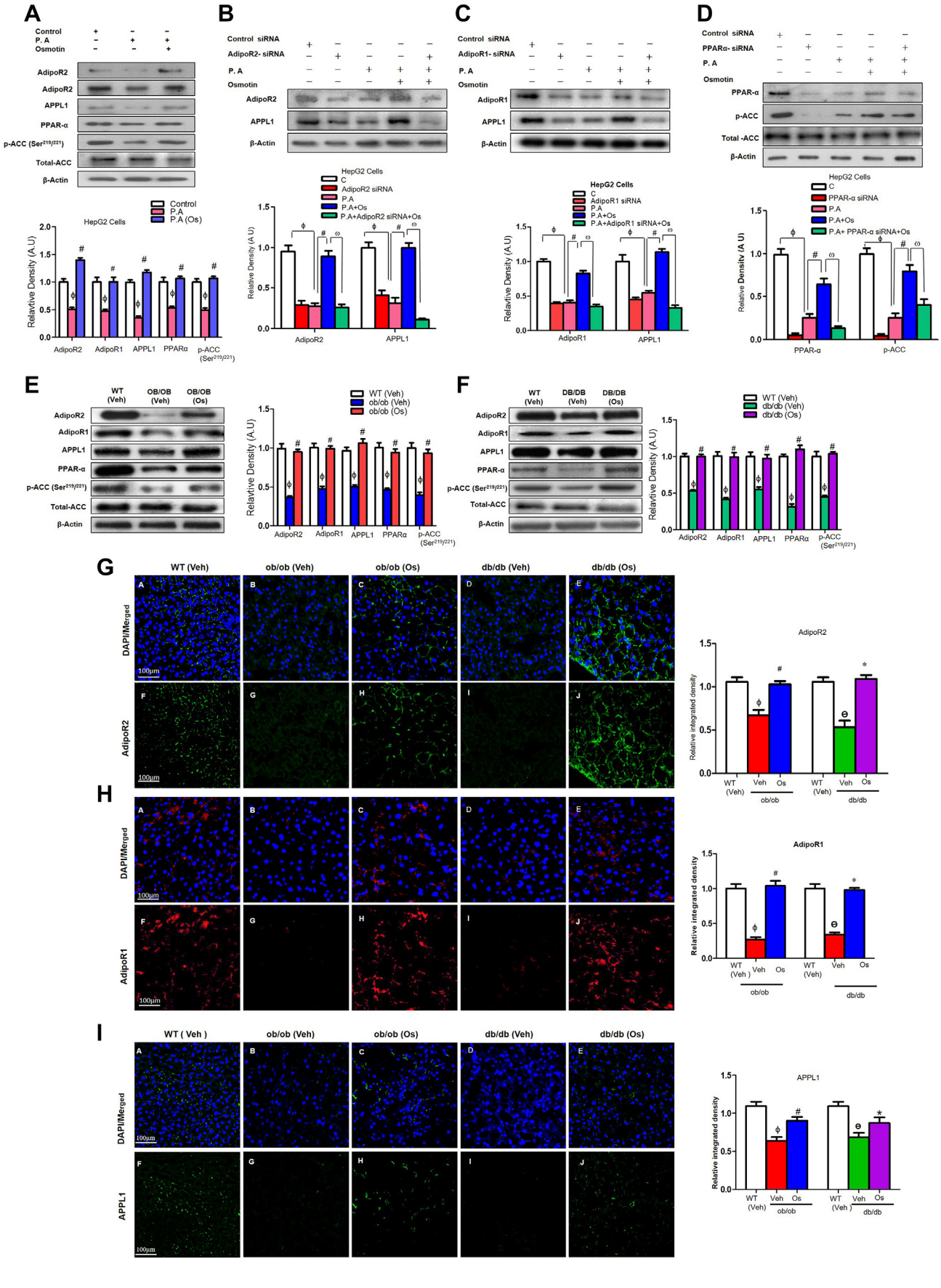


Fig. 3. Osmotin regulated the impaired metabolic parameters and hepatosteatosis in *ob*/*ob* and *db*/*db* mice. (A–E) Histograms show the TC, HDL, LDL, FFA and TG levels, respectively. (F–H) Histogram illustrates the measured blood glucose levels, GTT levels and HbA1C levels, respectively. (I, J) Histograms depict the serum levels of hepatic enzymes (AST, ALT), respectively. (K) The representative immunohistochemical (HE-stained) images of the liver of Veh-treated WT, Veh-treated *ob*/*ob* mice, osmotin-treated *ob*/*ob* mice, Veh-treated *db*/*db* mice, and osmotin-treated *db*/*db* mice. $n = 5$ pups/group, and the number of experiments = 3. Magnification 20×. Scale bar = 50 and 20 μm respectively. The biochemical data associated with metabolic parameters collected are expressed as the mean ± SEM (N = number 12 mice/group). Significance, $p < 0.05$. # compared to Veh-treated *ob*/*ob* mice and * compared to Veh-treated *db*/*db* mice.


A
Control
P. A
Osmotin
AdipoR2
AdipoR2
APPL1
PPAR-α
p-ACC (Ser219/221)
Total-ACC
β-Actin
HepG2 Cells
Control
P.A
P.A (Os)
Relavtive Density (A.U)
AdipoR2
AdipoR1
APPL1
PPARα
p-ACC (Ser219/221)
B
Control siRNA
AdipoR2- siRNA
P. A
Osmotin
AdipoR2
APPL1
β-Actin
HepG2 Cells
C
AdipoR2 siRNA
P.A
P.A+Os
P.A+AdipoR2 siRNA+Os
Relative Density (A.U)
AdipoR2
APPL1
C
Control siRNA
AdipoR1- siRNA
P. A
Osmotin
AdipoR1
APPL1
β-Actin
HepG2 Cells
C
AdipoR1 siRNA
P.A
P.A+Os
P.A+AdipoR1 siRNA+Os
Relavtive Density (A.U)
AdipoR1
APPL1
D
Control siRNA
PPARα- siRNA
P. A
Osmotin
PPAR-α
p-ACC
Total -ACC
β-Actin
HepG2 Cells
C
PPAR-α siRNA
P.A
P.A+Os
P.A+ PPAR-α siRNA+Os
Relative Density (A.U)
PPAR-α
p-ACC
E
WT (Veh)
OB/OB (Veh)
OB/OB (Os)
AdipoR2
AdipoR1
APPL1
PPAR-α
p-ACC (Ser219/221)
Total-ACC
β-Actin
WT (Veh)
ob/ob (Veh)
ob/ob (Os)
Relavtive Density (A.U)
AdipoR2
AdipoR1
APPL1
PPARα
p-ACC (Ser219/221)
F
WT (Veh)
DB/DB (Veh)
DB/DB (Os)
AdipoR2
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PPAR-α
p-ACC (Ser219/221)
Total-ACC
β-Actin
WT (Veh)
db/db (Veh)
db/db (Os)
Relavtive Density (A.U)
AdipoR2
AdipoR1
APPL1
PPARα
p-ACC (Ser219/221)
G
WT (Veh)
ob/ob (Veh)
ob/ob (Os)
db/db (Veh)
db/db (Os)
DAPI/Merged
AdipoR2
100μm
AdipoR2
Relative integrated density
WT (Veh)
Veh
Os
ob/ob
db/db
H
DAPI/Merged
AdipoR1
100μm
AdipoR1
Relative integrated density
I
WT (Veh)
ob/ob (Veh)
ob/ob (Os)
db/db (Veh)
db/db (Os)
DAPI/Merged
APPL1
100μm
APPL1
Relative integrated density

2.14. Statistical Analysis

Western blot bands were scanned, and the optical densities of the bands were measured using computer-based Sigma Gel Software (Jandel Scientific, San Rafael, Chicago, USA). ImageJ software was used for quantitative immunohistological analysis. All biochemical and Western blot band optical densities were analyzed by one-way ANOVA followed by *post hoc* Bonferroni multiple comparison test using GraphPad Prism 5 software. The expressed data are presented as the means ± SEM of the three independent experiments. The acceptance level for statistical significance of each group was $p < 0.05$.

3. Results

3.1. Osmotin Acted via AdipoR1/R2-dependent Mechanism to Reduce Palmitic Acid-induced Toxicity In Vitro

To optimize the palmitic acid *in vitro* model using human liver carcinoma HepG2 cells, we first treated HepG2 cells with various concentrations of palmitic acid for 12 h. Our results showed that palmitic acid at 100 μM, 200 μM, 500 μM and 1000 μM remarkably decreased HepG2 cell viability (Fig. 1A), while osmotin treatment alone at various concentrations (0.1 μM, 0.2 μM, 0.4 μM, 0.8 μM, and 1 μM) had no toxic effect and improved HepG2 cell viability (Fig. 1B). Further, we co-treated cells with different concentrations of osmotin (0.1, 0.2, 0.4, 0.8 and 1 μM) and palmitic acid (200 μm). The results indicated that osmotin at all concentrations showed a significant protective effect on HepG2 cell viability and abrogated the cytotoxicity induced by palmitic acid (Fig. 1C). To determine the mechanism of the protective effect of osmotin against palmitic acid in HepG2 cells, we used AdipoR1/R2 siRNAs. The cell viability and cytotoxicity results indicated that osmotin (4 μM) was unable to reduce the palmitic acid-induced toxicity in AdipoR1/R2 siRNA-treated HepG2 cells (Fig. 1D–G). These *in vitro* results conferred that osmotin induces its beneficial protective effect *via* AdipoR1/R2 signaling.

3.2. Beneficial Effect of Osmotin on Body Weight and BMI of ob/ob and db/db Mice

Several pieces of evidence have suggested that adiponectin and AdipoRs signaling induce beneficial effects on body weight and BMI [10,13,14]. Herein, we investigated whether osmotin treatment affected body weight and BMI in *ob*/*ob* and *db*/*db* mice. Interestingly, osmotin administration significantly reduced the body weight ($p < 0.05$) and BMI ($p < 0.05$) of the *ob*/*ob* and *db*/*db* mice relative to the body weight and BMI of the Veh-treated *ob*/*ob* and *db*/*db* mice (Fig. 2A–C).

3.3. Osmotin Regulated the Impaired Metabolic Parameters and Hepatosteatosis in ob/ob and db/db Mice

Numerous literature surveys have shown that adiponectin deficiency and AdipoR signaling impairment lead to abnormal plasma and hepatic biochemical parameters. Hence, next, we investigated the lipid parameters in the *ob*/*ob* and *db*/*db* mice. Our biochemical results showed elevated TC, LDL, FFA, and TG levels and reduced HDL content in the Veh-treated *ob*/*ob* and *db*/*db* mice compared to those in the Veh-treated WT mice, while osmotin-treated *ob*/*ob* and *db*/*db* mice exhibited a significantly improved TC clearance indicated by a lower TC level, a higher expression level of HDL ($p < 0.05$), and lower LDL ($p < 0.05$), FFA ($p < 0.05$), and TG ($p < 0.05$) levels compared to the respective Veh-treated mice (Fig. 3A–E). Further, to explore the protective effect of osmotin on the blood glucose levels, a glucose tolerance test (GTT) and glycated hemoglobin (HbA1C) analysis were performed. Our results showed increased blood glucose, GTT and HbA1C levels in the Veh-treated *ob*/*ob* and *db*/*db* mice compared to those in the Veh-treated WT mice (Fig. 3F–H), while osmotin treatment significantly ($p < 0.05$) reduced the blood glucose, GTT and HbA1C levels in the *ob*/*ob* and *db*/*db* mice. Further, we found higher serum levels of ALT and AST in the Veh-treated *ob*/*ob* and *db*/*db* mice than in the Veh-treated WT mice, while osmotin supplementation reversed the upregulated levels of AST ($p < 0.05$) and ALT ($p < 0.05$) in osmotin-treated *ob*/*ob* and *db*/*db* mice ($p < 0.05$) compared to those in the Veh-treated *ob*/*ob* and *db*/*db* mice (Fig. 3I, J). Further, these metabolic parameters associated with NAFLD were conferred by the histological examination of liver tissue (Fig. 3K). We observed hepatosteatosis in the Veh-treated *ob*/*ob* and *db*/*db* mice compared to the Veh-treated WT mice. Osmotin treatment to *ob*/*ob* and *db*/*db* mice intervene the severity of hepatosteatosis and prevents the hepatosteatosis in the *ob*/*ob* and *db*/*db* mice.

3.4. Osmotin Upregulated the AdipoRs/PPARα Pathway and Its Downstream Signaling Both in Palmitic Acid-exposed HepG2 Cells and in ob/ob and db/db Mice

According to the previous literature indicating that osmotin acts similar to adiponectin *via* AdipoRs, our *in vitro* results showing the protective effects of osmotin *via* AdipoRs, and the above beneficial effects of osmotin, we further evaluated the effect of osmotin on AdipoRs signaling and its downstream mediators *via* Western blotting of *in vitro* HepG2 cells exposed to palmitic acid. We found reduced expression levels of AdipoR1/R2 and APPL1 in palmitic acid (200 μM)-exposed groups compared to those in the control group. Interestingly, 12 h of osmotin (0.4 μM) treatment overcame the palmitic acid effect and significantly increased the expression levels of AdipoR1/R2 and APPL1 compared to exposure to palmitic acid alone (Fig. 4A–C). Furthermore, to biochemically examine the mechanistic approach and ability of osmotin to activate AdipoR1/R2 and APPL1, HepG2 cells were transfected with AdipoR1/R2 siRNA to knockdown the expression of AdipoR1/R2 in HepG2 cells. Notably, under the AdipoR1/R2 knockdown conditions, osmotin (0.4 μM) was unable to induce AdipoR1/R2 activation. Similarly, AdipoR1/R2 silencing further decreased the expression levels of APPL1 when cells were co-treated with osmotin (0.4 μM) and palmitic acid (200 μM) (Fig. 4B, C). Next, we examined expression of the downstream markers PPARα and phosphorylated acetyl-CoA carboxylase (p-ACC) in the HepG2 cells *in vitro*. The Western blot results revealed that cells treated with palmitic acid expressed lower levels of PPARα and p-ACC than non-treated HepG2 cells. Osmotin reversed the palmitic acid effect, and cells treated with osmotin had significantly higher expression levels of PPARα and p-ACC than those exposed to palmitic acid alone (Fig. 4A, D). Next, we used PPARα siRNA to evaluate the modulatory role of osmotin in the HepG2 cells exposed to palmitic acid. Knockdown of PPARα expression in HepG2 cells with PPARα siRNA significantly abrogated the effects of osmotin and further decreased PPARα expression when cells were co-treated with PPARα siRNA and palmitic acid (Fig. 4D). In addition, PPARα silencing

Fig. 4. Osmotin upregulated the AdipoRs/PPARα pathway and its downstream signaling both *in vitro* and *in vivo*. (A) Western blot and densitometric analysis of AdipoR1/R2, APPL1, PPARα, p-ACCβ and ACCβ in HepG2 cell lysates. β-Actin was used as the loading control. (B–D) Western blot analysis and their respective histograms of AdipoR1/R2/APPL1 and PPARα/p-ACC in the lysates of HepG2 cells exposed to palmitic acid and osmotin and subjected to siRNA-induced silencing of AdipoR1/R2 and PPARα. β-Actin was used as the loading control. The data collected are expressed as the mean ± SEM for the indicated proteins ($n = 5$/group). (E, F) Western blot analysis of the liver tissue homogenates of *ob*/*ob* and *db*/*db* mice using AdipoR1/R2, APPL1, PPARα and p-ACCβ/ACCβ antibodies. β-Actin was used as the loading control. All of the density values are expressed in arbitrary units (A.U.). The data collected are expressed as the mean ± SEM for the indicated proteins ($n = 7$ mice/group). (G–I) Shown are the immunofluorescence images of AdipoR1/R2 and APPL1 staining along with their relative integrated density histograms in the liver of the Veh-treated WT, Veh-treated *ob*/*ob* mice, osmotin-treated *ob*/*ob* mice, Veh-treated *db*/*db* mice, and osmotin-treated *db*/*db* mice. The magnification 40×. The images represents for $n = 5$ mice/group. Significance, $p < 0.05$. ϕ compared to WT Veh-treated and # compared to Veh-treated *ob*/*ob*; Θ compared to WT Veh-treated and * compared to Veh-treated *db*/*db* mice. ϕ compared to WT Veh-treated and # compared to Veh-treated *ob*/*ob*; Θ compared to Veh-treated WT and * compared to Veh-treated *db*/*db* mice. Similarly, for *in vitro* studies, ϕ compared to control; # compared to palmitic acid; ω compared to palmitic acid + osmotin.

further abolished the ability of osmotin to upregulate the expression level of the p-ACC protein in cells co-treated with palmitic acid (Fig. 4D). Similarly, in addition to the beneficial effects on body weight, BMI and biochemical parameters, the results of the *in vivo* studies indicated that *ob*/*ob* and *db*/*db* mice treated with osmotin at a dose of 5 μg/g had significantly higher expression levels of AdipoR1/R2 and APPL1 as well PPARα and p-ACC than the respective Veh-treated mice (Fig. 4E, F). The immunofluorescence results showed reduced reactivity of AdipoR1/R2 and APPL1 in both Veh-treated *ob*/*ob* and *db*/*db* mice ($p < 0.05$) compared to that in the Veh-treated WT mice, while *ob*/*ob* and *db*/*db* mice given osmotin had significantly higher expression levels of AdipoR1/R2 ($p < 0.05$) and APPL1 ($p < 0.05$) than the respective Veh-treated mice (Fig. 4G–I).

3.5. Osmotin Activated AMPK and Its Downstream Molecules (SIRT1, PGC-1α) Both in Palmitic Acid-exposed HepG2 Cells and in ob/ob and db/db Mice

Adiponectin acts through AdipoRs signaling to promote the activation of the central energy sensor p-AMPK and facilitate lipid metabolism in cultured bovine hepatocytes by promoting lipid oxidation, suppressing lipid synthesis and decreasing hepatic lipid accumulation [30]. To determine p-AMPK activation, we exposed HepG2 cells to palmitic acid (200 μM) and osmotin (0.4 μM) for 12 h. The Western blot results indicated that osmotin significantly increased the expression level of p-AMPK and its downstream markers SIRT1 and peroxisome proliferator-activated receptor-gamma coactivator 1 alpha (PGC-1α) compared to treatment with palmitic acid alone (Fig. 5A–C). Furthermore, to confirm that osmotin activated the p-AMPK/SIRT1/PGC-1α pathway *via* an AdipoR1/R2-dependent manner, we used AdipoR1/R2 siRNA in the HepG2 cells. The Western blot results showed that after silencing AdipoR2, the expression levels of p-AMPK and its downstream markers SIRT1/PGC-1α were significantly lower in the siRNA-transfected and palmitic acid-treated cells compared to those in the control group (Fig. 5B, C). In contrast, cells treated with osmotin and palmitic acid exhibited enhanced expression of p-AMPK and SIRT1/PGC1-α relative to those treated with AdipoR1/R2 siRNA, palmitic acid and osmotin (Fig. 5B, C). To correlate these results *in vitro*, we next investigated the p-AMPK, SIRT1, PGC-1α levels in the liver of *ob*/*ob* and *db*/*db* mice. Western blot results showed a significantly decreased ($p < 0.05$) expression level of p-AMPK, SIRT1 and PGC-1α in the Veh-treated *ob*/*ob* and *db*/*db* mice compared to that in the Veh-treated WT mice. However, osmotin-treated *ob*/*ob* and *db*/*db* mice expressed significantly ($p < 0.05$) higher levels of p-AMPK, SIRT1 and PGC-1α than Veh-treated *ob*/*ob* and *db*/*db* mice, respectively (Fig. 5D, E).

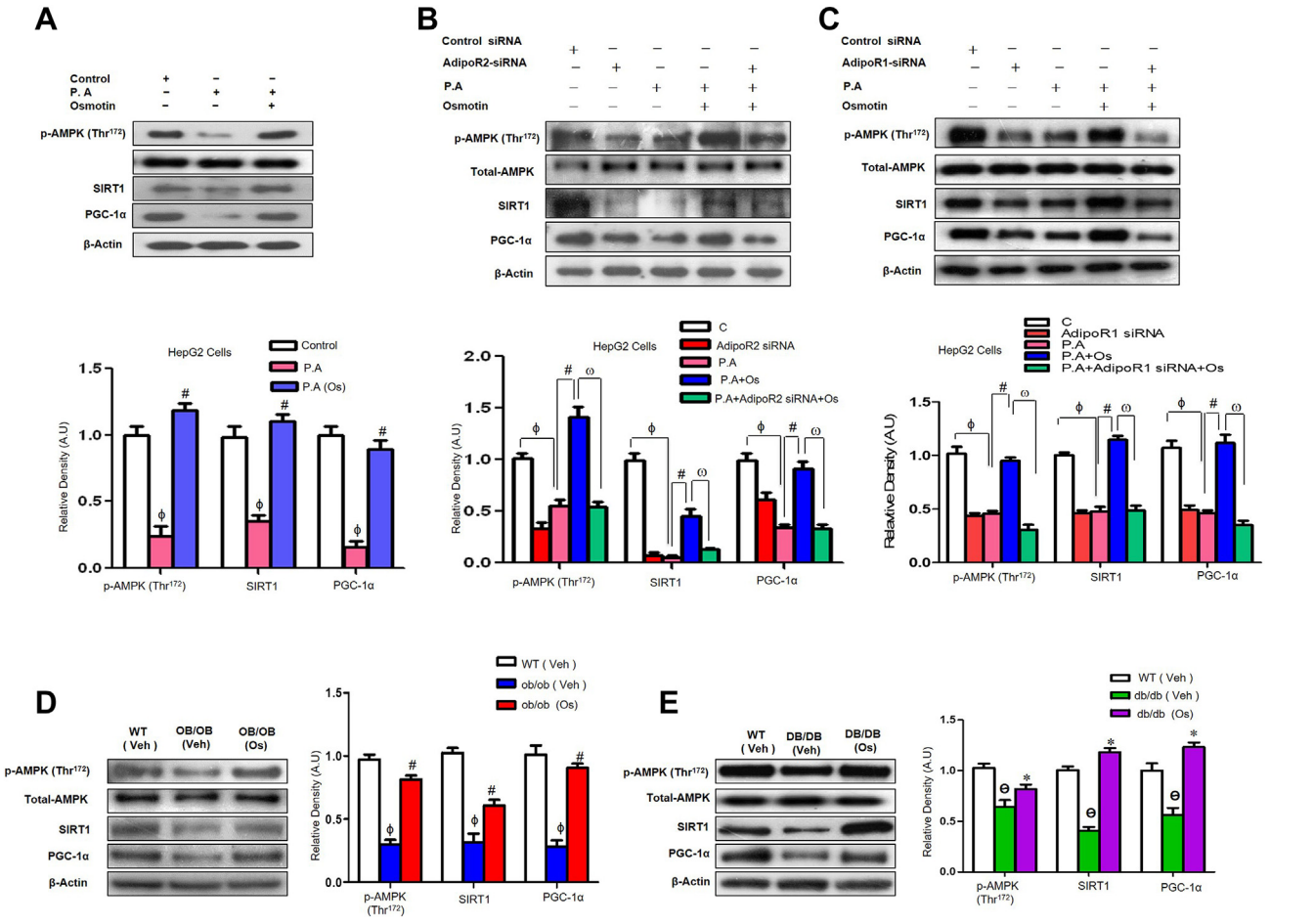

Fig. 5. Osmotin activated AMPK and its downstream molecules (SIRT1, PGC-1α) both *in vitro* and *in vivo*. (A) Western blot and densitometric analysis of p-AMPK, AMPK, SIRT1 and PGC-1α in HepG2 cell lysates. β-Actin was used as the loading control. (B, C) Western blot analysis and respective density histograms of p-AMPK/AMPK, SIRT1 and PGC-1α in lysates of HepG2 cells subjected to AdipoR1/R2 small interfering RNA (siRNA) targeting p-AMPK/AMPK, SIRT1 and PGC-1α. β-Actin was used as the loading control. Density values are expressed in arbitrary units (A.U.). The data collected are expressed as the mean ± SEM for the indicated proteins ($n = 5$/group). (D, E) Western blot analysis in the liver of *ob*/*ob* and *db*/*db* mice using anti-p-AMPK, anti-SIRT1 and anti-PGC-1α antibodies. β-Actin was used as the loading control. Significance, $p < 0.05$. ϕ compared to Veh-treated WT and # compared to Veh-treated *ob*/*ob*; Θ compared to WT Veh-treated and * compared to Veh-treated *db*/*db* mice. Similarly, for *in vitro* studies, ϕ compared to control; # compared to palmitic acid; ω compared to palmitic acid + osmotin.

3.6. Osmotin Reversed the Impaired Insulin Signaling and Activated the Downstream Markers Both in Palmitic Acid-exposed HepG2 Cells and in *ob*/*ob* and *db*/*db* Mice

Numerous well-known studies have reported that adiponectin acts as an insulin sensitizer and prevents insulin resistance [31,32]. To evaluate the effect of osmotin on insulin signaling, we initially investigated the phosphorylation of the insulin receptor substrate 1 (p-IRS1) and its downstream markers. Our *in vitro* results showed that palmitic acid (200 μM) significantly increased the phosphorylation of p-IRS-1 $^{(Ser\ 636)}$ and reduced the expression of downstream markers p-PI3K/Akt and transcription factor p-FOXO1 $^{(Ser\ 256)}$ protein levels compared to the control treatment. In contrast, cells treated with osmotin (0.4 μM) expressed significantly lower levels of p-IRS-1 $^{(Ser\ 636)}$ ($p < 0.05$) and higher levels of p-PI3K/Akt ($p < 0.05$) and p-FOXO1 Ser 256 ($p < 0.05$) than those treated with palmitic acid alone (Fig. 6A, B). Furthermore, our *in vivo* Western blot results showed higher expression of p-IRS-1 $^{(Ser\ 636)}$ and lower levels of p-PI3K, p-Akt and p-FOXO1 $^{(Ser\ 256)}$ in the Veh-treated *ob*/*ob* and *db*/*db* mice than in the Veh-treated WT mice. Osmotin administration significantly reversed the upregulated expression levels of p-IRS-1 $^{(Ser\ 636)}$ ($p < 0.05$) and enhanced the expression of the downstream protein markers in the osmotin-treated *ob*/*ob* and *db*/*db* mice relative to vehicle treatment (Fig. 6C, D). Furthermore, our immunofluorescence analysis indicated that osmotin-treated *ob*/*ob* and *db*/*db* mice expressed higher levels of p-FOXO1 $^{(Ser\ 256)}$ ($p < 0.05$) than the Veh-treated *ob*/*ob* and *db*/*db* mice (Fig. 6E).

3.7. Osmotin Downregulated the Expression of Gluconeogenic Genes (G-6-P/PEPCK) Both in Palmitic Acid-exposed HepG2 Cells and in *ob*/*ob* and *db*/*db* Mice

A reduction in glucose-6-phosphate dehydrogenase has recently been reported to improve insulin resistance in obesity [33]. To assess

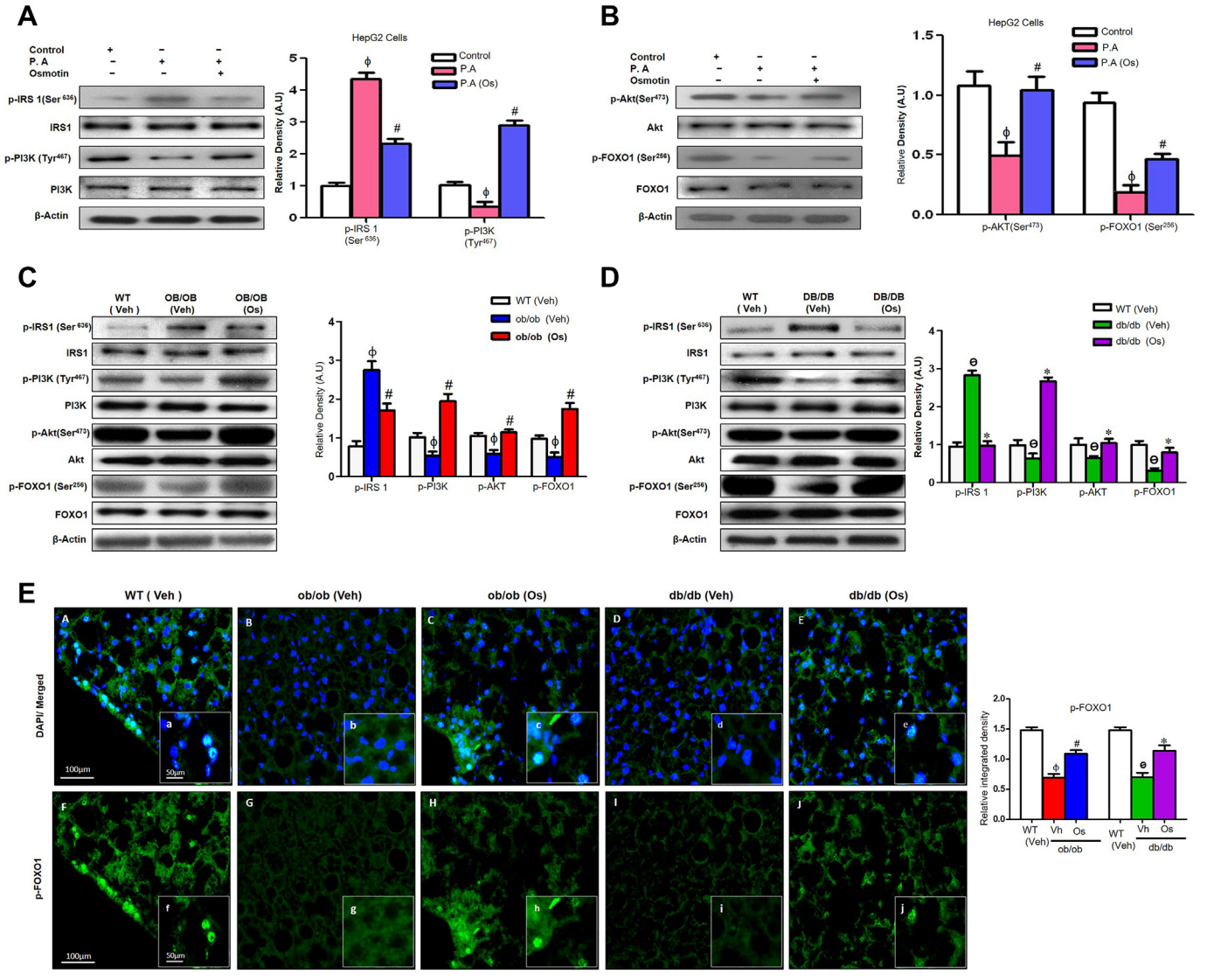

Fig. 6. Osmotin affected the activation of the p-IRS-1/PI3K/Akt signaling pathway both *in vitro* and *in vivo*. (A, B) Western blot and densitometric analysis of p-IRS-1 $^{(Ser\ 636)}$/IRS-1, p-PI3K $^{(Tyr\ 467)}$/PI3K, p-Akt $^{(Ser\ 473)}$/Akt and p-FOXO-1 $^{(Ser256)}$/FOXO-1 in the HepG2 cell lysates. β-Actin was used as the loading control. (C, D) Western blot analysis in the liver tissue homogenates of *ob*/*ob* and *db*/*db* mice using antibodies against p-IRS-1 $^{(Ser\ 636)}$/IRS-1, p-PI3K $^{(Tyr\ 467)}$/PI3K, p-Akt $^{(Ser\ 473)}$/Akt and p-FOXO-1 $^{(Ser256)}$/FOXO-1. β-Actin was used as the loading control. All density values are expressed in arbitrary units (A.U.). (E) Shown are the p-FOXO-1 $^{(Ser256)}$ staining immunofluorescence images along with their integrated density histogram in the liver of Veh-treated WT, Veh-treated *ob*/*ob*, osmotin-treated *ob*/*ob*, Veh-treated *db*/*db*, and osmotin-treated *db*/*db* mice. Panels (A–J) are of low magnification *i.e.*, 40×, while the panels (a–j) are a magnification of the 40× image. The data collected are expressed as the mean ± SEM ($n = 5$ mice/group). Significance, $p < 0.05$. ϕ compared to Veh-treated WT and # compared to Veh-treated *ob*/*ob* mice; Θ compared to Veh-treated WT and * compared to Veh-treated *db*/*db* mice. Similarly, for *in vitro* studies, ϕ compared to control; # compared to palmitic acid.

the role of osmotin on hepatic gluconeogenesis, we examined the expression of two rate-limiting enzymes, glucose-6-phosphate (G-6-P) and phosphoenol pyruvate carboxy kinase (PEPCK), both *in vitro* and *in vivo*. Initially, when we exposed HepG2 cells to palmitic acid (200 μM) for 12 h, the expression levels of G-6-P and PEPCK were significantly elevated in the palmitic acid group compared to those in the control. Our results showed that osmotin (0.4 μM) treatment effectively downregulated the elevated level of G-6-P and PEPCK relative to palmitic acid alone (Fig. 7A). To further explore the effect of osmotin on hepatic enzyme expression, we performed Western blotting analysis of *ob*/*ob* and *db*/*db* liver tissue. The protein expression levels of both G-6-P and PEPCK were significantly higher in the Veh-treated *ob*/*ob* and *db*/*db* mice ($p < 0.05$) than in the Veh-treated WT mice (Fig. 7B, C). Osmotin treatment significantly abrogated the elevated expression levels of G-6-P and PEPCK ($p < 0.05$) in the *ob*/*ob* and *db*/*db* mice compared to vehicle treatment. The Fig. 8 shows the proposed schematic diagram that illustrates the underlying mechanism of the protective effects of osmotin against obesity/diabetes-induced NAFLD in leptin-deficient transgenic *ob*/*ob* and *db*/*db* mice and in the *in vitro* palmitic acid model.

4. Discussion

Recently, numerous studies have reported that hepatic adiponectin and AdipoRs signaling play a key and pivotal protective role in the insulin resistance and obesity-associated hepatic diseases *e.g.* NAFLD [34–39]. Therefore the therapeutic approach to enhance the AdipoR1/R2 targets and its downstream signaling demonstrate a new therapeutic approach against obesity and diabetes-induced NAFLD. Here, our study focused on the effect of the natural plant-derived novel drug, osmotin, which has been considered a homolog of mammalian adiponectin, on the obesity- and diabetes-associated metabolic disorder NAFLD in both *in vivo* and *in vitro* studies. We found that osmotin stimulated AdipoR1/R2 and its downstream mediators such as APPL1/PPAR-α/AMPK/SIRT1 pathways, and regulated insulin signaling, consequently resulting in weight loss, reduced insulin resistance, and enhanced fatty acid oxidation and promoting mitochondrial biogenesis.

Adiponectin is an important insulin-sensitizing adipocytokine implicated in the prevention and reduction of obesity and diabetes-related NAFLD complications [19,40–42], and adiponectin pretreatment has been reported to induce beneficial effects in diabetic conditions [43].

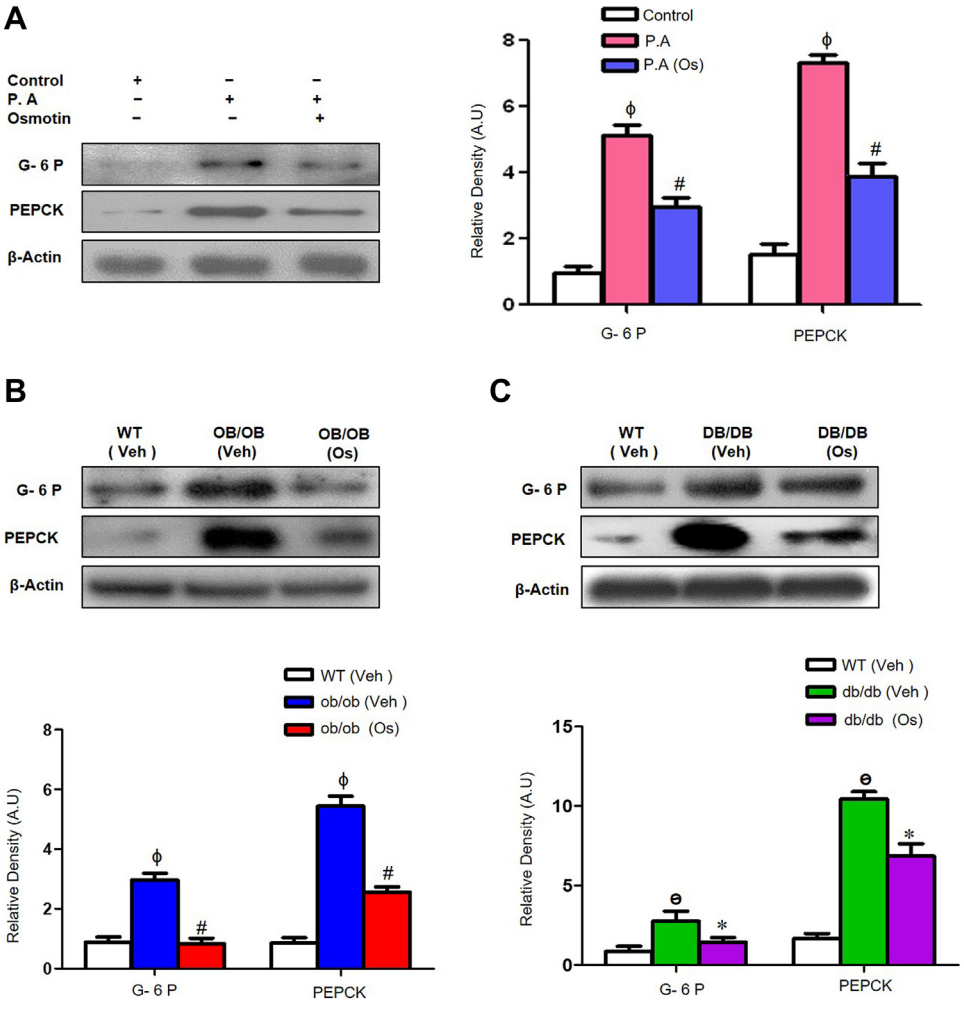

Fig. 7. Osmotin downregulated the expression of gluconeogenic genes (G-6 P and PEPCK) bot *in vitro* and *in vivo*. (A) Western blot analysis of G-6-P and PEPCK in the palmitic acid (200 μM)-exposed HepG2 cells with/without osmotin (0.4 μM) treatment for 12 h. β-Actin was used as the loading control. (B, C) Western blot analysis in the liver tissue homogenate of *ob*/*ob* and *db*/*db* mice with/without osmotin treatment using anti-G-6-P and anti-PEPCK antibodies, respectively. The bands were quantified using Sigma Gel software, and their differences are represented in the graphs. β-Actin was used as the loading control. The density values are expressed in arbitrary units (A.U.) as the mean ± SEM for the respective indicated proteins ($n = 7$/group). Significance, $p < 0.05$. ϕ $p < 0.05$ compared to Veh-treated WT, # compared to Veh-treated *ob*/*ob* mice; Θ $p < 0.05$ compared to Veh-treated WT and * $p < 0.05$ compared to vehicle-treated *db*/*db* mice. Similarly, for *in vitro* studies, ϕ compared to control; # compared to palmitic acid.

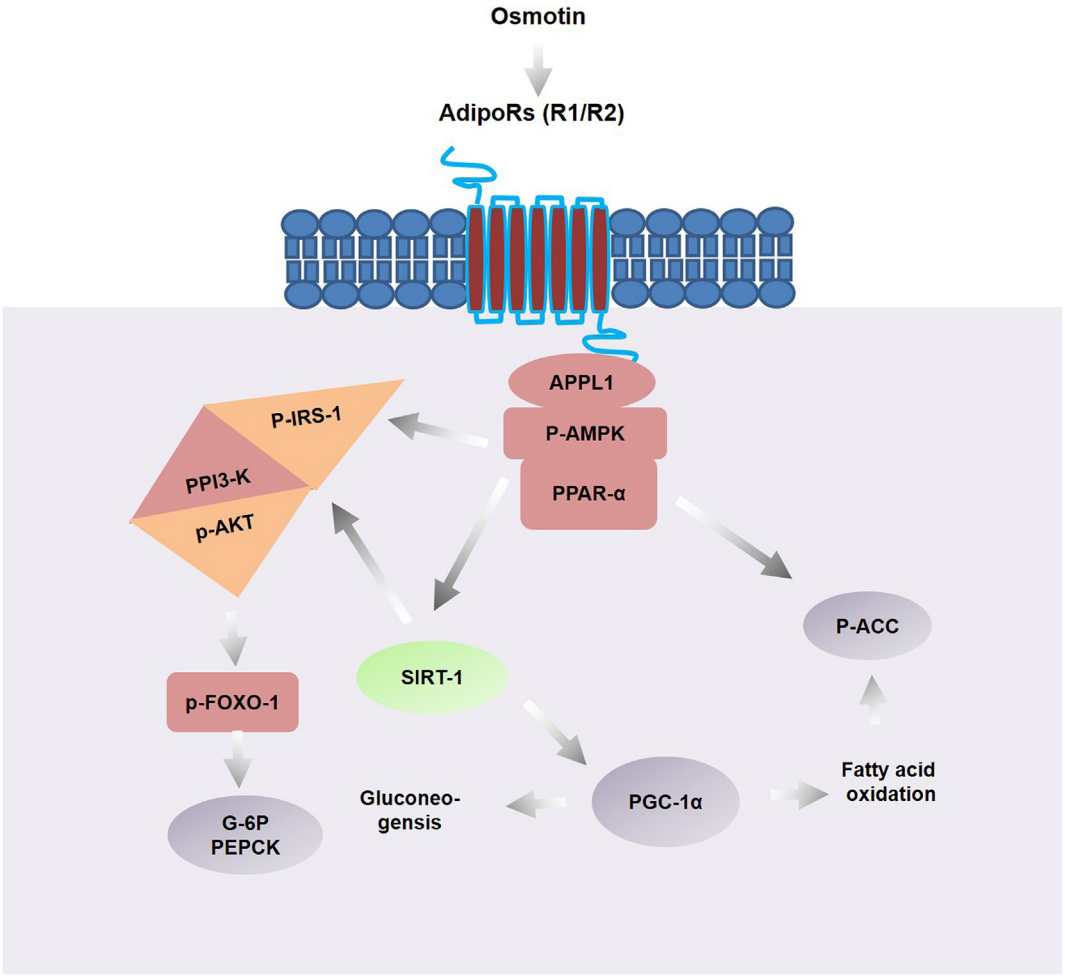

Fig. 8. Proposed mechanism for the effect of osmotin against obesity/diabetes-induced NAFLD. Graphical illustration indicates that osmotin exerts its beneficial therapeutic effects against NAFLD, including reduction in body weight, increase in fatty acid oxidation, and prevention of hepatic gluconeogenesis, and regulates mitochondria biogenesis *via* the activation of AdipoRs signaling and APPL1/PPAR-α/AMPK/SIRT1 pathways in both *in vitro* and in *ob*/*ob* and *db*/*db* mouse models.

The accumulation of visceral fats is a risk factor for insulin resistance and increased FFA release and plays a pivotal role in the onset and pathogenesis of hepatic steatosis and fibrosis [44]. These data revealed that osmotin treatment significantly reduced the body weight and BMI of obese and diabetic mice. This reduction in body weight by osmotin treatment indicated that the role of osmotin in metabolic dysfunction is related to lipogenesis and gluconeogenesis [45]. The fasting blood glucose, GTT and HbA1C levels were higher in the Veh-treated obese and diabetic mice than in the WT mice, but osmotin supplementation significantly reversed these biochemical parameters in the *ob*/*ob* and *db*/*db* mice. This significant reduction in the glucose and glycated hemoglobin level suggested that osmotin works as a mimetic of adiponectin, thus regulating gluconeogenesis [46]. Likewise, the lipid contents comprising the concentration of TC, TG, LDL, and FFA were significantly lower after osmotin treatment, while the HDL level was enhanced (Fig. 2A–E). Furthermore, the serum levels of the two hepatic enzymes, ALT and AST, are well-known key biochemical markers for the detection of liver damage, and a study has shown that they correlate with liver fat and diabetes [47]. Herein, our results revealed that the elevated ALT and AST levels were markedly reduced by osmotin treatment in the *ob*/*ob* and *db*/*db* mice (Fig. 2I, J). Thus, osmotin plays an effective role in reversing various metabolic phenotypic and biochemical parameters in the same manner as adiponectin as previously reported [48].

The AdipoR1 and AdipoR2 are very important for the normal physiological liver function and in the liver diseases. Initially, the studies indicated that AdipoR2 is the main signaling for the adiponectin-mediated protection in the hepatic disorder [35–37]. However, recently several line of investigation reported the expression AdipoR1 and their importance physiological and pharmacological function in the human hepatocytes as well as in rodent's. Notably, in the diabetes and obesity mouse models, the expression levels of adiponectin and its receptor AdipoR1/R2 were lower than in the WT mice. Hepatic AdipoR1/R2 signaling activation has been shown to protect against the progression of NAFLD. Moreover, decreased adiponectin signaling due to weight gain results in nonalcoholic steatohepatitis through disrupted mitochondrial biogenesis [12,14,38,48–51]. Our current studies suggested that osmotin administration enhanced the expression level of AdipoR1/R2 both *in vivo* and *in vitro*, suggesting that osmotin modulated and activated hepatic AdipoR1/R2 signaling. Recently, well-established studies have reported that APPL1 is an important downstream mediator of adiponectin signaling; it binds to AdipoRs, mediating the functions of adiponectin, and is particularly implicated in obesity and diabetes patients [52]. Additionally, APPL1 is also involved in the increase in insulin sensitivity by potentiating IRS-1, which binds with the insulin receptor [53]. In addition to AdipoR1/R2 activation, osmotin administration increased the expression level of APPL1 in the HepG2 cells exposed to palmitic acid and in the *ob*/*ob* and *db*/*db* mice. Herein, we found that osmotin not only significantly reduced the protein expression of p-IRS1 ^{Ser636} but subsequently activated the downstream effectors p-PI3K and p-Akt $^{(Ser473)}$. Moreover, osmotin supplementation also enhanced the expression levels of the transcription factor p-FOXO-1 $^{(Ser256)}$ and subsequently inhibited the expression of two key

gluconeogenic genes, G-6 P and PEPCK, in the HepG2 cells exposed to palmitic acid and in the *ob*/*ob* and *db*/*db* mice. Hence, these results suggested that osmotin plays a role in regulating gluconeogenesis through insulin sensitization to minimize the risk of fatty liver and NAFLD [54].

AMPK is a key and very important cellular regulator of lipid and glucose metabolism [53]. This multifaceted enzyme is involved in the initiation of hepatic unsaturated fat oxidation and ketogenesis, inhibition of cholesterol synthesis, induction of lipogenesis, enhancement of triglyceride deposition, restraint of adipocyte lipolysis and lipogenesis, initiation of skeletal muscle unsaturated fat oxidation and muscle glucose uptake, and adjustment of insulin discharge by pancreatic beta cells [54,55]. We found that osmotin treatment activated the p-AMPK levels both in palmitic acid-treated cells *in vitro* and in *ob*/*ob* and *db*/*db* leptin-deficient mice *in vivo*. Activated AMPK further activated SIRT1, one of the key markers in the treatment of diabetes-related fatty liver as well as alcoholic fatty liver disease [56], and recently Xu et al. found that the anti-diabetic drug exenatide rescues hepatic steatosis *via* SIRT1 activation [57]. Previous reports have shown that SIRT1 mediates the activation of PGC-1α, a transcription factor that regulates energy homeostasis by mitochondrial biogenesis [58]. PGC-1α is also reported to be involved in maintaining blood pressure and regulating cellular cholesterol homeostasis and the development of obesity, which leads to NAFLD. Both our *in vitro* and *in vivo* results confirmed that osmotin treatment regulates mitochondrial biogenesis through activation of the AMPK/SIRT1/PGC-1α pathway. AMPK is known to be involved in the regulation of fatty acid metabolism by activating PPAR-α, a transcription factor that regulates fatty acid synthesis and lipid metabolism in the liver [59]. Osmotin played a key role in the regulation of fatty acid oxidation, as it enhanced the expression of PPAR-α and p-ACC in the HepG2 cells exposed to palmitic acid and in the *ob*/*ob* and *db*/*db* mice. p-ACC has already been reported to be an important regulator and key player in fatty acid oxidation as a downstream target of AMPK [60]. Fatty acid consumption leads to the utilization of body fats that were responsible for obesity and NAFLD. These results proposed that osmotin has the potential to regulate adiponectin signaling, enhance the phosphorylation of AMPK ^{Th172}, and activate downstream protein markers PPARα and p-ACC involved in fatty acid oxidation.

5. Conclusion

Taken together, our *in vivo* and *in vitro* results suggest that osmotin protects against NAFLD *via* activation of AdipoRs signaling and APPL1/PPAR-α/AMPK/SIRT1 pathways and reduces body weight, insulin resistance and hepatic glucogenesis, resulting in increased fatty acid oxidation and mitochondrial function. In conclusion, we proposed that supplementation of plant protein osmotin, which has been considered to be a homolog of mammalian adiponectin, is a potential novel therapeutic agent to prevent NAFLD in genetically induced obese and diabetic mice. Further, future mechanistic and complete pharmacological studies are needed to elucidate the beneficial therapeutic efficacy of osmotin for the translational and clinical studies as a potent anti-diabetic and anti-obesity agent.

Acknowledgements

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Conflict of Interest

No potential conflicts of interest declared.

Author Contribution

AA designed the experimental work, arranged the data and wrote the manuscript. AA, MWK, AK, MI, and RSU performed western blot analysis. TA, SK, MHJ, NBA, MK, RU and MGJ performed confocal microscopy and *in vitro* experiments. TA contributed equally in finalizing the whole manuscript. The MOK is a corresponding author, reviewed and approved the manuscript and holds all the responsibilities related to this manuscript. All authors reviewed the manuscript. MOK is a guarantor of this work and, as such had full access to all data in the study and take responsibility for the integrity of the data.

Appendix A. Supplementary Data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.metabol.2018.10.004.

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