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High molecular weight adiponectin reduces glucolipotoxicity-induced inflammation and improves lipid metabolism and insulin sensitivity via APPL1-AMPK-GLUT4 regulation in 3T3-L1 adipocytes

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HIGHLIGHTS

- *In vitro* intricacies of HMW adiponectin (HMW Ad) in 3T3-L1 adipocytes are less studied.
- HMW Ad treatment improves glucose homeostasis and results in reduced lipolysis, inflammation and insulin resistance.
- Beneficial modulation and regulation of APPL1 and AMPK signals by HMW Ad observed in this study are a novel mechanism.
- Raising endogenous HMW Ad levels either by pharmacological or lifestyle modification will have a therapeutic value.

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ABSTRACT

Background and aims: Although the importance of adipokines in modulating the disease process of type 2 diabetes is well recognized, there is dearth of data on the specific role of high molecular weight adiponectin (HMW Ad) on insulin resistance and obesity. Therefore, we tested the effects of HMW Ad on glucolipotoxcity-induced inflammation and insulin resistance in 3T3-L1 adipocytes.
Methods: 3T3-L1 adipocytes were subject to glucolipotoxicity with and without HMW Ad treatment. Real-time PCR and Western-blot experiments were performed to analyse gene and protein expressions, respectively. Lipolysis, adipored staining, and glucose uptake assay were performed to evaluate alterations in lipid and glucose metabolism.
Results: Adipocytes subject to glucolipotoxicity showed significantly ($p < 0.05$) decreased mRNA expression of adiponectin, AdipoR2, GLUT4, and increased inflammation, lipid accumulation as well as lipolysis. Treatment with HMW Ad beneficially modulated lipid metabolism, reduced inflammation and improved glucose uptake in adipocytes. HMW Ad also beneficially regulated APPL1 and AMPK signaling in adipocytes. Silencing of *APPL1* gene in adipocytes significantly reduced the effects of HMW Ad on pAMPK protein expression, indicating that HMW Ad plays an important role in regulating AMPK phosphorylation via APPL1 in 3T3-L1 adipocytes.
Conclusions: HMW Ad treatment improved glucose homeostasis and resulted in reduced lipolysis, inflammation and insulin resistance in adipocytes subject to glucolipotoxicity. The beneficial modulation and regulation of APPL1 and AMPK signals by HMW Ad observed in this study represent a novel mechanism. Raising endogenous HMW Ad levels either by pharmacological or lifestyle modification could have a therapeutic value.

1. Introduction

Coexistent type 2 diabetes (T2DM) and obesity, often termed "diabesity" is an emerging epidemic in the developing and the developed nations [1]. It is believed that elucidation of adipocyte biology and its molecular intricacies will facilitate the development of strategies to combat obesity and/or T2DM. Adipose tissue has important endocrine, autocrine, and paracrine functions and is reported to play a key role in

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the pathogenesis of diabetes and cardiovascular disease [2]. Adipose tissue secretes adipokines that regulate insulin sensitivity in adipocytes and other peripheral tissues critical to glucose metabolism. Among adipokines, adiponectin is a key regulator of insulin sensitivity and inflammation, through its direct actions on liver and skeletal muscle, improving hepatic insulin sensitivity, increasing fuel oxidation, and decreasing vascular inflammation [3,4].

Adiponectin is released into the circulation in various multimer complexes like trimer as low molecular weight (LMW), hexamer as middle molecular weight, octamer as high molecular weight (HMW) and proteolytically cleaved globular Adiponectin [5,6]. Each adiponectin form has tissue specific activity and thus generates distinct biological response [7]. HMW adiponectin (HMW Ad) has been shown to be biologically more active in conferring protection against T2DM [8,9] and correlates well with improved insulin sensitivity and fat metabolism than total adiponectin [10].

Studies have reported that HMW Ad is related to ectopic fat, thus possibly representing the form of adiponectin regulating lipid oxidation in the liver and skeletal muscle [4]. Our earlier studies demonstrated that oxidative stress markers, visceral fat and inflammation are associated with total and HMW Ad levels in Asian Indians [11,12]. However, there is lack of studies on the beneficial actions of HMW Ad in terms of its molecular effects.

APPL1 (adaptor protein containing phosphotyrosine binding, pleckstrin homology domains and leucine zipper 1) appears to be a key signaling molecule that couples adiponectin receptors and its downstream molecule AMPK, leading to the activation of the AMPK pathway [13,14]. AMPK activation influences several pathways, including glucose metabolism, lipid metabolism and inflammation. In addition, APPL1 in mouse potentiates insulin sensitivity, as APPL1 deleted mice consistently exhibit systemic insulin resistance, and a significant reduction in insulin-stimulated IRS1/2 tyrosine phosphorylation [15]. However, an understudied question in this paradigm is whether the effect of HMW Ad in 3T3-L1 adipocytes is mediated via APPL1. Moreover, given the increasing importance of adipokines in modulating the disease process of type 2 diabetes, there is dearth of data on the specific role of HMW Ad, with special reference to insulin resistance and obesity. Therefore, we tested the effects of HMW Ad on glucolipotoxicity (GLT)-induced inflammation and insulin resistance in 3T3-L1 adipocytes.

2. Materials and methods

2.1. 3T3-L1 fibroblasts culture, differentiation and treatment

3T3-L1 mouse fibroblasts were procured from the American Type Culture Collection (ATCC) and were induced to differentiate into mature adipocytes by standard procedures [16]. Briefly, cells were grown to 80% confluence in DMEM containing 10% calf serum. Differentiation was induced by incubating the cells in fresh DMEM containing 1 μM dexamethasone, 10 μg/ml insulin (91077C, Sigma, USA), 0.5 mM isobutylmethylxanthine (IBMX) (Sigma, USA), and 10% fetal bovine serum (FBS) for 3 days. Then cells were maintained for another 6 days in maintenance medium containing DMEM, 10% FBS, and 10 μg/ml insulin. The cells used for experimentation were more than 80% differentiated as determined visually. For GLT treatment, cells were exposed to high glucose (25 mM) and palmitate (500 μM) in the presence and absence of HMW Ad (5 μg/ml) for 6 h. Palmitate was prepared in complex with albumin. Briefly, sodium palmitate was dissolved in preheated NaOH (0.1 M) and then complexed with 10% BSA at 55 °C as described elsewhere [17]. Human recombinant adiponectin HEK293, Tag free was procured from Biovendor, USA (Catalog # RD172023100-C).

2.2. Quantitative real-time PCR

Total RNA was prepared from the adipocytes using TRIzol reagent (TaKaRa) according to the manufacturer's instructions. 1 μg of total RNA was reverse transcribed using reverse transcriptase enzyme (Thermoscientific), and random hexamer primers (IDT). Real time quantitative PCR was performed on a Light-cycler (Roche) with appropriate cycle conditions using SYBR Premix Ex Taq II (TaKaRa) and using appropriate sets of forward and reverse primers (Supplementary Table 1). Relative quantification of gene transcripts was calculated using $2^{-\Delta\Delta Ct}$ method and normalized to β-actin transcripts. Negative controls were run as well to ensure the absence of contamination.

2.3. Western blot analysis

Cells were harvested from the culture plate using RIPA (Thermoscientific) lysis buffer supplemented with protease and phosphatase inhibitors. 30 μg of protein per lane and known molecular weight markers (Puregene, Genetix Biotech) were separated by SDS-PAGE transferred to Polyvinylidene difluoride (PVDF) membrane (Millipore) and detected with specific primary and secondary antibodies. Proteins were detected by chemiluminescence molecular doc (BioRad, USA) and bands were analyzed by using Image J software. Primary anti-phospho-AMPKα (Thr172), anti-AMPKα, anti-APPL1 were purchased from Cell Signaling (USA) and used at 1:2000 dilutions. Primary anti-adiponectin, anti-TNFα, anti-IL-6, anti-MCP-1, anti-GLUT-4 were purchased from Santa Cruz (USA) and used at 1:1000 dilutions. Anti-PPARγ were purchased from Abcam (UK) and used at 1:2000 dilutions. HRP-conjugated secondary antibodies were purchased from Santa Cruz (USA) and used at 1:5000 dilutions.

2.4. Lipolysis assay

Differentiated 3T3-L1 cells were pre-exposed to GLT with and without HMW Ad (5 μg/ml) for 6 h. Lipolysis was induced by 0.5 mM IBMX for 4 h in appropriate wells. Lipolysis assay was performed with the Adipolysis Assay Kit (EnzyChrom, BioAssay Systems, USA) according to the manufacturer's protocol.

2.5. Adipored staining

Differentiated 3T3-L1 cells were treated with GLT in the presence and absence of HMW Ad for 24 h. Cells were washed twice with PBS and fixed with 4% paraformaldehyde for 30 min. Cell were washed with PBS three times and permeabilized using 0.25% triton X. Cells were then washed again followed by addition of adipored (AdipoRed, Lonza) for 15 min in the dark, finally washed with PBS and mounted on the slides using DAPI-mounting medium (Fluoroshield with DAPI, Sigma, USA). The fluorescent images were obtained using a Confocal Laser Scanning microscope (Carl-Zeiss LSM 700) with a 60X objective.

2.6. Glucose uptake assay

Cells were seeded in 12-well plates (2×10^4 cells/well) and differentiated with standard protocol. Cells were pre-treated with GLT for 6 h, and then stimulated with HMW Ad for 2 h prior to the start of glucose uptake experiment. Cells were then washed with Krebs-Ringer Buffer (KRB) without glucose (supplemented with 0.1% BSA and $CaCl_2$), and stimulated with 100 nM insulin for 30 min in appropriate wells. Cells were then treated with 40 μM 2- 2-(*N*-(7-Nitrobenz-2-oxa-1,3-diazol-4-yl)Amino)-2-Deoxyglucose (NBDG, BioVision) in the same media for 1 h [18]. After incubation, cultures were washed out of free 2-NBDG with KRH buffer, lysed with lysis buffer (20 mM Tris, 1% Sodium deoxycholate, 40 mM KCL) and gently homogenised with 100% DMSO. The contents of the lysed cell were transferred to 96-well fluorescence plate and read at excitation wavelength of 485 nm and emission wavelength of 535 nm.

2.7. APPL1 gene silencing and treatment with HMW adiponectin

3T3-L1 adipocytes were grown to 40–60% confluence in 6-well plates, and then transfected for 36 h with 50 nM control or APPL1 siRNA (Catalog # 1027416, Qaigen) using FuGENE Transfection reagent (Catalog #E4983, Promega, USA) according to the manufacturer's instructions. HMW Ad was treated at 36 h post-transfection. The transfection efficiency was determined using GFP-tagged plasmid under a fluorescence microscope and APPL1 knockdown efficiency was assessed by Western blot (Supplementary Fig. 1).

2.8. Statistical analysis

Data were expressed as Mean ± SEM. Differences were evaluated by one and two way analysis of variance (ANOVA) using Graph Pad Prism, Version 6 (GraphPad Software, Inc., La Jolla, CA, USA) and a *p*-value < 0.05 was considered statistically significant.

3. Results

3.1. Effect of HMW Ad on glucolipotoxicity (GLT) impaired Ad signaling in 3T3-L1 adipocytes

We first studied the effects of GLT on adiponectin signaling in 3T3-L1 adipocytes. GLT treatment showed significant ($p < 0.05$) reduction in both mRNA and protein expression of adiponectin compared to untreated cells (Fig. 1A–C). mRNA expression of adiponectin receptor AdipoR2 showed significant ($p < 0.05$) reduction under GLT treatment (Fig. 1E). *AdipoR1* mRNA expression was also reduced under GLT treatment, but it did not reach statistical significance (Fig. 1D). Treatment with HMW Ad in the presence of GLT did not increase endogenous adiponectin expression but appeared to facilitate adiponectin signaling by increasing the expression of its receptors, AdipoR2 ($p < 0.05$) and AdipoR1 (Fig. 1D and E).

3.2. HMW Ad beneficially modulates lipid metabolism in 3T3-L1 adipocytes

Since adiponectin plays an important role in lipid metabolism, we tested the effects of GLT and HMW Ad on lipid accumulation and lipolysis in 3T3-L1 adipocytes. PPARγ is the key regulator of lipogenesis and responsible for intracellular lipid accumulation, whereas ATGL and HSL are responsible for lipolysis. Both mRNA and protein expression of PPARγ (Fig. 2A, C, and D), *HSL*, and *ATGL* gene expression (Fig. 2E and F) were up-regulated ($p < 0.05$) in cells treated with GLT, indicating an increase in the process of lipogenesis and lipolysis. However, in the presence of HMW Ad, GLT mediated increase in mRNA profiles of *PPARγ*, *ATGL*, and *HSL* was significantly ($p < 0.05$) reduced. HMW Ad also significantly reduced PPARγ protein expression compared to GLT treated cells (Fig. 2C and D). We also investigated the effects of GLT on *leptin* gene expression, and observed that GLT up-regulates *leptin* gene expression, however, HMW Ad treatment in the presence of GLT did not produce any effect on *leptin* gene expression (Fig. 2B).

Effect of HMW Ad on lipolysis was also studied by measuring the levels of glycerol released in the cell culture medium. HMW Ad treatment significantly ($p < 0.05$) reduced IBMX and GLT mediated lipolysis in 3T3-L1 adipocytes (Fig. 2G), indicating reduced rate of lipolysis, in accordance with the mRNA levels of *ATGL*. We further studied the extent of intracellular lipid accumulation in 3T3-L1 adipocytes by Adipored staining. Compared to untreated cells, intracellular lipid accumulation was increased in GLT treated adipocytes. Treatment with HMW Ad in the presence of GLT significantly ($p < 0.05$) reduced lipid accumulation in 3T3-L1 adipocytes (Fig. 2H and I).

3.3. HMW Ad reduces the inflammation caused by GLT

Dysregulation in lipid metabolism might be associated with increased pro-inflammation. Therefore, we next examined the effects of GLT and HMW Ad on inflammatory signals in 3T3-L1 adipocytes. Gene expression studies revealed increased pro-inflammatory markers, TNF-α (Fig. 3A), IL-6 (Fig .3B), and MCP-1 (Fig. 3C) in 3T3-L1 adipocytes when they were subject to GLT, and this was significantly ($p < 0.05$) reversed under HMW Ad treatment. Consistent with the mRNA profiles, cells under GLT conditions also exhibited increased protein expression of TNF-α, IL-6, and MCP-1 ($p < 0.05$ each) (Fig. 3D). GLT induced increase in protein expression of inflammatory markers IL-6 and MCP-1 was also significantly ($p < 0.05$ each) attenuated by HMW Ad treatment (Fig. 3D, F and G).

Nucleotide binding oligomerization domain (NOD) like receptors (NLRs), the cytosolic proteins of the innate immunity, are known to propagate inflammatory signals through NFκB. We tested the effects of GLT on NOD receptors in the absence and presence of HMW Ad. GLT up-regulates ($p < 0.05$) inflammation by inducing the expression of NOD1 and NOD2, and this was reduced by HMW Ad (Fig. 3H and I). Since RIPK2 is a critical mediator of NOD1/2 signaling and an important trigger for NFκB mediated pro-inflammatory responses, we then analyzed mRNA expression of *RIPK2* and *NFκB*. mRNA expression of both *RIPK2* (Fig. 3J) and *NFκB* (Fig. 3K) was significantly ($p < 0.05$) up-regulated in GLT treated cells and this was reduced by HMW Ad treatment. We also checked mRNA expression of *SOCS-3*, an important immune regulatory signal, which was found to be up-regulated ($p < 0.05$) under GLT treatment, and was significantly ($p < 0.05$) reduced when treated with HMW Ad in the presence of GLT (Fig. 3L).

3.4. HMW Ad increased both GLUT-4 expression and insulin stimulated glucose uptake in mature 3T3-L1 adipocytes as revealed by 2-NBDG glucose uptake assay

We observed that GLT significantly reduced glucose uptake in 3T3-L1 adipocytes. This could be attributed to the reduced mRNA and protein expression of GLUT-4 in the adipocytes on treatment with GLT. Treatment with HMW Ad even in the presence of GLT results in the increased ($p < 0.05$) gene and protein expression of GLUT-4 (Fig. 4A–C).

We also performed glucose uptake assay in 3T3-L1 adipocytes using 2-NBDG fluorescent glucose analog. Insulin significantly ($p < 0.05$) increased the glucose uptake in 3T3-L1 adipocytes, however, when adipocytes were pretreated with GLT, the effect of insulin was weakened, resulting in the reduction of glucose uptake. Adipocytes, when pre-treated with HMW Ad before insulin stimulation, showed a significant ($p < 0.05$) increase in glucose uptake even in the presence of GLT (Fig. 4D). This shows that HMW Ad resists the effect of GLT on glucose uptake, rendering the adipocytes to respond to insulin normally, most likely working as insulin-sensitizer.

3.5. GLT impairs insulin signaling by reducing APPL1 and pAMPK protein expression in 3T3-L1 adipocytes and HMW Ad reverses this phenomenon

We further investigated the effects of HMW Ad on insulin signaling pathway with reference to APPL1 and AMPK in 3T3-L1 adipocytes. As shown in Fig. 5A, GLT treatment significantly ($p < 0.05$) reduced pAMPK and APPL1 protein expression in 3T3-L1 cells. Treatment with HMW Ad in the presence of GLT significantly ($p < 0.05$) up-regulated the expression of both pAMPK and APPL1 proteins (Fig. 5A–C). These results demonstrate that HMW Ad beneficially modulates APPL1 and AMPK expression in 3T3-L1 adipocytes.

To further investigate whether activation of AMPK by HMW Ad is APPL1 dependent, we performed *APPL1* gene silencing in 3T3-L1 adipocytes. It was observed that in the absence of APPL1, effect of HMW Ad on pAMPK expression was significantly ($p < 0.05$) reduced (Fig. 5D

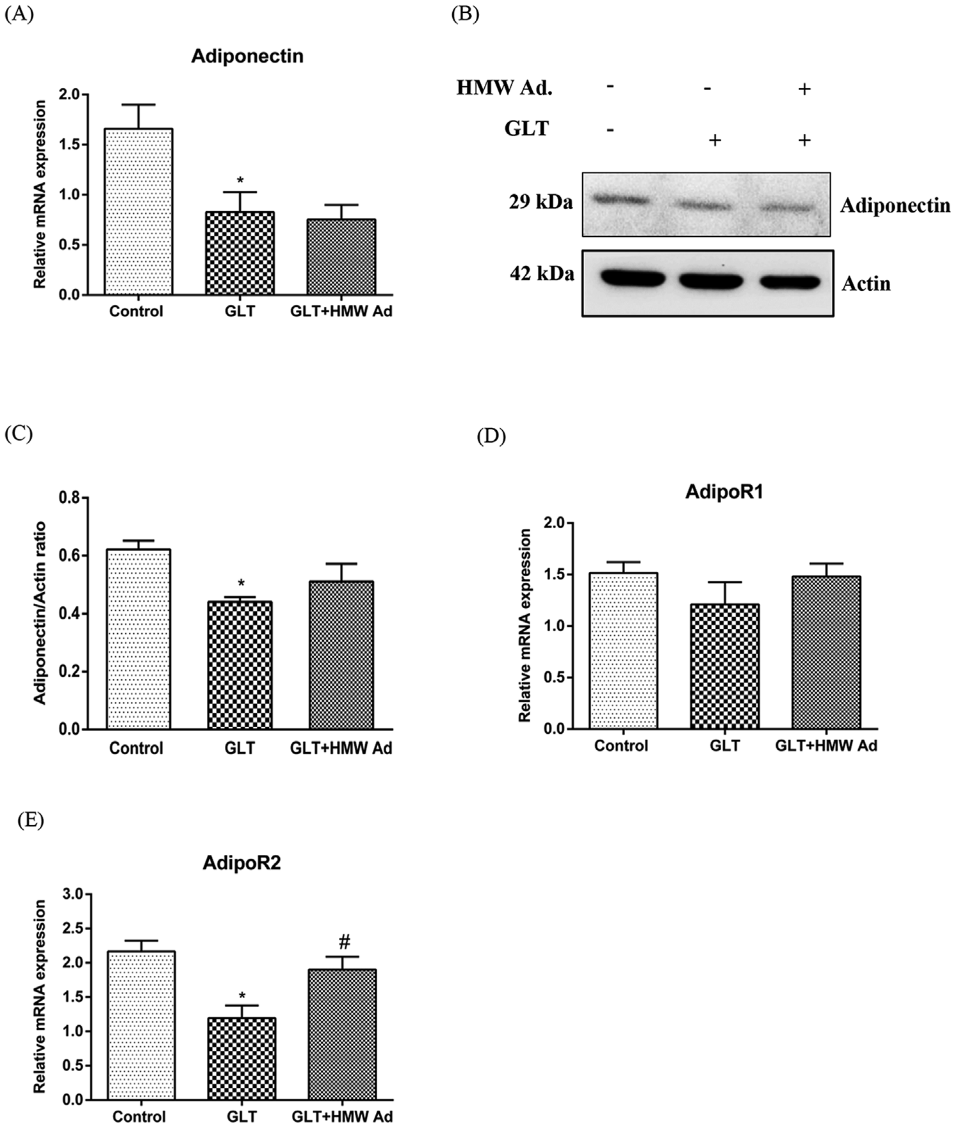

Fig. 1. Effect of GLT and HMW adiponectin on adiponectin signaling in 3T3-L1 adipocytes.
mRNA expression patterns of (A) adiponectin, (D)AdipoR1, and (E) AdipoR2 in 3T3-L1 adipocytes under different experimental conditions. (B) Representative protein expression blot of adiponectin along with β-Actin. (C) Cumulative protein expression data of adiponectin. *$p < 0.05$ compared to control; #$p < 0.05$ compared to GLT. Results are expressed as mean ± SEM of minimum three independent determinations.

and E). Thus, our results confirm that HMW Ad regulation of AMPK phosphorylation in 3T3-L1 adipocytes is APPL1 dependent.

4. Discussion

The role of adiponectin in regulating lipid and glucose metabolism both in *in vivo* and *in vitro* is well documented. However, the autocrine effects of HMW Ad have not been adequately studied. In the present study, we demonstrated that HMW Ad protects 3T3-L1 adipocytes from GLT by activating AMPK-APPL1 signaling axis. It is of interest that 3T3-L1 adipocytes treated with HMW Ad reduce cellular inflammation, increase glucose uptake and improve lipid metabolism.

Adiponectin mediates its response mainly via two receptors, known as AdipoR1 and AdipoR2, indicating the existence of autocrine/paracrine effects of adiponectin. AdipoR1 is a high-affinity receptor for globular adiponectin, and a low affinity receptor for full length adiponectin and high molecular weight adiponectin [19,20]. AdipoR2 mainly recognizes full length adiponectin and high molecular weight adiponectin and is abundantly expressed in the liver and adipose tissue [4,19]. Reduced expression of both AdipoR1 and AdipoR2 was observed in patients with type 2 diabetes and found to be positively correlated with insulin sensitivity [21]. Simultaneous disruption of both AdipoR1 and AdipoR2 abolished adiponectin binding and actions, resulting in increased tissue triglyceride content, inflammation, and oxidative stress, leading to insulin resistance and glucose intolerance in mice [22]. In the present study, GLT treatment in adipocytes impaired adiponectin signaling by reducing the expression of endogenous adiponectin as well as its receptor AdipoR2. We have shown that treatment with HMW Ad did not increase the endogenous adiponectin expression in adipocytes but facilitated appropriate adiponectin signaling by increasing the expression of receptor AdipoR2. As AdipoR2 is more reactive to bind to HMW adiponectin [4,19], our results are consistent with this observation.

Nucleotide oligomerization domains (NOD)-like receptors (NLRs) are a type of cytoplasmic pattern-recognition receptors that play an important role in innate immunity. NODs activate the nuclear factor-kB (NF-kB) pathway to induce the production of inflammatory cytokines, which requires polyubiquitination of receptor interacting protein kinase 2 (RIPK2, also called RIP2). Our earlier studies demonstrated increased expression of NODs in patients with type 2 diabetes [23]. Our *in*

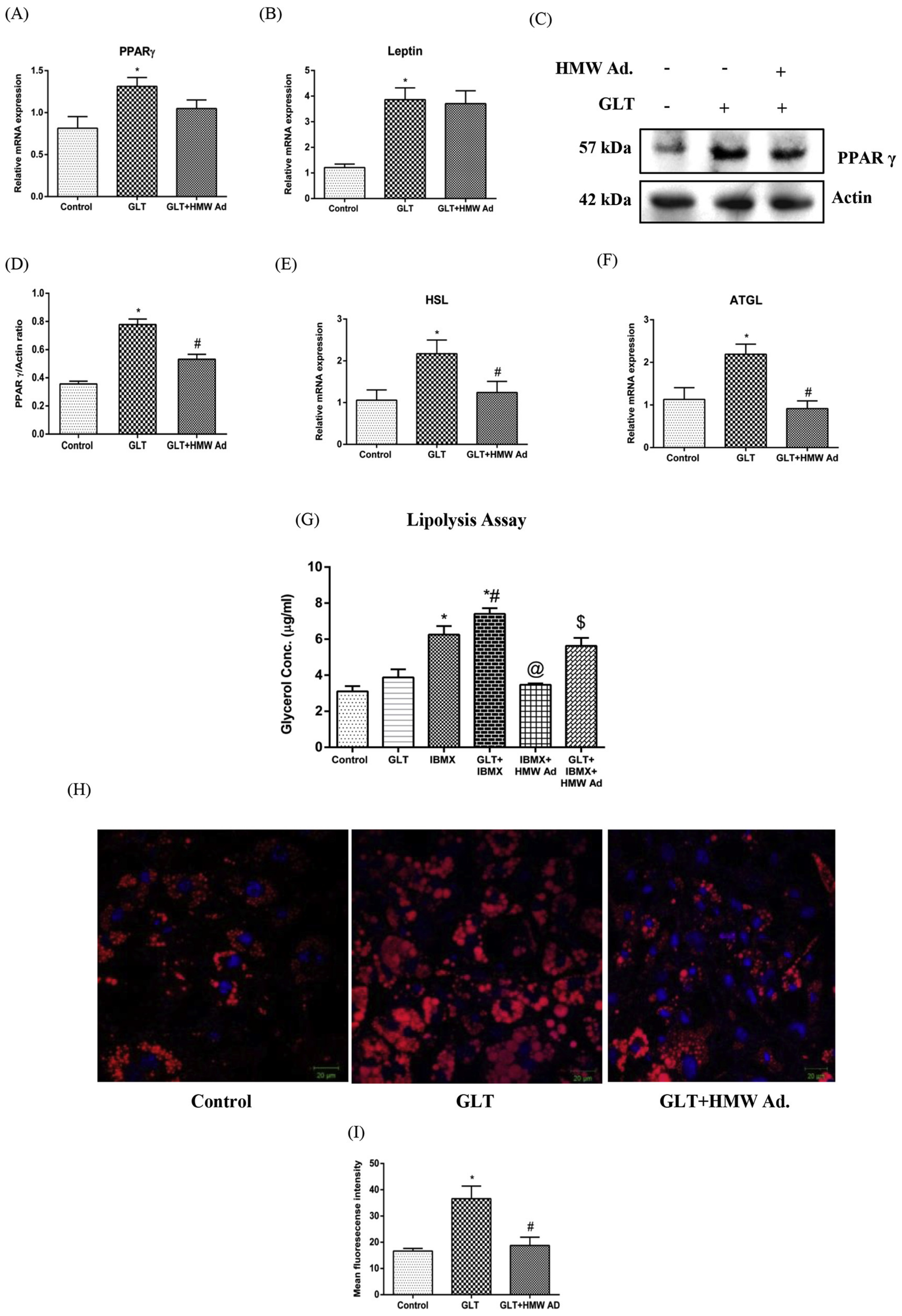

Fig. 2. Effect of HMW Ad on GLT altered lipid metabolism in 3T3-L1 adipocytes.

mRNA expression patterns of (A) *PPAR*γ, (B) *Leptin*, (E) *ATGL*, and (F) *HSL*. (C) Representative protein expression blot of PPARγ along with β-Actin. (D) Cumulative protein expression data of PPARγ. $*p < 0.05$, compared to control; $\#p < 0.05$, compared to GLT. (G) Effect of HMW Ad on lipolysis. 3T3-L1 cells were pre-treated with GLT with and without HMW Ad or HMW Ad alone for 6 h before lipolysis stimulation by IBMX. $*p < 0.05$, compared to control; $\#p < 0.05$, compared to GLT; $^{@}p < 0.05$, compared to IBMX; $^{\$}p < 0.05$, compared to GLT + IBMX. (H) Effect of HMW Ad on intracellular lipid accumulation. 3T3-L1 cells were differentiated and treated with GLT with and without HMW Ad for 24 h and processed for adipored staining. $*p < 0.05$, compared to control; $\#p < 0.05$, compared to GLT. (I) Mean fluorescence intensity. Results are expressed as mean ± SEM of minimum three independent determinations.

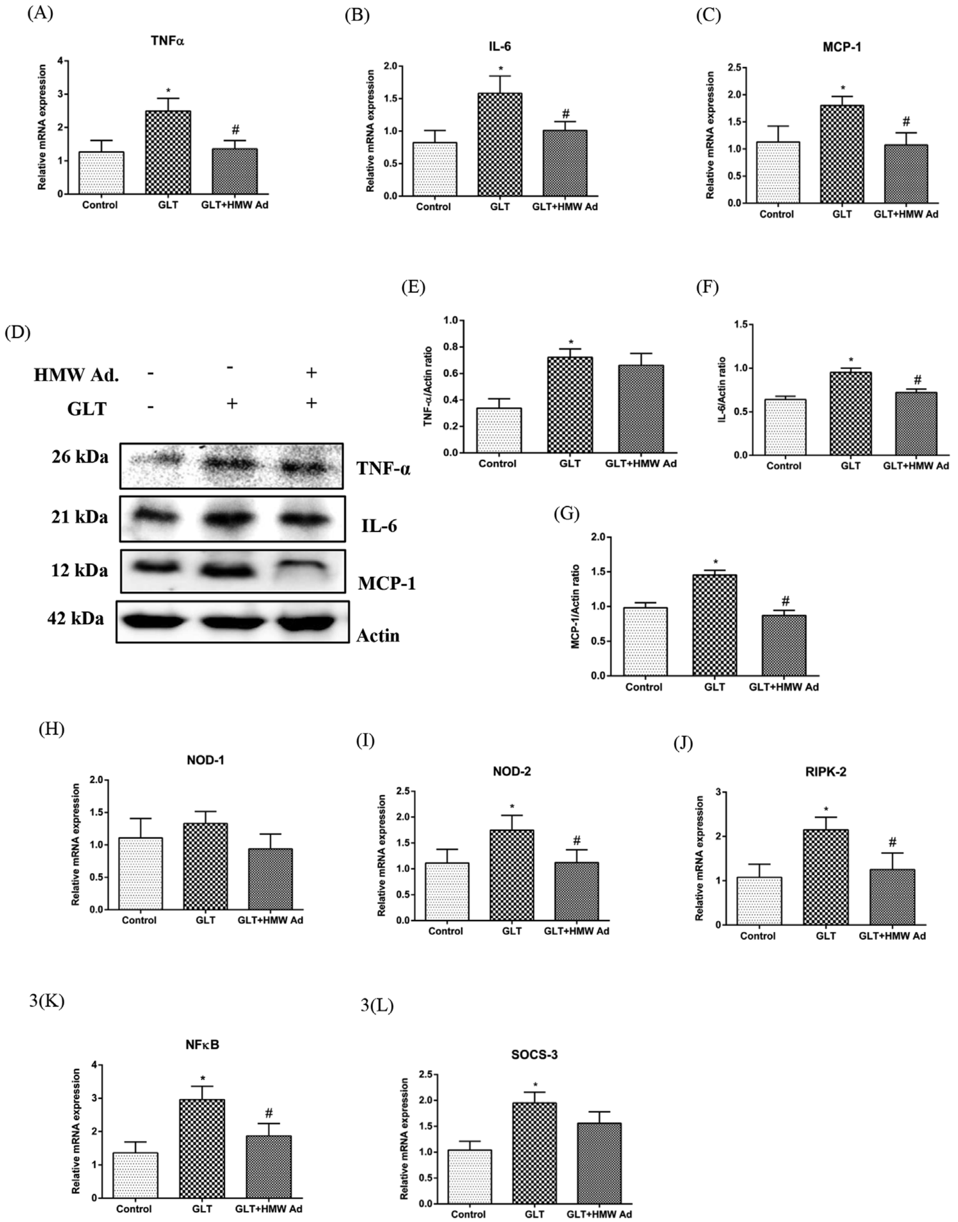

Fig. 3. Effect of HMW adiponectin on expression of GLT induced pro-inflammatory cytokines and Pattern Recognition Receptors (PRR) in 3T3-L1 adipocytes. mRNA expression patterns of (A) *TNFα*, (B) *IL-6*, and (C) *MCP-1*. (D) Representative protein expression blots of TNFα, IL-6, and MCP-1 along with β-Actin. Cumulative protein expression data of (E) TNFα, (F) IL6, and (G) MCP-1. mRNA expression profiles of (H) *NOD-1*, (I) *NOD-2*, (J) *RIPK-2*, (K) *NFκB*, and (L) *SOCS-3*. $*p < 0.05$ compared to control; $\#p < 0.05$ compared to GLT. Results are expressed as mean ± SEM of minimum three independent determinations.

vitro work supports this, as 3T3-L1 adipocytes treated with GLT exhibited increased expression of pattern recognition molecules like NOD1 and NOD2. In addition to the elevated expression of NOD1/2, our study also demonstrated increased expression of downstream signaling mediators RIPK2 and NFκB under GLT treatment. GLT treated adipocytes exhibited increased mRNA expression of inflammatory markers IL-6, TNF-α and MCP-1. This was also corroborated by the increased protein expression of these markers. It is interesting to observe that HMW Ad treatment normalizes all of these modulations in 3T3-L1 adipocytes, which supports the previous clinical observations that HMW Ad is negatively correlated with various inflammatory mediators [24].

Increased *SOCS-3* gene expression in adipocytes subject to GLT treatment is an interesting finding in this study. SOCS-3 can directly suppress insulin signaling by interacting with phosphorylated tyrosine of the insulin receptor and insulin receptor substrate-1 [25,26]. Indeed, one study showed that acriflavine (ACF), an inhibitor of HIF1α, induced the expression of adiponectin and reduced the expression of SOCS-3 in cultured 3T3-L1 adipocytes to counteract insulin resistance [27]. We also observed that augmentation of SOCS-3 expression under GLT was normalized on treatment with HMW Ad in 3T3-L1 adipocytes. As the adipose tissue in obesity is characterized by pro-inflammatory cell infiltration, causing chronic low-grade inflammation, which has significant systemic adverse consequences, such as insulin resistance and the progression of atherogenic processes [28], reduction in adipose inflammation by HMW Ad is an important observation. The underlying causes, which mediate inflammation and insulin resistance in adipocytes, are still not well understood, but could be attributed to the impairment in triglyceride management of adipose tissue. Glucolipotoxicity can alter genes involved in lipid metabolism altering the synergy between triglyceride synthesis and its breakdown [29]. While many important functions have been attributed to adiponectin, little is known

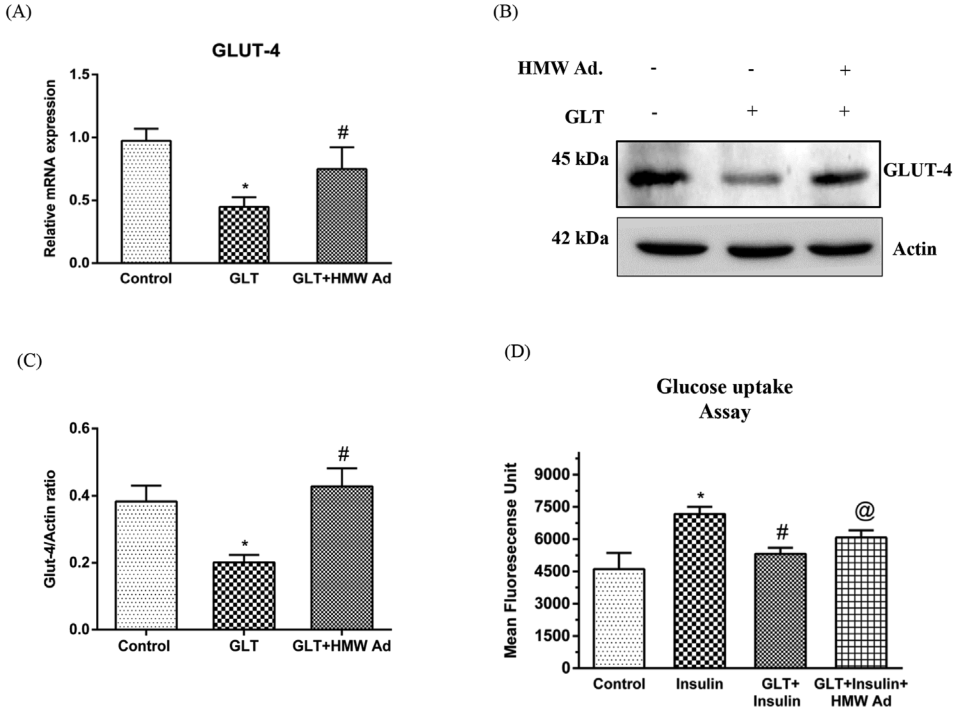

Fig. 4. Effect of HMW adiponectin on glucose homeostasis in 3T3-L1 adipocytes.
(A) mRNA expression pattern of *GLUT-4* receptors under different experimental conditions. (B) Representative protein expression blot of GLUT-4 along with β-Actin. (C) Cumulative protein expression data of GLUT-4. (D) 2-NBDG glucose uptake assay. 3T3-L1 adipocytes were pre-treated with HMW Ad for 2 h, and then stimulated with 100 nM insulin for 1 h. 40 μM 2-NBDG was then added to the cells for 30 min in the dark. Cells were washed, lysed and fluorescence was measured in the multimode reader. Results are expressed as mean ± SEM of minimum three independent determinations. $^*p < 0.05$ compared to control; $^\#p < 0.05$ compared to Insulin; $^@p < 0.05$ compared to GLT + Insulin.

about the role of HMW Ad in regulating lipid homeostasis. We found that HMW Ad treatment reduces the lipid content of adipocytes, as indicated by Adipored staining, which is also supported by reduced mRNA and protein expression of PPARγ. PPARγ plays an important role in lipid synthesis and accumulation, leads to adipocyte differentiation and fatty-acid storage and is directly linked to obesity and type 2 diabetes [30,31,32].

Leptin, which increases satiety and mediates energy expenditure, is a suppressor of lipogenesis, stimulates triglyceride hydrolysis, as well as fatty acid and glucose oxidation [33]. In our study, GLT up-regulates leptin gene expression in differentiated 3T3-L1 adipocytes; however, HMW Ad does not have any effect on leptin gene expression. Increase in leptin gene expression under GLT treatment might be the reason for the increased lipolysis in our cells, as supported by the elevated levels of the expression of two key lipolytic enzymes, ATGL and HSL in 3T3-L1 adipocytes under GLT regime. Upon treatment with HMW Ad, these adipocytes significantly exhibited lower mRNA expression of ATGL, HSL as well as reduced lipolysis of fat molecules. These results indicated that HMW Ad attenuates ectopic lipid accumulation, and causes reduced lipolysis, suggesting an overall reduction in the turnover of lipids, thus beneficially improving lipid metabolism in 3T3-L1 adipocytes and protecting the adipocytes from the glucolipotoxicity.

Adiponectin promotes glucose uptake through increased *GLUT-4* gene expression and increased GLUT-4 translocation to the plasma membrane [34]. It has been reported that adiponectin increases glucose uptake in skeletal muscle, apparently via activation of AMP kinase [35]. Studies have also shown that TNF-α contributes to insulin resistance by inhibiting the expression of GLUT-4 in 3T3-L1 adipocytes. We found that HMW Ad increases protein expression of GLUT-4 in addition to decreasing the expression of inflammatory markers on GLT treatment. Importantly, our study has demonstrated that HMW Ad offered a sensitizing effect on insulin-stimulated glucose uptake in adipocytes treated with GLT. Significant increased expression of GLUT-4 mRNA and protein receptors accompanied by increased glucose uptake and reduction in gene expression of inflammatory markers under HMW Ad treatment implies that improvement in peripheral insulin sensitivity is linked to HMW Ad.

We further investigated the mechanisms that lead to the pleotropic effects of HMW Ad. Overexpression of adiponectin has been shown to stimulate the adaptor protein APPL1 expression, which in turn improves adiponectin mediated downstream events such as lipid oxidation, glucose uptake and the membrane translocation of glucose transport 4 (GLUT-4) [36–38]. APPL1 interacts with both adiponectin receptors, AdipoR1 and AdipoR2, and positively mediates adiponectin signaling [39]. Binding of APPL1 to adiponectin receptors is necessary for adiponectin-induced AMP-activated protein kinase (AMPK) activation in muscle cells [40]. However, the role of HMW Ad in APPL1-AMPK signaling has not been studied in 3T3-L1 adipocytes. We showed that HMW Ad stimulated phosphorylation of AMPK was significantly reduced in APPL1-silenced 3T3-L1 adipocytes compared to the scrambled siRNA-treated cells. These results indicate that HMW Ad regulates the expression of pAMPK via APPL1, and APPL1, apart from its adaptor function, has the ability to regulate the expression of the proteins involved in the adiponectin signaling pathway. Together, these findings emphasize that HMW Ad plays a major role in regulating lipid metabolism, curtailing pro-inflammation, and improving the glucose uptake in adipocytes (Supplementary Fig. 2). While our study demonstrated the mechanistic action of HMW Ad, further studies are needed to document the beneficial effects of HMW Ad in preclinical and clinical studies.

In conclusion, this study demonstrates that HMW Ad treatment reduces lipolysis, pro-inflammation and insulin resistance in adipocytes subject to GLT. The beneficial modulation and regulation of APPL1 and AMPK signals by HMW Ad observed in this study is a novel finding and it has translational applications. It suggests that raising endogenous HMW Ad levels either by pharmacological or lifestyle modifications could have some therapeutic value in metabolic disorders.

Conflict of interest

The authors declared they do not have anything to disclose regarding conflict of interest with respect to this manuscript.

Author contributions

KG conceptualized the work; KG and GKP designed & executed the

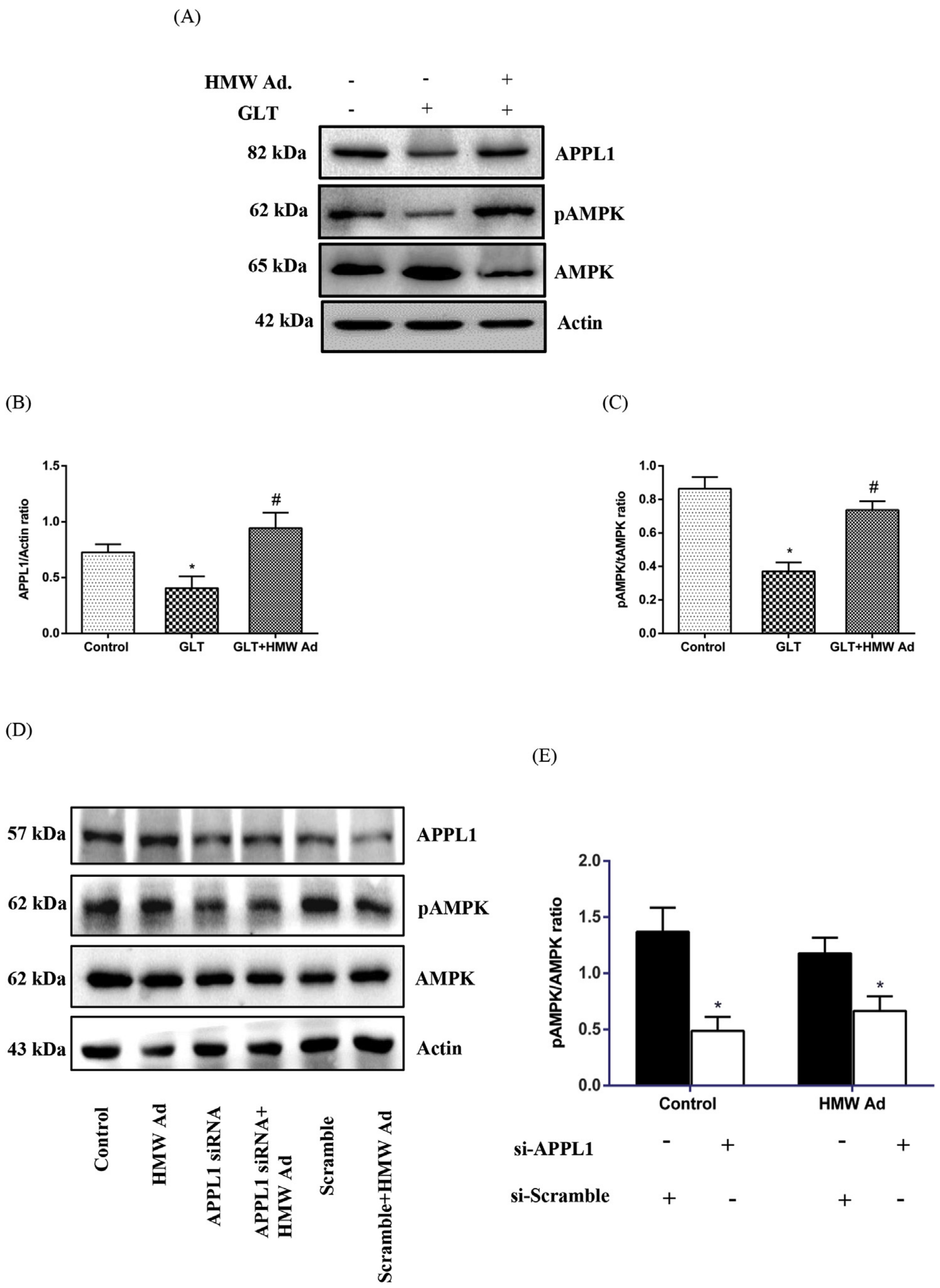

Fig. 5. Effect of HMW adiponectin on GLT impaired insulin signaling in 3T3-L1 adipocytes. (A) Representative protein expression blots of APPL1 and pAMPK, along with total APMK and β-Actin, in 3T3-L1 adipocytes, under different experimental conditions. Cumulative protein expression data of (B) APPL1 and (C) p-AMPK from 3T3-L1 adipocytes. $^{*}p < 0.05$ compared to control; $^{\#}p < 0.05$ compared to GLT. (D and E) Adiponectin activates AMPK via APPL1. (D) Representative protein expression blots of APPL1 and pAMPK, along with total APMK and β-Actin, in 3T3-L1 adipocytes, under different experimental conditions. (E) Cumulative protein expression data of pAMPK. $^{*}p < 0.05$, compared to scramble. Results are expressed as mean ± SEM of minimum three independent determinations.

study methods, analyzed data, interpreted the results and drafted the manuscript. GKP, SV and SR performed experiments and cell-culture work. VM and MB interpreted the results and helped draft the manuscript.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.atherosclerosis.2019.07.011.

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