A collagen domain–derived short adiponectin peptide activates APP1 and AMPK signaling pathways and improves glucose and fatty acid metabolisms

Mohd Sayeed, Sudeep Gautam, Devesh Pratap Verma, Tayyab Afshan, Tripti Kumari, Arvind Kumar Srivastava, and Jimut Kanti Ghosh

From the Molecular and Structural Biology Division and the Biochemistry Division, CSIR-Central Drug Research Institute, Sector 10, Jankipuram Extension, Sitapur Road, Lucknow–226 031, India

Edited by Jeffrey E. Pessin

Adiponectin is a fat tissue–derived adipokine with beneficial effects against diabetes, cardiovascular diseases, and cancer. Accordingly, adiponectin-mimetic molecules possess significant pharmacological potential. Oligomeric states of adiponectin appear to determine its biological activity. We identified a highly conserved, 13-residue segment (ADP-1) from adiponectin’s collagen domain, which comprises GXXG motifs and has one asparagine and two histidine residues that assist in oligomeric protein assembly. We therefore hypothesized that ADP-1 promotes oligomeric assembly and thereby mediates potential metabolic effects. We observed here that ADP-1 is stable in human serum and oligomerizes in aqueous environments. We also found that ADP-1 activates AMP-activated protein kinase (AMPK) in an adaptor protein, phosphotyrosine interacting with PH domain and leucine zipper 1 (APP1)-dependent pathway and stimulates glucose uptake in rat skeletal muscle cells (L6 myotubes). ADP-1–induced glucose transport coincided with ADP-1–induced biosynthesis of glucose transporter 4 and its translocation to the plasma membrane. ADP-1 induced an interaction between APP1 and the small GTPase Rab5, resulting in AMPK phosphorylation, in turn leading to phosphorylation of p38 mitogen-activated protein kinase (MAPK), acetyl-CoA carboxylase, and peroxisome proliferator–activated receptor α. Similar to adiponectin, ADP-1 increased the expression of the adiponectin receptor 1 (AdipoR1) gene. Of note, ADP-1 decreased blood glucose levels and enhanced insulin production in pancreatic β cells in db/db mice. Further, ADP-1 beneficially affected lipid metabolism by enhancing lipid globule formation in mouse 3T3-L1 adipocytes. To our knowledge, this is the first report on identification of a short peptide from adiponectin with positive effects on glucose or fatty acid metabolism.

Type 2 diabetes is caused either by the decline in the ability of β-pancreatic cells to produce insulin or the organism itself becoming resistant to insulin, which is termed as insulin resistance. As a result of not effectively responding to secreted insulin, cellular uptake of glucose decreases during type 2 diabetes.

Several proteins, directly or indirectly, play important roles in combating various metabolic disorders, and adiponectin is one of them (1, 2). Adiponectin, also known as Acrp30 (3), AdipoQ (4), GBP-28 (5), and apM1 (6), was originally cloned as an adipocyte-enriched protein, highly induced upon 3T3-L1 adipocyte differentiation (7, 8). The human adiponectin gene encodes a 244-amino acid protein (247 amino acids for the mouse ortholog) of 30 kDa, which consists of a signal peptide, a variable region, a collagen-like domain at the N terminus, and a globular domain at the C terminus (7).

Adiponectin is found in human serum at a concentration range of 2–20 μg/ml (9). However, circulating adiponectin level decreases in obese individuals, and a decrease in adiponectin level contributes to the development of diabetes and metabolic syndromes (8, 10, 11).

In obese rhesus monkey models, plasma adiponectin level often decreases with the development of type 2 diabetes (12). Replacement of adiponectin ameliorates high fat–induced insulin resistance and hypertriglyceridemia (13). The thiazolidinedione class of anti-diabetic drugs seem to exert their therapeutic effects partly by increasing the secretion of adiponectin from adipocytes, suggesting an anti-diabetic activity of this protein (14).

A proteolytic cleavage product of adiponectin, which structurally resembles globular adiponectin, increases fatty-acid oxidation in muscle, decreases plasma glucose, and causes weight loss in mice (15). Globular adiponectin transgenic ob/ob mice showed partial amelioration of insulin resistance and diabetes (16). Several studies have been carried out to understand the possible role of the globular domain of adiponectin in the metabolic activity of the protein (17, 18). However, a 10-residue peptide mimetic, ADP-355, derived from the globular domain of adiponectin inhibited proliferation of adiponectin receptor–positive cancer cell lines (9). Also, ADP-355 inhibited proliferation of chronic myeloid leukemia cells and differentiation of renal myofibroblasts and reduced thioacetamide-induced necroinflammation and liver fibrosis (19). However, there is no report on the metabolic effect of any of the peptides/peptide...
Metabolic effect of collagen domain of adiponectin

mimetics derived from the globular domain of adiponectin. Interestingly, the full-length collagen domain peptide showed a positive effect on osteoblastic differentiation (20). Because osteoblast differentiation and type 2 diabetes are closely associated with respect to biochemical pathways/molecular mechanisms (21–24), we hypothesized that it would be worthwhile to explore a peptide with metabolic effects of adiponectin from its collagen domain. Toward this end, we have identified a region corresponding to amino acid residues 42–54 from the collagen domain of adiponectin that possesses the amino acids and structural motif for oligomeric assembly. Our detailed characterization with in vitro studies in rat skeletal muscle cells (L6 myotubes), ex vivo studies in primary skeletal muscle cells of db/db mice, and in vivo experiments in db/db mice revealed a strong metabolic effect of this 13-residue collagen domain–derived peptide.

Results

Identification of adiponectin fragments

The ability of adiponectin to oligomerize plays an essential role in its interaction with adiponectin receptor and in its metabolic effects (25, 26). Hexameric adiponectin seems to interact with adiponectin receptor 1, whereas its higher oligomers interact with both adiponectin receptor 1 and 2 (8). Therefore, we presumed that a small peptide with the ability to oligomerize could be an ideal candidate to interact with the adiponectin receptor and demonstrate its metabolic effects. Only limited work hitherto has been reported toward the biological activity of the peptides derived from the collagen domain of adiponectin (20). However, the observed differentiation of osteoblasts in the presence of the collagen domain of adiponectin (20) prompted us to further explore the identification of a peptide from this domain with metabolic effects. We found a conserved (Fig. 1A) and unique sequence (amino acid region 42–54) in the collagen domain of adiponectin that possesses a couple of histidine residues and an asparagine residue (Fig. 1B) that are known to contribute to the oligomeric structure of globular proteins (27). In addition, this identified segment (Fig. 1B) contains four continuous and overlapping GXXG motifs that could assist in the oligomerization. Further, this segment contains three proline residues that frequently occur in biologically active peptides and are known to provide them proteolytic stability (28). Thus, the peptide, ADP-1, comprising the amino acid region, residues 42–54 of the collagen domain (1B) of adiponectin, was synthesized, purified, and characterized structurally, functionally, and biologically with respect to its metabolic effects. The observed mass of ADP-1 from the MALDI-TOF experiment is close to its calculated mass, and its HPLC retention time by a C-18 column is shown in Fig. 1B.

ADP-1 showed oligomerization properties

To investigate its oligomeric nature, different amounts of ADP-1 (10, 20, and 30 µg), dissolved in milli-Q water, were incubated with 0.2% glutaraldehyde (cross-linker) at 37 °C for 30 min. Then each of these different amounts of ADP-1 were run in SDS-PAGE for resolving their sizes and also stained with Coomassie Blue for their visualization. A broad band appeared, which corresponded to ~7 kDa. Because the size of 13-residue ADP-1 is ~1.2 kDa, this peptide seems to oligomerize into a hexamer (Fig. S1A).

Structural properties of ADP-1

The FTIR spectrum of ADP-1 showed prominent peaks (low frequency) at around 1621.93 and 1659.60 cm⁻¹, respectively, corresponding to amide I region (1600–1700 cm⁻¹), which are indicative of parallel β-sheet (1615–1637 cm⁻¹) and α-helical (1649–1660 cm⁻¹) structure, respectively (Fig. S1B) (29). Further secondary structure of ADP-1 was investigated in the presence of trifluoroethanol (40% (v/v)), PBS (pH 7.4), and SDS (1% (w/v)) (Fig. S1C, i–iii) by CD studies. The CD spectra of ADP-1 indicated enhancement of its helical and β-sheet structures in SDS and trifluoroethanol as compared with that in PBS (Table S1).

Proteolytic stability

Proteolytic stability of ADP-1 was investigated in human serum at 37 °C as described in the supporting information. Analyses by HPLC indicated that ~75% of ADP-1 remained intact after 60 min of incubation with human serum (Fig. 1C and Fig. S2). Thus, ADP-1 offered significant resistance against the proteolytic enzymes that are present in the human serum, which was remarkable for such a short peptide and exhibited its in vivo potential.

ADP-1 is nontoxic in nature

A hemolytic activity assay suggested that ADP-1 exhibited insignificant lysis of human red blood cells at up to 200 µM peptide concentration (Fig. S3A). Dose-dependent determination of viability of 3T3 cells in the presence of ADP-1 by a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay suggests that this peptide does not possess significant cytotoxicity toward these cells at up to 200 µM concentration as evidenced by more than 90% viability of the cells (Fig. S3B). Overall, the data suggested a significant noncytotoxic nature of the ADP-1.

ADP-1–enhanced glucose uptake in L6 myotubes

It was of interest to examine whether adiponectin-derived peptide, ADP-1, can induce glucose uptake in L6 myotubes. For this purpose, ADP-1 was incubated with these cells for varying incubation periods, and peptide-induced glucose uptake was determined for different incubation times. It was observed that at an incubation period of 3 h, ADP-1 exhibited the maximum glucose uptake in L6 myotubes (Fig. 2A). In this experiment, insulin was used as a positive control and incubated with L6 myotubes for 20 min at a concentration of 100 nM, which were reported as its optimum concentration and incubation period in a previous study (30, 31). The data indicate that ADP-1 requires a longer incubation period to cause optimum glucose uptake in L6 myotubes compared with insulin. Then dose response of ADP-1–induced glucose uptake in L6 myotubes was determined by incubating different concentrations of the peptide with these cells for 3 h. It was observed that pretreatment of L6 myotubes with ADP-1 increased glucose uptake in these cells in a concentration-dependent manner, and ADP-1 enhanced optimum glucose uptake in these cells at a concen-
tration of 14.3 μg/ml (1.8-fold). Insulin enhanced a significant increase (2-fold) in glucose uptake at a concentration of 100 nM after an incubation of 20 min with L6 myotubes (Fig. 2B).

**Effect of ADP-1 on glucose uptake in TNFα-treated L6 myotubes**

Proinflammatory cytokine TNFα2 is known to induce insulin resistance in the peripheral tissues, such as skeletal muscle cells and adipose tissues (32). TNFα attenuates both GLUT-4 translocation and glucose uptake in skeletal muscle cells (33). Therefore, we investigated whether ADP-1 could break TNFα-induced insulin resistance in L6 myotubes. For this purpose, L6 myotubes were treated with TNFα (10 ng/ml) and incubated for 24 h, followed by treatment with ADP-1 (14.3 μg/ml) for 3 h. We observed that ADP-1 significantly induced glucose uptake in TNFα-treated L6 myotubes, suggesting its ability to break the insulin resistance (Fig. 2C).

**Effect of ADP-1 on glucose uptake in the presence of inhibitors**

To investigate the molecular mechanism of ADP-1–induced glucose uptake in L6 myotubes, these cells were pretreated with different inhibitors corresponding to different molecular pathways like AKT-i (specific AKT1/2 inhibitor) and indinavir (specific GLUT-4 inhibitor), and then uptake of glucose in treated and untreated cells was measured. Uptake of glucose in these...

---

**Figure 1.** A, sequence alignment of various vertebrate adiponectin using software Clustal Omega (version 1.2.4). The N-terminal region of adiponectin features a secretion signal peptide, a variable region followed by a collagen-like domain. ADP-1 peptide derived from the collagen-like domain of human adiponectin is underlined, and its sequence alignment is marked in red in various vertebrates (asterisks, colons, and periods indicate fully conserved, strongly similar, and weakly similar residues, respectively). B, sequence of peptide ADP-1 and its calculated and observed mass and HPLC retention time. Glycine residues of the GXXG motif are marked in red. C, the proteolytic stability of the peptide ADP-1 was evaluated by incubating it in fresh human serum (25%) for different time periods at 37 °C. The amount of intact peptide was estimated by HPLC after removing the bulk of proteins by methanol precipitation. The values correspond to the in vitro half-life, which is defined as the time needed for 50% degradation of the peptide.
Metabolic effect of collagen domain of adiponectin

Figure 2. Effects of ADP-1 on glucose uptake in L6 myotubes. A, L6 myotubes were treated with ADP-1 at a concentration of 14.3 μg/ml for different time intervals. A subset of cells was also treated with a 100 nM concentration of insulin (as a standard) for 20 min. At 180 min, peptide induced maximum glucose uptake by 2-fold (**, p < 0.001 versus control basal) in L6 myotubes. Results shown are mean ± S.D. (error bars), of three independent experiments. B, 6 L6 myotubes were treated with different concentrations of ADP-1 for 3 h. A subset of cells was also treated with a 100 nM concentration of insulin (as a standard) for 20 min. At a concentration of 14.3 μg/ml ADP-1, peptide induced maximum glucose uptake by 1.8-fold (**, p < 0.001 versus control basal) in L6 myotubes. Results shown are mean ± S.D. of three independent experiments. C, TNF-α-induced L6 myotubes. L6 myotubes were treated with either TNFα (10 ng/ml for 24 h), ADP-1 (14.3 μg/ml for 3 h), or insulin (100 nM for 20 min), followed by determination of 2-deoxyglucose uptake. D, effect of ADP-1 and metformin on ADP-1–induced glucose uptake in L6 myotubes. Cells were incubated with ADP-1 (14.3 μg/ml for 3 h), AKT-i (420 nM for 30 min), and indinavir (100 μM for 30 min), followed by determination of the glucose uptake. Results shown are mean ± S.D. of three independent experiments. *, p < 0.05; **, p < 0.001 versus control.

The results indicate that ADP-1 increased the expression of phosphorylated AMPK protein by 2.9-fold (p < 0.001) (Fig. 3A, i and ii). ADP-1-induced expression of APP1L was increased by 3-fold (p < 0.01), and metformin increased the expression of the same by 4.1-fold (p < 0.01) (Fig. 3B, i and ii). ADP-1 increased the total GLUT-4 protein synthesis by 1.9-fold (p < 0.001), whereas metformin increased its expression by 2-fold (p < 0.01) (Fig. 3C, i and ii).

Further, ADP-1 increased the expression of phosphorylated ACC protein by 3.1-fold (p < 0.001), whereas metformin enhanced the same by 3.0-fold (p < 0.001) (Fig. 3D, i and ii).

ADP-1–induced mRNA expression of Glut-4 gene

To obtain more insight into ADP-1–induced glucose transport, transcription of the Glut-4 gene was investigated in the presence of this peptide in L6 myotubes. To investigate the effect of ADP-1 on Glut-4 gene expression, L6 cells were seeded in 6-well plates, and after 4–6 days, confluent cells were treated with ADP-1 (14.3 μg/ml for 3 h), and as a positive control, cells were treated with metformin (20 μM) for 12 h. Real-time PCR results suggested that the treatment with ADP-1 increased Glut-4 gene expression by 3.5-fold (p < 0.01 versus control basal) in L6 cells. Metformin also augmented the expression of the Glut-4 gene by 3.8-fold (p < 0.001 versus control basal) (Fig. S4).

ADP-1 stimulates basal GLUT-4 translocation in L6 myotubes

To examine whether ADP-1–induced glucose transport was involved with GLUT-4 translocation, the translocation of GLUT-4 protein was studied in L6 myotubes in the presence of ADP-1. For this purpose, plasma and cytoplasmic fractions of proteins of L6 cells were separately run on SDS-PAGE and probed with GLUT-4 antibody. It is evident that ADP-1 treatment significantly enhanced the translocation of GLUT-4 protein to the plasma membrane (Fig. 4A, i) of L6 myotubes as compared with that observed in the untreated cells (3-fold, p < 0.001 versus nontreated) (Fig. 4A, i and ii), whereas positive control, insulin, caused an increase in GLUT-4 translocation by 4.1-fold (p < 0.001 versus nontreated) (Fig. 4A, i and ii). However, analysis of the cytoplasmic proteins of the treated and untreated cells indicated that more GLUT-4 protein resides in the cytoplasm of untreated cells compared with ADP-1– and insulin–treated cells (Fig. 4A, iii and iv). Overall, the data suggested that ADP-1 augmented the translocation of GLUT-4 protein from the cytoplasm to the plasma membrane of L6 myotubes.

To further understand the basis of ADP-1–stimulated GLUT-4 translocation in L6 myotubes, expression of phosphorylated AS160 and TBC1D1 were measured (Fig. 4B). ADP-1 increased the expression of p-AS160 and p-TBC1D1 by 3.8-fold (p < 0.001 versus nontreated) (Fig. 4B, i and ii) and 5.5-fold (p < 0.001 versus nontreated) (Fig. 4B, iii and iv), respectively. Significant phosphorylation of AS160 and TBC1D1 suggested the direct involvement of these phosphorylated proteins in ADP-1–induced GLUT-4 translocation.

ADP-1 stimulates the interaction of APP1L and Rab5 proteins in L6 myotubes

An association between Rab5 and APP1L is a key event in adiponectin-induced glucose uptake in cells (35). Therefore, we examined the effect of ADP-1 on the association of Rab5 with APP1L in L6 myotubes. Lysate of L6 cells after treatment with ADP-1 and metformin was immunoprecipitated with APP1L antibody and a nonspecific antibody (IgG isotype) at the same
concentration. The same immunoprecipitations were also carried out with the lysate of the cells that were not treated with either ADP-1 or metformin (basal control). The formed immune complexes were eluted as described under “Experimental procedures,” resolved by SDS-PAGE, and then probed with both APPL1 and Rab5 (Fig. 5A, i). When the lysate of L6 cells pretreated with ADP-1 was immunoprecipitated with APPL1 antibody, prominent bands of Rab5 and APPL1 were observed after probing with the respective antibody. However, no band of Rab5 was observed in the eluate of basal control cells after probing with this antibody (Fig. 5A, i). The results indicated that ADP-1 induced the interaction between APPL1 and Rab5 (Fig. 5A, i). Metformin, employed as a positive control, also induced interaction between APPL1 and Rab5 (Fig. 5A, i).

Figure 3. Effect of ADP-1 on different downstream markers of adiponectin signaling pathways by studying their expressions in the presence of the peptide by Western blot analysis. A (i), Western blot analysis of the phosphorylation of AMPK (p-AMPK) protein in L6 myotubes. A (ii), densitometric quantification of p-AMPK relative to β-actin. Results shown are mean ± S.D. (error bars) of three independent experiments. ***, p < 0.001, relative to nontreated. B (i), Western blot analysis of the expression of APPL1 proteins in L6 myotubes. B (ii), densitometric quantification of APPL1 relative to β-actin. Results shown are mean ± S.D. of three independent experiments. **, p < 0.01, relative to nontreated. C (i), Western blot analysis of the expression of GLUT-4 protein in L6 myotubes. C (ii), densitometric quantification of total GLUT-4 protein relative to β-actin. Results shown are mean ± S.D. of three independent experiments. **, p < 0.01, relative to nontreated. D (i), Western blot analysis of the phospho-ACC protein. D (ii), densitometric quantification of p-ACC relative to β-actin. Results shown are mean ± S.D. of three independent experiments. ***, p < 0.001, relative to nontreated. All proteins were probed from the same lysate and while probing the individual proteins, β-actin blots were quite comparable with each other; hence, a single β-actin blot has been used for these proteins.
Metabolic effect of collagen domain of adiponectin

in SDS-PAGE and probed with both APPL1 and Rab5 antibodies (Fig. 5A, i).

Overall, the results indicated that Rab5 bands appeared in the anti-APPL1 immunoprecipitated lysates of the L6 myotubes, pretreated with ADP-1 or metformin as a result of specific interaction with APPL1 and Rab5.

ADP-1–induced expression of AdipoR1 gene in L6 myotubes

One of the most crucial events in adiponectin-induced glucose uptake in muscle cells is the interaction of adiponectin with its receptor. It has been mentioned that relatively lower aggregates (hexameric) of adiponectin interact with adiponectin receptor 1 (8). Therefore, we examined whether ADP-1 altered expression of AdipoR1. For this purpose, L6 cells were seeded in 6-well plates, and after 4–6 days, confluent cells were treated with ADP-1 for 3 h; as a positive control, cells were treated with metformin for 12 h. Real-time PCR results suggested that the treatment with ADP-1 significantly increased AdipoR1 gene expression in L6 cells compared with the non-treated negative control cells (Fig. 5A, ii). Metformin also augmented the expression of AdipoR1 (Fig. 5A, ii).

**Activation of AMPK leads to the phosphorylation of p38 MAPK and activation of peroxisome proliferator–activated receptor–α (PPAR-α) in the nucleus**

Adiponectin enhances the phosphorylation of AMPK, which further phosphorylates p38 MAPK (36). Because ADP-1 activated the AMPK pathway, we investigated whether phosphorylation of p38 MAPK could occur in the presence of this peptide in L6 myotubes. As shown in Fig. 5B (i and ii), ADP-1 also induced the phosphorylation of p38 MAPK. Interestingly, when the activity of AMPK was inhibited by incubating the L6
cells with compound C (dorsomorphin) for 6 h, the expression level of p-p38 MAPK was found to be reduced (Fig. 5B, i and ii), indicating that the phosphorylation of p38 MAPK depends on AMPK activation. PPARs are involved in lipid homeostasis, diabetes, and cancer (37). We observed that the treatment with ADP-1 increased the expression of PPAR-α inside the nucleus of L6 cells. When AMPK was inhibited by incubating the L6 cells with compound C (dorsomorphin) for 6 h, a decrease in the expression of PPAR-α was also observed (Fig. 5B, iii and iv).

Thus, ADP-1–induced phosphorylation of AMPK played a crucial role in the activation of PPAR-α into the nucleus of these cells.

**ADP-1 restores phosphorylation of p38 MAPK and AMPK in TNFα-treated insulin-resistant cells**

TNFα inhibits insulin signaling pathway by increasing Ser-307 phosphorylation of IRS-1, resulting in the enhancement of insulin resistance in L6 myotubes (32). However, we observed...
Metabolic effect of collagen domain of adiponectin

Figure 6. ADP-1 enhanced adipogenesis. A, 3T3-L1 preadipocytes were cultured in DMEM and MDI containing 14.3 μg/ml ADP-1 for 8 days. Images were acquired before lipid droplet staining with ORO and were taken with a Nikon Eclipse Ti. B, absorbance of extracted ORO accumulated in lipid droplets of 3T3-L1–derived adipocytes was measured spectrophotometrically at 492 nm, and percentage of lipid accumulation was determined in the presence of ADP-1 (**, p < 0.01 versus control). All values are presented as the mean ± S.D. (error bars) (n = 3).

Figure 8, A (B and C) shows the effect of ADP-1 on oral glucose tolerance in db/db and nontreated basal) (Fig. 8, B and C). Fig. 8 (B and C) data and graphical presentation depicts an overall glucose-lowering effect of ADP-1 treatment at a 30-mg/kg dose on db/db mice after 30 days of the experiment compared with the nontreated mice. ADP-1 caused a significant decline in the hyperglycemia compared with the nontreated control group of db/db mice (Fig. 8, B and C). Fig. 8 (B and C) shows the effect of ADP-1 on oral glucose tolerance in db/db mice after 30 days of ADP-1 treatment on every alternate day at a 30-mg/kg dose. The overnight-fasted db/db mice were subjected to an oral glucose tolerance test after a 3.0-g oral glucose load. The fasting baseline of blood glucose values at 3 min (3 min before glucose load) were found to be lowered significantly (δδδδ, p < 0.001) in all of the treated mice as compared with the nontreated control group at the same time, suggesting an anti-hyperglycemic effect of the peptide (Fig. 8, B and C). Metformin (50 mg/kg) also significantly reduced blood glucose level (**, p < 0.001) compared with the nontreated mouse group (Fig. 8, B and C). After giving the glucose load (3.0 g), blood glucose levels were checked after every 30-min interval up to 120 min (Fig. 8, B and C). After 120 min, we observed that the ADP-1–treated mouse group possessed significantly lower blood glucose levels as compared with the nontreated mouse group (δδδδ, p < 0.01) (Fig. 8, B and C). Metformin-treated mice also exhibited a significant decrease in the blood glucose levels (**, p < 0.001) (Fig. 8, B and C). In terms of AUC (area under the curve) values, ADP-1 and metformin showed 25.5 and 32.57% blood glucose–lowering effect, respectively, in db/db mice with respect to the nontreated controls (Fig. 8B). Experimental values are expressed as mean ± S.D. (n = 4).

that this chronic effect of TNFα did not influence the ADP-1–induced glucose uptake in L6 cells, as already described before (Fig. 2C). Because ADP-1 induced phosphorylation of AMPK and p38 MAPK, it was of interest to examine whether these phosphorylation events occurred in TNFα–treated, insulin-resistant L6 myotubes in the presence of ADP-1. For this purpose, L6 myotubes, pretreated with TNFα (10 ng/ml, 24 h) (38), were incubated with or without ADP-1 (14.3 μg/ml) for 3 h. Nontreated cells were taken as basal control. Results of Western blot analysis indicated that ADP-1 could restore the phosphorylation of p38 MAPK and AMPK in TNFα–treated insulin-resistant L6 myotubes (Fig. S5).

ADP-1–enhanced adipocyte differentiation and lipid accumulation

We further examined whether ADP-1 had any influence in fatty acid metabolism. Preadipocytes (3T3-L1) form lipid globules following the treatment with differentiating medium (MDI) by the process of adipogenesis. To examine whether ADP-1 can influence lipid globule formation, it was added to the cells with MDI during differentiation. The standard drug rosiglitazone was used as a positive control. Microscopic observation suggested that ADP-1 increased lipid droplet accumulation in the differentiated 3T3-L1 adipocytes (Fig. 6A). The absorbance of extracted oil red stain, accumulated onto lipid droplets, confirmed that ADP-1 induced adipogenesis in these cells (**, p < 0.01 versus control) (Fig. 6B).

Ex vivo metabolic effect of ADP-1 on primary skeletal muscle cells

To investigate whether ADP-1 shows a similar metabolic effect on primary skeletal muscle cells (isolated from adult db/db mice) (Fig. 7A) as it has on L6 myotubes (in vitro), we performed Western blot analysis of the total proteins isolated from these cells pretreated with ADP-1 and metformin (positive control) and measured the effect of ADP-1 on the expressions of GLUT-4 and phospho-AMPK proteins (Fig. 7B).

We found that ADP-1 significantly increased the expressions of GLUT-4 (p < 0.001 versus nontreated basal) (Fig. 7B, i and ii) and p-AMPK proteins (p < 0.001 versus nontreated basal) (Fig. 7B, iii and iv). The results suggest that ADP-1 also shows ex vivo metabolic effect on primary skeletal muscle cells.

Treatment with ADP-1 peptide increases the level of insulin in blood serum of db/db mice

30 mg/kg ADP-1 was administered to db/db mice (n = 4) on every alternate day for 20 days through intraperitoneal injection (IP). 50 mg/kg metformin was injected in the positive control mouse group through IP on every alternate day for 20 days. The third mouse group was of untreated mice. During administration of the peptide or drug, blood samples from each group of db/db mice were collected on the first, fifth, 10th, 15th, and 20th day. Serum was collected from each blood sample and stored at −20 °C. Levels of insulin were examined in these samples using an insulin ELISA kit. We observed that the insulin levels in the sera of peptide–treated mice increased progressively, and on the 20th day, significant increases in these levels (**, p < 0.001 versus nontreated, n = 4) were observed as compared with the nontreated mice. Metformin also increased the level of insulin significantly on the 20th day (δδδδ, p < 0.001 versus nontreated) (Fig. 8A).

In vivo effect of ADP-1 on glucose tolerance in db/db mice

Metformin (50 mg/kg) also significantly reduced blood glucose level (**, p < 0.001) compared with the nontreated mouse group (Fig. 8, B and C). After giving the glucose load (3.0 g), blood glucose levels were checked after every 30-min interval up to 120 min (Fig. 8, B and C). After 120 min, we observed that the ADP-1–treated mouse group possessed significantly lower blood glucose levels as compared with the nontreated mouse group (δδδδ, p < 0.01) (Fig. 8, B and C). Metformin-treated mice also exhibited a significant decrease in the blood glucose levels (**, p < 0.001) (Fig. 8, B and C). In terms of AUC (area under the curve) values, ADP-1 and metformin showed 25.5 and 32.57% blood glucose–lowering effect, respectively, in db/db mice with respect to the nontreated controls (Fig. 8B). Experimental values are expressed as mean ± S.D. (n = 4).
Discussion

Despite promising therapeutic prospects of adiponectin, only limited knowledge has been gained of its structure–function relationships. Also, the potential role of various segments of adiponectin contributing to the biological activities remains obscure. In the present investigation, we have identified and characterized a 13-residue peptide derived from the collagen domain of adiponectin, namely ADP-1, that showed versatile metabolic effects, including glucose uptake (Fig. 2) and fatty acid oxidation (Fig. 3D), in rat muscle cell L6 myotubes. Further, ADP-1 induced lipid droplet formation in adipocytes (Fig. 6), increased the insulin level of blood serum (Fig. 8A), and augmented glucose tolerance (Fig. 8, B and C) in db/db mice. ADP-1 showed significant nontoxic nature (Fig. S3) and stability in the presence of human serum (Fig. 1C and Fig. S2) and thus qualified for its use in animal experiments. The identified segment possesses significant amino acid sequence conservation among the selected vertebrate family members (Fig. 1A). FTIR and CD studies indicated that ADP-1 adopted both α-helical and β-sheet secondary structures (Fig. S1, B and C). Previous studies suggest that adiponectin circulates in multiple oligomeric forms of which hexameric and higher oligomeric forms (12–18-mer) are the active forms that interact with AdipoR1 and further stimulate downstream signaling events. Remarkably, ADP-1 adopted a hexameric form in an aqueous environment (Fig. S1A). Adiponectin possesses a much higher molecular weight than the short peptide; therefore, it is hard to corroborate the interaction of oligomeric adiponectin and adiponectin receptor with that of the hexameric form of ADP-1 and the adiponectin receptor. Nevertheless, the data suggest the capability of only ~1.2-kDa ADP-1 to adopt the oligomeric state (Fig. S1A). Adiponectin possesses a much higher molecular weight than the short peptide; therefore, it is hard to corroborate the interaction of oligomeric adiponectin and adiponectin receptor with that of the hexameric form of ADP-1 and the adiponectin receptor. Nevertheless, the data suggest the capability of only ~1.2-kDa ADP-1 to adopt the oligomeric state (Fig. S1A). Adiponectin possesses a much higher molecular weight than the short peptide; therefore, it is hard to corroborate the interaction of oligomeric adiponectin and adiponectin receptor with that of the hexameric form of ADP-1 and the adiponectin receptor. Nevertheless, the data suggest the capability of only ~1.2-kDa ADP-1 to adopt the oligomeric state (Fig. S1A). Adiponectin possesses a much higher molecular weight than the short peptide; therefore, it is hard to corroborate the interaction of oligomeric adiponectin and adiponectin receptor with that of the hexameric form of ADP-1 and the adiponectin receptor. Nevertheless, the data suggest the capability of only ~1.2-kDa ADP-1 to adopt the oligomeric state (Fig. S1A). Adiponectin possesses a much higher molecular weight than the short peptide; therefore, it is hard to corroborate the interaction of oligomeric adiponectin and adiponectin receptor with that of the hexameric form of ADP-1 and the adiponectin receptor. Nevertheless, the data suggest the capability of only ~1.2-kDa ADP-1 to adopt the oligomeric state (Fig. S1A). Adiponectin possesses a much higher molecular weight than the short peptide; therefore, it is hard to corroborate the interaction of oligomeric adiponectin and adiponectin receptor with that of the hexameric form of ADP-1 and the adiponectin receptor. Nevertheless, the data suggest the capability of only ~1.2-kDa ADP-1 to adopt the oligomeric state (Fig. S1A). Adiponectin possesses a much higher molecular weight than the short peptide; therefore, it is hard to corroborate the interaction of oligomeric adiponectin and adiponectin receptor with that of the hexameric form of ADP-1 and the adiponectin receptor. Nevertheless, the data suggest the capability of only ~1.2-kDa ADP-1 to adopt the oligomeric state (Fig. S1A). Adiponectin possesses a much higher molecular weight than the short peptide; therefore, it is hard to corroborate the interaction of oligomeric adiponectin and adiponectin receptor with that of the hexameric form of ADP-1 and the adiponectin receptor. Nevertheless, the data suggest the capability of only ~1.2-kDa ADP-1 to adopt the oligomeric state (Fig. S1A). Adiponectin possesses a much higher molecular weight than the short peptide; therefore, it is hard to corroborate the interaction of oligomeric adiponectin and adiponectin receptor with that of the hexameric form of ADP-1 and the adiponectin receptor. Nevertheless, the data suggest the capability of only ~1.2-kDa ADP-1 to adopt the oligomeric state (Fig. S1A). Adiponectin possesses a much higher molecular weight than the short peptide; therefore, it is hard to corroborate the interaction of oligomeric adiponectin and adiponectin receptor with that of the hexameric form of ADP-1 and the adiponectin receptor. Nevertheless, the data suggest the capability of only ~1.2-kDa ADP-1 to adopt the oligomeric state (Fig. S1A).
inhibited ADP-1–induced glucose uptake in L6 myotubes, suggesting that AKT is not involved with this peptide-induced glucose uptake event (Fig. 2D).

ADP-1 in L6 myotubes stimulated phosphorylation of AMPK, which was comparable with that of metformin (Fig. 3A, i and ii). Moreover, ADP-1 showed remarkable similarity with the whole protein, adiponectin, with respect to activation of oxidation of fatty acid, which was evident from the phosphorylation of ACC (acetyl-CoA carboxylase) (Fig. 3D, i and ii). Adaptor protein APPL1 mediates most of the crucial events in the metabolic activity of adiponectin. Therefore, we investigated whether ADP-1 was capable of activating the adaptor protein APPL1. Results indicated that ADP-1 stimulated the expression of APPL1 in L6 myotubes (Fig. 3B, i and ii). Biosynthesis and translocation of GLUT-4 protein into the plasma membrane of the concerned cells are crucial to the glucose uptake in these cells. Our results suggested that ADP-1 stimulated both mRNA (Fig. S4) and protein expression of GLUT-4 (Fig. 3C, i and ii). In addition, ADP-1 also caused translocation of GLUT-4 protein from the cytoplasm to the plasma membrane of L6 myotubes (Fig. 4A). Thus, ADP-1–induced glucose uptake in L6 myotubes could be involved with both enhanced biosynthesis and translocation of GLUT-4 protein from the cytoplasm to the plasma membrane of these cells. Further, translocation of GLUT-4 to the plasma membrane of L6 cells was corroborated by ADP-1–induced phosphorylations of two related proteins, namely AS160 and TBC1D1 (Fig. 4B).

The interaction between APPL1 and Rab5 is a crucial upstream event that occurs in adiponectin–induced metabolic pathways (39). Both Rab5 and APPL1 bands were visible in APPL1 antibody–immunoprecipitated cell lysate eluates of L6 myotubes that were pretreated with ADP-1 (Fig. 5A, i). However, the Rab5 band was not visible in the absence of ADP-1 treatment of these cells (Fig. 5A, i). These observations indicate that ADP-1 induced specific interaction between the adaptor protein APPL1 and Rab5. The results may further imply that
ADP-1 activates the adiponectin-mediated glucose uptake pathway by inducing the interaction between APPL1 and Rab5 (Fig. 5A, i). Following the interactions between APPL1 and Rab5, phosphorylation of p38 MAPK occurs, which could be associated with increased glucose uptake in the cells (40). Our data suggested that ADP-1 stimulated phosphorylation of p38 MAPK (Fig. 5B, i and ii). Interestingly, inhibitor of AMPK (compound C) inhibited phosphorylation of p38 MAPK, suggesting that this event could be dependent on kinase activity of AMPK (Fig. 5B, i and ii).

To understand the basis of glucose transport in TNFα-treated, insulin-resistant L6 myotubes, phosphorylations of p38 MAPK and AMPK were investigated. The results suggested that ADP-1 treatment of these insulin-resistant cells restored phosphorylations of both AMPK and p38 MAPK that were attenuated as a result of TNFα treatment (Fig. S5). Overall, ADP-1 along with glucose transport in both regular and insulin-resistant L6 myotubes caused phosphorylation of AMPK and p38 MAPK in both of these cells. The data indicated a significant efficacy of ADP-1 in causing glucose transport in the insulin-resistant L6 myotubes and a possible interrelation among these phosphorylations and glucose transport events. The role of ADP-1 in fatty acid oxidation was further proven by the nuclear expression of PPAR-α, which is a downstream signaling transcription factor of ACC (Fig. 5B, iii and iv). Inhibitor of AMPK attenuated ADP-1–induced nuclear expression of PPAR-α in L6 myotubes (Fig. 5B, iii and iv), suggesting that AMPK is a key protein in fatty acid oxidation caused by ADP-1. Further, ADP-1 induced lipid droplet formation in adipocytes, suggesting that, like adiponectin, ADP-1 facilitated both the catabolic (Figs. 3D and 5B (iii)) and anabolic metabolism of lipid (Fig. 6).

After observing significant in vitro metabolic activity of ADP-1 in L6 myotubes, we examined the activity of the peptide toward the primary skeletal muscle cells isolated from the adult db/db mice. Our results showed significant expressions of GLUT-4 and AMPK proteins in these primary cells in the presence of ADP-1 compared with the nontreated cells, suggesting ex vivo metabolic activity of this peptide (Fig. 7). Finally, in vivo activity of ADP-1 was investigated in db/db mice. Our results indicated that ADP-1 enhanced the secretion of insulin in db/db mice (Fig. 8A), suggesting its insulin-sensitizing nature like that of its parent protein (7). Considering the in vitro metabolic effect of ADP-1 in L6 myotubes and primary skeletal muscle cells from db/db mice, its low cytotoxicity, and appreciable stability in the presence of human serum, the in vivo efficacy of the peptide was examined in db/db mice in a glucose tolerance model. A 30-day treatment with ADP-1 at a dose of 30 mg/kg showed significant efficacy to attenuate the blood glucose level in db/db mice against oral engulfment of glucose (Fig. 8, B and C), which further proved the in vivo anti-diabetic property of this peptide.

ADP-1 shows similarity with the well-known anti-diabetic drug metformin. For example, both of them induce signaling through the AMPK-dependent pathway (41) and activate PPARs through phosphorylation of p38 MAPK. ADP-1 also exhibits similarity with the thiazolidinedione drug rosiglitazone in their adipogenesis properties by inducing the formation of lipid globules in 3T3-L1 cells (42).

Overall, ADP-1 showed appreciable resemblance to its parent protein, adiponectin, which includes the stimulation of expression of AdipoR1; promotion of the interaction between APPL1 and Rab5; phosphorylations of AMPK, ACC, and p38 MAPK; enhancement of the expression of GLUT-4; and its translocation to the plasma membrane.

Insolubility of adiponectin is a major obstacle to its use as a therapeutic agent (9) despite its significant potential in diabetes and other metabolic disorders. No peptides nor their derivatives from the globular domain of adiponectin have been implicated in the anti-diabetic activity, and not many peptides have been characterized from the collagen domain of adiponectin to our knowledge. In this context, the 13-residue, water-soluble ADP-1, identified from the collagen domain of adiponectin, showed remarkable metabolic effects, as described here. Taking into account its in vitro, ex vivo, and in vivo activities, low cytotoxicity, and stability in the presence of human serum, ADP-1 possesses significant potential for anti-diabetic therapy, and the present study could assist in the design of peptides with beneficial metabolic effects.

Experimental procedures

Peptide synthesis and purification

The methodology has been described in the supporting information.

Cell culture

Rat skeletal muscle cells (L6)—The L6 cell line was obtained from the CSIR-Central Drug Research Institute, Lucknow cell line repository and cultured in high-glucose DMEM (Sigma) supplemented with 10% fetal bovine serum (FBS; Gibco), 10 mM HepES (Invitrogen), and Anti-Anti 100 X (antibiotic-antimycotic, 15240, Invitrogen) in a 5% CO2 and 37 °C environment. For differentiation, L6 cells were transferred to DMEM with 2% FBS for 8–8 days post-confluence (43, 44).

Mouse adipocytes (3T3-L1)—3T3-L1 is a cell line derived from (mouse) 3T3 cells that is used in biological research on adipose tissue. 3T3-L1 cells have a fibroblast-like morphology, but under appropriate conditions, the cells differentiate into an adipocyte-like phenotype (45–48). 3T3-L1 preadipocytes (obtained from CSIR-Central Drug Research Institute, Lucknow cell line repository) were cultured in DMEM (Sigma) with 10% FBS and Anti-Anti 100 X (antibiotic-antimycotic, 15240, Invitrogen) in a 5% CO2 and 37 °C environment. 3T3-L1 preadipocytes were grown in 12-well plates until 2 days post-confluence, and the cells were induced by the differentiation medium (combination of 0.5 mmol/liter isobutylmethyloxanthine, 0.25 μmol/liter dexamethasone, and 1 mg/liter insulin in DMEM medium with 10% FBS) to differentiate into adipocytes (49). Three days after induction, the differentiation medium was replaced with medium containing 1 mg/ml insulin alone. The medium was subsequently replaced again with fresh culture medium (DMEM with 10% FBS), and after 2 days, the extent of differentiation was measured by monitoring the formation of lipid globules in cells.
Metabolic effect of collagen domain of adiponectin

Animals

Animals were procured from the animal colony of the Central Drug Research Institute, Lucknow, India. db/db mice of body weight 40 ± 5 g and of age 5–6 weeks were procured and housed in the animal housing facility by maintaining the standard conditions of temperature, relative humidity, and a 12-h light/dark cycle. The animals had free access to diet and water unless stated otherwise. The study was approved by the CDRI Institutional Animal Ethical Committee (approval IAEC/2015/90), and all research work on animals was conducted in accordance with the guidelines of Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA).

Chemicals

2-Deoxyglucose, insulin, and metformin were purchased from Sigma-Aldrich, and 3H-labeled 2-deoxyglucose was from Amersham Biosciences. All antibodies used were of rabbit origin, and the dilution factor was 1:1000: anti-glucose transporter GLUT-4 (catalogue no. ab654) and anti-PPARα (catalogue no. ab8934) from Abcam; anti-APPL1 (catalogue no. ABC469) and anti-Rab5 (catalogue no. 552108) from Merk; anti-phospho-AMPKα (catalogue no. 25355), anti-phospho-ACC (catalogue no. 118185), anti-phospho-p38 MAPK (Thr-180/Tyr-182) (catalogue no. 9211S), anti-phospho-AS160 (catalogue no. 6929), and anti-β-actin (catalogue no. 4970L) from Cell Signaling Technology; and anti-Pax7 (catalogue no. ARP32742) from Aviva Systems Biology. Inhibitors like AKT-i and indinavir Z95% (HPLC) and dorsomorphin (compound C) were purchased from Cayman Chemicals. The mouse insulin ELISA kit was purchased from ImmunoTag. TNFα lyophilized powder was obtained from MP Biomedical. Protein A-Sepharose beads and Protein G-Sepharose beads were purchased from Biovison.

Assay of hemolytic activity of the peptides

A hemolytic activity assay of the peptide against human red blood cells has been described in the supporting information.

Cell viability assay

Cytotoxicity of the peptide was examined by determining the viability of murine 3T3 in its presence by a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay as described in the supporting information.

Measurement of [3H]2-deoxyglucose uptake in L6 muscle cell lines

L6 myoblasts grown in a 24-well plate were subjected to glucose uptake. Briefly, L6 myotubes were incubated with different concentrations of the peptide and the standard drug for different time periods with the final 3 h in serum-deprived medium, and a subset of cells was stimulated with 100 nm insulin for 20 min. Glucose uptake was assessed for 5 min in HEPES-buffered saline (140 mM NaCl, 20 mM HEPES, 5 mM KCl, 2.5 mM MgSO₄, 1 mM CaCl₂ (pH 7.4)) containing 10 μM 2-deoxyglucose (0.5 μCi/ml [3H]2-deoxyglucose) at room temperature. Subsequently, cells were rinsed with an ice-cold stop solution containing 0.9% NaCl and 20 mM D-glucose. To quantify the radioactivity incorporated, cells were lysed with 0.05 N NaOH, and lysates were counted with scintillation fluid in a β-counter (Beckman Coulter) (50, 51).

Oligomerization of ADP-1 by Tris-Tricine SDS-gel electrophoresis

Different concentrations of peptide, ADP-1 (30, 20, and 10 μg), dissolved in milli-Q water were incubated at 37 °C for 30 min. After that, these peptide samples were treated with freshly prepared 0.2% glutaraldehyde solution (linker) at 37 °C for 15 min. The reaction was stopped by adding one-third volume of 100 mM Tris (pH 8) (52). Each reaction mixture was treated with PAGE loading buffer containing 1% SDS and 5% mercaptoethanol. These peptide mixtures were subjected to SDS-PAGE in 16.5% Tris-Tricine acrylamide gel (53). Images of Coomassie Blue-stained gels were taken and analyzed with respect to peptide marker.

Western blot analysis

L6 myotubes grown in a 6-well plate were treated as indicated and lysed with the PBS containing 1% Nonidet P-40, 5 mM EDTA, phosphatase inhibitor (1 mM NaOVO₄ and 1 mM NaF), and protease inhibitor mixture (1:1,000) (RIPA lysis buffer). Lysates were centrifuged at 10,000 rpm for 15 min at 4 °C, and protein concentration was determined using BCA assay reagent. Lysates having equal protein concentrations were heated for 10 min at 70 °C in Laemmli sample buffer supplemented with 10% β-mercaptoethanol. Proteins were resolved by 10% SDS-PAGE and transferred to nitrocellulose membranes. After blocking, the blots were incubated with the indicated antibodies followed by incubation with appropriate HRP-conjugated secondary antibodies. Immunoblots were developed with chemiluminescent reagent according to the manufacturer's instructions (GE Healthcare). Densitometric quantification of protein bands was performed using ImageJ software (National Institutes of Health).

Cytosolic and plasma membrane protein isolation

The control (nontreated), insulin-treated, and ADP-1–treated L6 myotubes were lysed with lysis buffer (0.25 M sucrose, 10 mM Tris HCl, pH 7.4, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride) and centrifuged at 800 × g for 2 min at 4 °C, and protein concentration was determined using BCA assay reagent. Lysates containing equal protein concentrations were heated for 10 min at 70 °C in Laemmli sample buffer supplemented with 10% β-mercaptoethanol. Proteins were resolved by 10% SDS-PAGE and transferred to nitrocellulose membranes. After blocking, the blots were incubated with the indicated antibodies followed by incubation with appropriate HRP-conjugated secondary antibodies. Immunoblots were developed with chemiluminescent reagent according to the manufacturer’s instructions (GE Healthcare). Densitometric quantification of protein bands was performed using ImageJ software (National Institutes of Health).

Immunoprecipitation

L6 myotubes (pretreated with peptide) were lysed by adding 0.8 ml of cold lysis buffer (RIPA lysis buffer) containing protease inhibitor. The primary antibody (anti-APPL1 and nonspecific antibody (IgG isotype) of 1:100 dilution) was incubated with 60 μl of the protein A bead slurry at 4 °C for 4 h on a rocking platform. After centrifugation at 3,000 × g for 2 min at 4 °C, the supernatant was discarded, and then the cell lysate proteins (250 μg) were added. The lysate/bead/antibody conjugate mixture was incubated at 4 °C under rotary agitation over-
night. After incubation, the conjugate mixture was washed with 0.8 ml of lysis buffer and 0.8 ml of washing buffer two times each. After the last wash, the beads were heated at 90 °C for 10 min and loaded onto the gel for Western blot analysis.

**RNA extraction and quantification for real-time PCR**

L6 cells grown in 6-well plates were subjected to RNA isolation. Two days after cell fusion into myotubes, the cells were treated with different concentrations of peptide and incubated for the desired time. After incubation, total RNA was isolated from L6 myotubes using an RNA isolation kit (Nucleospin RNA, Macherey Nagel). Quantity was determined by $A_{260}$ and $A_{280}$ measurement. All samples had $A_{260}/A_{280}$ ratios of 1.8 – 2.1. Total RNA (2 µg) was reverse-transcribed to synthesize cDNA using a high-capacity cDNA reverse transcription kit (Applied Biosystems). cDNA levels were quantified by real-time PCR with SYBR Green using the Agilent Stratagene Mx3005P PCR detection system.

The primer sequences used were as follows: AdipoR1 (rat), 5'-AGACCCAGGAGAGAGAG-3' (left primer) and 5'-GGTTACAACAACCTCA-3' (right primer); Glut-4 (rat), 5'-GACGGGACTCTCATGTTG-3' (left primer) and 5'-GCCAACGATGGGAGCATAGC-3' (right primer).

**Adipogenesis assay (Oil Red O (ORO) staining method)**

Differentiated 3T3-L1 (with or without peptide treatment) adipocytes were rinsed in PBS (pH 7.4). The adipocyte lipid globules were stained with ORO (0.36% in 60% isopropyl alcohol) for 30 min. Accumulated dye was extracted using 100% isopropyl alcohol, and absorbance was measured at 492 nm (55).

**CD analysis**

The methodology has been described in the **supporting information**.

**ATR-FTIR**

ATR-FTIR spectroscopy was carried out on a single-beam Nicolet iS5 FT-IR spectrometer with the following scan parameters: scan range, 4,000 to 500 cm$^{-1}$; number of scans, 16; resolution, 4.0 cm$^{-1}$. The average of the peaks was observed. The run was carried out at 37 °C.

**Serum stability**

The proteolytic stability of ADP-1 peptide in the presence of human serum has been described in the **supporting information**.

**Ex vivo studies**

Primary myoblast cells were isolated from adult db/db mice according to the protocol of Hindi et al. (56). Immunostaining of primary myoblasts was also done with Pax7 antibody. After differentiating the primary myoblast cells in a 6-well cultured plate as per the above-mentioned protocol, treatment with ADP-1 (14.3 µg/ml for 3 h) was given, and metformin (20 µM for 12 h) was used as a positive control. After the treatment, cells were lysed by adding 0.8 ml of cold lysis buffer (RIPA lysis buffer) containing protease inhibitor, and total protein concentration of the cell lysate of each sample was quantified using the Bradford method. To investigate the metabolic effect of ADP-1 on primary myoblast cells of db/db mice (ex vivo), we performed Western blot analysis of each sample.

**Biochemical analysis**

During treatment (days 1, 5, 10, 15, and 20), blood samples were collected from the retro-orbital plexus, allowed to clot at 4 °C, and centrifuged for 20 min at 3000 rpm at 4 °C. The serum was separated and frozen at −20 °C until assayed for measuring insulin. The level of insulin was determined by an ELISA kit (immunotag, G-Biosciences).

**In vivo studies**

db/db mice were selected for this study and were randomly divided into three groups of four mice in each group. Group 1 served as diabetic and was given vehicle, whereas the other experimental groups were administered (intraperitoneally) with peptide (ADP-1). The fasting blood glucose levels were measured after animals were fasted for 4 h (starting from 9:00 a.m.) on day 0 (before treatment); days 5, 10, 15, 20, and 25 (during treatment); and day 30 (last day of treatment). Blood glucose levels were measured by a glucometer (ACCU-CHEK II, Roche Diagnostics) as per the manufacturer’s instructions. Peptide treatment was started from day 1. Peptide solution was prepared in water and was given through the IP route at the desired dose (30 mg/kg body weight). All of the animals were dosed on alternate days with the test peptide at a fixed time from day 1 to day 30. Animals of the positive control group were given metformin (50 mg/kg body weight). Blood glucose was measured by a glucometer using a glucostrip (Roche Applied Science).

**Statistical analysis**

For statistical evaluation, data were analyzed using Prism version 5 (GraphPad Software). Quantitative data are presented as the mean ± S.D.

**Author contributions**—J. K. G. and M. S. conceptualization; M. S. data curation; M. S., S. G., D. P. V., T. A., T. K., A. K. S., and J. K. G. formal analysis; M. S., S. G., A. K. S., and J. K. G. investigation; M. S. S. G., A. K. S., and J. K. G. methodology; M. S. and J. K. G. writing-original draft; J. K. G. supervision; J. K. G. project administration.

**Acknowledgments**—We are very thankful to Dr. Satyakam Patnaik and the HOD analytical chemistry division, CSIR-IITR, for allowing us to record FTIR spectra and use the liquid scintillation β counter, respectively. We are also very thankful to Dr. Jagdishwar Reddy Thota, Dr. Toofan, and R. K. Purshottam (Sophisticated Analytical Instrumentation Facility (SAIF), CSIR-CDFI) for MALDI-TOF mass spectra and assistance in purifying peptides by HPLC, respectively. We are very grateful to Dr. Raj Kamal Tripathi (Toxicology Division, CDRI), Dr. Jayanta Sarkar (Biochemistry division, CDRI), and Mohammad Shafeeq (Pharmacology Division, CDRI) for assisting and guiding us in the ex vivo experiment. We acknowledge Dr. Rituraj Konwar (Endocrinology Division, CDRI) and Dr. Sabyasachi Sanjal (Biochemistry Division, CDRI) for providing the reagents essential for our ex vivo studies. We are extremely thankful to Prof. Surajit Bhattacharya (NTU, Singapore) for editing the manuscript. We are thankful to the present and past directors of this Institute for their support.
Metabolic effect of collagen domain of adiponectin

References

1. Hui, X., Lam, K. S., Vanhoutte, P. M., and Xu, A. (2012) Adiponectin and cardiovascular health: an update. Br. J. Pharmacol. 165, 574–590 CrossRef Medline

2. Dalamaga, M., Diakopoulos, K. N., and Mantzoros, C. S. (2012) The role of adiponectin in cancer: a review of current evidence. Endocr. Rev. 33, 547–594 CrossRef Medline

3. Scherer, P. E., Williams, S., Fogliano, M., Baldini, G., and Lodish, H. F. (1995) A novel serum protein similar to C1q, produced exclusively in adipocytes. J. Biol. Chem. 270, 26746–26749 CrossRef Medline

4. Hu, E., Liang, P., and Spiegelman, B. M. (1996) AdipoQ is a novel adipose-specific gene dysregulated in obesity. J. Biol. Chem. 271, 10697–10703 CrossRef Medline

5. Nakano, Y., Tobe, T., Choi-Miura, N. H., Kihara, S., and Tomita, M. (1996) Isolation and characterization of GBP28, a novel gelatin-binding protein purified from human plasma. J. Biochem. 120, 803–812 CrossRef Medline

6. Maeda, K., Okubo, K., Shimomura, I., Funahashi, T., Matsuzawa, Y., and Matsubara, K. (1996) cDNA cloning and expression of a novel adipose specific collagen-like factor, apoM1 (AdiPose Most abundant Gene transcript 1). Biochem. Biophys. Res. Commun. 221, 286–289 CrossRef Medline

7. Ruan, H., and Dong, L. Q. (2016) Adiponectin signaling and function in insulin target tissues. J. Mol. Cell Biol. 8, 101–109 CrossRef Medline

8. Kadowaki, T., Yamauchi, T., Kubota, N., Hara, K., Ueki, K., and Tobe, K. (2006) Adiponectin and adiponectin receptors in insulin resistance, diabetes, and the metabolic syndrome. J. Clin. Invest. 116, 1784–1792 CrossRef Medline

9. Otvos, L., Jr., Haspinger, E., La Russa, F., Maspero, F., Graziano, P., Kovalszky, I., Lovas, S., Nama, K., Hoffmann, R., Knappe, D., Cassone, M., Wade, J., and Surmacz, E. (2011) Design and development of a peptide-based adiponectin receptor agonist for cancer treatment. BMC Biotechnol. 11, 90 CrossRef Medline

10. Kumada, M., Kihara, S., Sumitsuji, S., Kawamoto, T., Matsumoto, S., Ouchi, N., Arita, Y., Okamoto, Y., Shimomura, I., Hiraoka, H., Nakamura, T., Funahashi, T., Matsuzawa, Y., and Osaka CAD Study Group Coronary Artery Disease (2003) Association of hypoadiponectinemia with coronary artery disease in men. Arterioscler. Thromb. Vasc. Biol. 23, 85–89 CrossRef Medline

11. Ouchi, N., Ohishi, M., Kihara, S., Funahashi, T., Nakamura, T., Nagaretani, H., Kumada, M., Ohashi, K., Okamoto, Y., Nishizawa, H., Kishida, K., Maeda, N., Nagasawa, A., Kobayashi, H., Hiraoka, H., et al. (2003) Association of hypoadiponectinemia with impaired vasoreactivity. Hypertension 42, 231–234 CrossRef Medline

12. Hotta, K., Funahashi, T., Bodkin, N. L., Oertmeier, H. K., Arita, Y., Hansen, B. C., and Matsuzawa, Y. (2001) Circulating concentrations of the adipocyte protein adiponectin are decreased in parallel with reduced insulin sensitivity during the progression to type 2 diabetes in rhesus monkeys. Diabetes 50, 1126–1133 CrossRef Medline

13. Yamauchi, T., Kamon, J., Waki, H., Terauchi, Y., Kubota, N., Hara, K., Morii, Y., Ide, T., Murakami, K., Tsuboyama-Kasaoka, N., Ezaki, O., Akanuma, Y., Gavrilova, O., Vinson, C., Reitman, M. L., et al. (2001) The fat-derived hormone adiponectin reverses insulin resistance associated with both lipatrophy and obesity. Nat. Med. 7, 941–946 CrossRef Medline

14. Yu, J. G., Javorschi, S., Hevener, A. L., Kruszynska, Y. T., Scala, R., and Goldstein, B. J. (2003) Involvement of AMP-activated protein kinase in glucose uptake stimulated by the globular domain of adiponectin in primary rat adipocytes. Diabetes 52, 1355–1363 CrossRef Medline

15. Almner, G., Saba-Lepek, M., Haji-Yahya, S., Rohde, E., Strunk, D., Frohlich, E., Prass, R., and Manghe, B. J. (2015) Global domain of adiponectin: promising target molecule for detection of athero-ocerotic lesions. Diabetes 64, 95–105 CrossRef Medline

16. Wang, H., Zhang, H., Zhang, Z., Huang, B., Cheng, X., Wang, D., Li, Y., Zou, G., Fu, X., and Zhang, R. (2016) Adiponectin-derived active peptide ADP355 exerts anti-inflammatory and anti-fibrotic activities in thiacetamide-induced liver injury. Sci. Rep. 6, 19445 CrossRef Medline

17. Yamauchi, T., Kamada, A., and Ikeo, T. (2013) Acceleratory effect of novel synthesized collagen-like peptide from adiponectin on osteoblastic differentiation. J. Oral Tissue Eng. 11, 9–16 CrossRef Medline

18. Vianna, A. G. D., Sanches, C. P., and Barreto, F. C. (2017) Review article: effects of type 2 diabetes therapies on bone metabolism. Diabetol. Metab. Syndr. 9, 75 CrossRef Medline

19. Jiao, H., Xiao, E., and Graves, D. T. (2015) Diabetes and its effect on bone and fracture healing. Curr. Osteoporos. Rep. 13, 327–335 CrossRef Medline

20. Rubin, M. B. (2015) Bone cells and bone turnover in diabetes mellitus. Curr. Osteoporos. Rep. 13, 186–191 CrossRef Medline

21. Ito, Y., Takaoka, K., Hara, K., and Kihara, S. (2017) Adiponectin as an adipose-derived hormone. J. Clin. Invest. 127, 11999–12008 CrossRef Medline

22. Oikawa, S., Ishida, T., Hatakeyama, T., Takahashi, R., Kihara, S., Ueki, K., and Tobe, K. (2002) Adiponectin reverses insulin resistance associated with the fat-derived hormone adiponectin in primary rat adipocytes. Diabetologia 45, 736–742 CrossRef Medline

23. Yamauchi, T., Kamon, J., Waki, H., Imai, Y., Shimozawa, N., Hioki, K., Uchida, S., Ito, Y., Takakawa, K., Matsui, J., Takata, M., Eto, K., Terauchi, Y., Komeda, K., Tsunoda, M., et al. (2003) Globular adiponectin protected ob/ob mice from diabetes and ApoE-deficient mice from atherosclerosis. J. Biol. Chem. 278, 2461–2468 CrossRef Medline

24. Masuzaki, H., Ogawa, Y., Hosoda, K., Inoue, G., Hayashi, T., and Nakao, K. (2001) Troglitazone induces GLUT4 translocation in 6L myotubes. Diabetes 50, 1093–1101 CrossRef Medline

25. Yonemitsu, S., Nishimura, H., Shintani, M., Inoue, R., Yamamoto, Y., Masuzaki, H., Ogawa, Y., Hosoda, K., Inoue, G., Hayashi, T., and Nakao, K. (2001) Troglitazone induces GLUT4 translocation in 6L myotubes. Diabetes 50, 1093–1101 CrossRef Medline

26. Jiao, H., Xiao, E., and Graves, D. T. (2015) Diabetes and its effect on bone and fracture healing. Curr. Osteoporos. Rep. 13, 327–335 CrossRef Medline

27. Jiao, H., Xiao, E., and Graves, D. T. (2015) Diabetes and its effect on bone and fracture healing. Curr. Osteoporos. Rep. 13, 327–335 CrossRef Medline

28. Imai, Y., Shimozawa, N., Hioki, K., Uchida, S., Ito, Y., Takakawa, K., Matsui, J., Takata, M., Eto, K., Terauchi, Y., Komeda, K., Tsunoda, M., et al. (2003) Globular adiponectin protected ob/ob mice from diabetes and ApoE-deficient mice from atherosclerosis. J. Biol. Chem. 278, 2461–2468 CrossRef Medline
 stood adiogenesis, adipose dysfunction, and obesity. J. Cell Biochem. 110, 564–572 CrossRef Medline
46. Ruiz-Ojeda, F. J., Rupe´rez, A. L., Gomez-Llorente, C., Gil, A., and Aguilera, C. M. (2016) Cell models and their application for studying adipogenic differentiation in relation to obesity: a review. Int. J. Mol. Sci. 17, E1040 Medline
47. Green, H., and Meuth, M. (1974) An established pre-adipose cell line and its differentiation in culture. Cell 3, 127–133 CrossRef Medline
48. Green, H., and Kehinde, O. (1976) Spontaneous heritable changes leading to increased adipose conversion in 3T3 cells. Cell 7, 105–113 CrossRef Medline
49. Caprio, M., Fève, B., Claës, A., Viengchareun, S., Lombe`es, M., and Zennaro, M. C. (2007) Pivotal role of the mineralocorticoid receptor in cortico-steroid-induced adipogenesis. FASEB J. 21, 2183–2194 CrossRef Medline
50. Klip, A., and Ramlal, T. (1987) Protein kinase C is not required for insulin stimulation of hexose uptake in muscle cells in culture. Biochem. J. 242, 131–136 CrossRef Medline
51. Sawada, K., Kawabata, K., Yamashita, T., Kawasaki, K., Yamamoto, N., and Ashida, H. (2012) Ameliorative effects of polyunsaturated fatty acids against palmitic acid-induced insulin resistance in L6 skeletal muscle cells. Lipids Health Dis. 11, 36 CrossRef Medline
52. Nayak, A. R., Karade, S. S., Srivastava, V. K., Rana, A. K., Gupta, C. M., Sahasrabuddhe, A. A., and Pratap, J. V. (2016) Structure of Leishmania donovani coronin coiled coil domain reveals an antiparallel 4 helix bundle with inherent asymmetry. J. Struct. Biol. 195, 129–138 CrossRef Medline
53. Srivastava, S., and Ghosh, J. K. (2013) Introduction of a lysine residue promotes aggregation of temporin L in lipopolysaccharides and augmentation of its antiendotoxin property. Antimicrob. Agents Chemother. 57, 2457–2466 CrossRef Medline
54. Gautam, S., Ishtark, N., Singh, R., Narender, T., and Srivastava, A. K. (2015) Aegeline from Aegle marmelos stimulates glucose transport via Akt and Rac1 signaling, and contributes to a cytoskeletal rearrangement through PI3K/Rac1. Eur. J. Pharmacol. 762, 419–429 CrossRef Medline
55. Varshney, S., Shankar, K., Beg, M., Balaramnavar, V. M., Mishra, S. K., Jagdale, P., Srivastava, S., Chlonker, Y. S., Lakshmi, V., Chaudhari, B. P., Bharta, R. S., Saxena, A. K., and Gaikwad, A. N. (2014) Rohitukine inhibits in vitro adipogenesis arresting mitotic clonal expansion and improves dyslipidemia in vivo. J. Lipid Res. 55, 1019–1032 CrossRef Medline
56. Hindi, L., McMillan, J. D., Afroze, D., Hindi, S. M., and Kumar, A. (2017) Isolation, culturing, and differentiation of primary myoblasts from skeletal muscle of adult mice. Bio Protoc. 7, e2248 CrossRef Medline