Metformin increases the novel adipokine adipolin/CTRP12: role of the AMPK pathway

Bee K Tan, Jing Chen, Raghu Adya, Manjunath Ramanjaneya, Vanlata Patel and Harpal S Randeva
Warwick Medical School, University of Warwick, Coventry CV4 7AL, UK

Abstract

Adipolin is a novel adipokine with anti-inflammatory and glucose-lowering properties. Lower levels of adipolin are found in obese and diabetic mice. Polycystic ovary syndrome (PCOS) is a pro-inflammatory state associated with obesity and diabetes. To date, there are no human studies on adipolin. Therefore, we measured serum (ELISA) and adipose tissue adipolin mRNA expression (RT-PCR) and protein concentrations (western blotting) in PCOS and control subjects. We also investigated the ex vivo effect of glucose and metformin on adipolin protein production in human subcutaneous adipose tissue explants. We report novel data that serum and subcutaneous adipose tissue adipolin mRNA expression and protein concentrations were significantly lower in women with PCOS compared with control subjects. Furthermore, Spearman’s rank analysis showed that serum adipolin concentrations were significantly negatively correlated with BMI, waist-to-hip ratio, and glucose ($P<0.05$). However, when subjected to multiple regression analysis, none of these variables were predictive of serum adipolin concentrations ($P>0.05$). Also, subcutaneous adipose tissue adipolin mRNA expression and protein concentrations were only significantly negatively correlated with glucose ($P<0.05$). No significant correlations were found with omental adipose tissue adipolin mRNA expression and protein concentrations ($P>0.05$). Moreover, glucose profoundly reduced and metformin significantly increased adipolin protein production in human adipose tissue explants respectively. Importantly, metformin’s effects appear to be via the AMP-activated protein kinase signaling pathway.

Key Words

- adipokine
- adipolin
- metformin
- polycystic ovary syndrome

Introduction

Polycystic ovary syndrome (PCOS) is the most common endocrine disorder found in women, affecting between 5 and 10% of women of reproductive age (Dunaif 1997). PCOS is a pro-inflammatory state characterized by menstrual problems, hyperandrogenism, and associated with features of the metabolic syndrome, particularly, obesity and diabetes (Dunaif 1997, Diamanti-Kandarakis 2006, Diamanti-Kandarakis et al. 2006).

The metabolic syndrome is associated with excessive accumulation of central/visceral body fat. Adipose tissue produces several cytokines termed ‘adipokines’ that have extensive effects, which are crucial for the development of...
insulin resistance, diabetes, and atherosclerosis (Kershaw & Flier 2004). Furthermore, the accumulation of visceral adipose tissue poses a higher cardiometabolic risk than subcutaneous adipose tissue (Wajchenberg 2000) as removal of visceral rather than subcutaneous adipose tissue has been shown to enhance insulin sensitivity (Thorne et al. 2002). The connection between inflammation and insulin resistance in obesity and diabetes has been established (Hotamisligil et al. 2002). The connection between inflammation and insulin resistance in obesity and diabetes has been established (Hotamisligil et al. 1993, Kubaszek et al. 2003).

Recently, there has been intense interest in the adipokines of the C1q complement/TNF-related protein (CTRP) superfamily. Adipolin (CTRP12 or FAM132A) is a novel adipokine, richly expressed by adipose tissue with insulin-sensitizing and anti-inflammatory effects (Enomoto et al. 2011, Wei et al. 2012). Indeed, lower circulating and adipose tissue adipolin levels were noted in rodent models of obesity and diabetes (Enomoto et al. 2011, Wei et al. 2012).

As there is no literature with regard to adipolin in humans, we measured serum adipolin, mRNA expression, and protein concentrations in subcutaneous and omental adipose tissue(s) of women with PCOS. Furthermore, we studied the effects of glucose and metformin on adipolin protein production in human subcutaneous adipose tissue explants.

Subjects and Methods

Subjects

All women with PCOS met all three criteria of the revised 2003 Rotterdam ESHRE/ASRM PCOS Consensus Workshop diagnostic criteria. The three criteria are i) oligo- and/or anovulation, ii) clinical and/or biochemical signs of hyperandrogenism, and iii) polycystic ovaries (Fauser 2004). Furthermore, all subjects in the control arm had no discernible cause for infertility (unexplained infertility). No subjects were amenorrheic. Exclusion criteria for the study included age over 40 years, endometriosis, known cardiovascular disease, thyroid disease, neoplasms, current smoking, diabetes mellitus, hypertension (blood pressure >140/90 mmHg), and renal impairment (serum creatinine >120 µmol/l). None of these subjects had taken medications for at least 6 months prior to the study, including oral contraceptives, glucocorticoids, ovulation induction agents, anti-diabetic and anti-obesity drugs, and estrogenic, anti-androgenic, or anti-hypertensive medication. Also, the presence of other endocrinopathies was ruled out by measuring basal serum 17-hydroxyprogesterone, prolactin, and by measuring 0800–0900 h cortisol after 1.0 mg (2300 h) overnight dexamethasone suppression (value below 30 nmol/l was considered to rule out Cushing’s syndrome). All subjects suppressed cortisol below 30 nmol/l.

Subjects were initially seen at the infertility clinic and then scheduled for laparoscopy in order to assess Fallopian tube(s) patency. All subjects underwent anthropometric measurements. After an overnight fast, blood samples and subcutaneous and omental adipose tissues were obtained (0800–1000 h) from adult female patients undergoing elective surgery for infertility investigation. Serum was immediately aliquoted on ice and stored at −80 °C. The same fat pad was divided equally into two halves. Each half was either immediately frozen in liquid nitrogen and stored at −80 °C or placed into a sterile container containing Medium 199 (Sigma–Aldrich) for primary adipose tissue culture. Samples that were snap frozen were transported on dry ice (−80 °C) and stored at −80 °C in the laboratories of the University of Warwick. A total of 73 subjects were recruited consecutively from the infertility clinic in accordance with the inclusion/exclusion criteria (PCOS, n=19; controls, n=54). Of the 19 PCOS subjects recruited, five withdrew before the study could be completed. In the control group, seven subjects did not complete the study. From the remaining 47 control subjects, 14 control subjects matched for age, BMI, and waist-to-hip ratio (WHR) were included in the final analysis. The Local Research Ethics Committee approved the study, and all patients involved gave their informed consent, in accordance with the guidelines in The Declaration of Helsinki 2000.

Biochemical and Hormonal Analysis

Assays for glucose, insulin, cholesterol, triglycerides, prolactin, 17β-estradiol (E2), progesterone, 17-hydroxyprogesterone, testosterone, androstenedione, DHEA-S, and sex hormone binding globulin (SHBG) were performed using an automated analyzer (Abbott Architect, Abbott Laboratories). The estimate of insulin resistance by homeostasis model assessment (HOMA)-IR score was calculated as $\text{HOMA-IR} = \frac{\text{Insulin} \times \text{Glucose}}{22.5}$, where insulin is the fasting insulin and glucose is the fasting glucose, as described by Matthews et al. (1985). Circulating leptin and adiponectin levels were measured with a coated-tube IRMA kit (Diagnostic Systems Laboratories, Sinsheim, Germany) and by a commercially available RIA Kit (Millipore, Watford, UK) respectively according to the manufacturer’s protocol.
Adipolin concentrations in sera and conditioned media were measured using a commercially available ELISA Kit (Aviscera, Santa Clara, CA, USA), according to the manufacturer’s protocol, with an intra-assay coefficient of variation (CV) of <8%.

**Concentration of conditioned media**  Conditioned media were collected into sterile eppendorfs and spun to remove debris. Afterward, the supernatant was subjected to vacuum centrifugation in a CentriVap Centrifugal Concentrator as per manufacturer’s instructions (Labconco, Kansas City, MO, USA). Protein levels in concentrated samples of conditioned media were quantified using a bicinchoninic acid protein quantification assay kit as per manufacturer’s protocol (Thermo Scientific, Rockford, IL, USA). Conditioned media, equalized for protein concentrations, were subsequently assayed for adipolin concentrations. This step was necessary given the low concentrations of adipolin in conditioned media.

**Primary explant culture**

Adipose tissue organ explants were cultured using a protocol that was a modification of the method described by Fried & Moustaid-Moussa (2001). Briefly, 1–3 g adipose tissue were minced into 5–10 mg (~1 mm³) fragments, passed through a 230 μm mesh (filter no. 60, Sigma–Aldrich), and rinsed with sterile PBS warmed to 37°C. Samples were then transferred to six-well plates (~50 mg/well) containing 3 ml Media 199 (Invitrogen) supplemented with 50 μg/ml gentamicin and 1% FCS (containing insulin at a concentration of 10⁻¹⁴ M) and cultured for 24 h with or without the addition of D-glucose (Sigma–Aldrich), D-mannitol (Sigma–Aldrich), or metformin (Sigma–Aldrich) or AMP-activated protein kinase (AMPK) inhibitor (Compound C; Sigma–Aldrich) in a 37°C incubator under an atmosphere of 5% CO₂/95% air.

**Optimization experiments**  Concentration- and time-dependent experiments were performed for insulin, rosiglitazone, and the AMPK inhibitor – Compound C (data not shown).

**Total RNA extraction and cDNA synthesis**

Total RNA was extracted from adipose tissue samples using Qiagen RNeasy Lipid Tissue Mini Kit according to the manufacturer’s guidelines (Qiagen). The purity of the extracted RNA was measured by a NanoDrop spectrophotometer (Labtech International, Ringmer, UK). A set concentration of RNA was reverse transcribed into cDNA, using M-MuLV Reverse Transcriptase (Fermentas, York, UK) and random hexamers (Promega) as primers.

**RT-PCR**

Quantitative PCR of adipolin was performed on a Roche Light Cycler system (Roche Molecular Biochemicals). PCRs were carried out in a reaction mixture consisting of 5.0 μl reaction buffer (Fermentas) and 2.0 mM MgCl₂ (Biogene, Kimbolton, UK), 1.0 μl each primer (10 ng/μl), 2.5 μl cDNA, and 0.5 μl Light Cycler DNA Master SYBR Green I (Roche). Protocol conditions consisted of denaturation at 95°C for 15 s, followed by 40 cycles of 94°C for 1 s, 58°C for 10 s, and 72°C for 12 s, followed by melting curve analysis. For analysis, quantitative amounts of genes of interest were standardized against the housekeeping gene β-actin. The RNA levels were expressed as a ratio, using the ‘Delta–delta method’ for comparing relative expression results between treatments in real-time PCR (Pfaffl 2001). The sequences of the sense and anti-sense primers used were adipolin (112 bp) 5’-GGACGTGGTGTGTGTTCTCA-3’ and 5’-CT-GCACCTGTAGCGTGAAGA-3’; β-actin (216 bp) 5’-AAG-GAGGCATCCCTACCCT-3’ and 5’-TACATGGCTGGGT-CTTGAAA-3’. Ten microliters of the reaction mixture(s) were subsequently electrophoresed on a 1% agarose gel and visualized by ethidium bromide (Sigma–Aldrich), using a 1 kb DNA ladder (Invitrogen) in order to estimate the band sizes. As a negative control for all the reactions, preparations lacking RNA or reverse transcriptase were used in place of the cDNA. RNAs were assayed from three independent biological replicates.

**Western blotting**

Protein lysates were prepared by homogenizing adipose tissue in radioimmunoprecipitation lysis buffer (Upstate, Lake Placid, NY, USA) according to the manufacturer’s instructions. Protein samples (35 μg/lane) containing SDS-sample buffer (5 M urea, 0.17 M SDS, 0.4 M dithiothreitol, and 50 mM Tris–HCl, pH 8.0) were subjected to SDS–PAGE (10% resolving gel) and transferred to PVDF membranes (Millipore). PVDF membranes were blocked in Tris-buffered saline (TBS) containing 0.1% Tween 20 and 5% BSA for 2 h. The PVDF membranes were then incubated with polyclonal primary rabbit-anti-human antibody for adipolin (LifeSpan Biosciences, Seattle, WA, USA; 1:500 dilution) or monoclonal primary rabbit-anti-human antibody for β-actin (Cell Signaling Technology, Inc., Beverly, MA, USA) (1:1000 dilution) overnight at 4°C.
The membranes were washed thoroughly for 60 min with TBS–0.1% Tween before incubation with the secondary anti-rabbit HRP-conjugated immunoglobulin (Dako, Ely, UK; 1:2000), for 1 h at room temperature. Antibody complexes were visualized using chemiluminescence (ECL; GE Healthcare, Little Chalfont, UK). Human adipolin (Aviscera, Santa Clara, CA, USA) was used as a positive control and water as a negative control (data not shown).

**Data analysis**  The densities were measured using a scanning densitometer coupled to scanning software Scion Image (Scion Corporation, Frederick, MD, USA). Standard curves were generated to ensure linearity of signal intensity over the range of protein amounts loaded into gel lanes. Comparisons of densitometric signal intensities for adipolin and β-actin were made only within this linearity range.

**Statistical analysis**

Data were analyzed by the Mann–Whitney U test, Kruskal–Wallis ANOVA (*post hoc* analysis: Dunn’s test), and Friedman’s ANOVA (*post hoc* analysis: Dunn’s test), according to the number of groups compared. Data are medians (interquartile range). Spearman’s rank correlation was used for calculation of associations between variables. Subsequently, if individual bivariate correlations achieved statistical significance, variables were entered into a linear regression model and multiple regression analysis with adipolin as a dependent variable was performed to test the joint effect of different parameters on adipolin. All statistical analyses were performed using SPSS version 21.0 (SPSS, Inc.). *P*<0.05 was considered significant.

**Results**

**Demographic data**

Table 1 shows the anthropometric, biochemical, and hormonal data in all subjects. Glucose, HOMA-IR, triglycerides, E₂, testosterone, androstenedione, DHEA-S concentrations, and free androgen index were significantly higher whereas SHBG was significantly lower in women with PCOS. Serum adipolin concentrations were significantly lower in women with PCOS compared with control subjects (416.3 (322.4–568.4) vs 805.2 (570.2–961.4) pg/ml; *P*<0.05: Table 1). Serum progesterone concentrations in all subjects confirmed follicular phase of the menstrual cycle.

**mRNA expression and protein concentrations of adipolin in adipose tissue of normal and women with PCOS**

We detected adipolin mRNA in adipose tissue of all subjects and subsequent sequencing of the PCR products

Table 1  Clinical, hormonal, and metabolic features of women with PCOS and control subjects. Free androgen index (FAI) = testosterone (nmol/l)/SHBG (nmol/l) × 100. Data are medians (interquartile range). Group comparison by Mann–Whitney U test

<table>
<thead>
<tr>
<th>Variable</th>
<th>PCOS (n=14)</th>
<th>Controls (n=14)</th>
<th>Significance (<em>P</em>)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>29.5 (28–38)</td>
<td>32.5 (29–35)</td>
<td>NS</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>30.5 (27.8–30.9)</td>
<td>28.8 (28–30.5)</td>
<td>NS</td>
</tr>
<tr>
<td>WHR</td>
<td>0.89 (0.78–0.99)</td>
<td>0.84 (0.81–0.96)</td>
<td>NS</td>
</tr>
<tr>
<td>Glucose (mmol/l)</td>
<td>5.5 (4.8–6.0)</td>
<td>4.5 (4.3–5.2)</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Insulin (pmol/l)</td>
<td>78.9 (42.0–91.1)</td>
<td>57.3 (48.5–66.0)</td>
<td>NS</td>
</tr>
<tr>
<td>HOMA-IR</td>
<td>3.0 (2.1–3.6)</td>
<td>2.0 (1.4–2.2)</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>Total cholesterol (mmol/l)</td>
<td>5.1 (4.1–5.7)</td>
<td>5.0 (4.8–5.5)</td>
<td>NS</td>
</tr>
<tr>
<td>Triglycerides (mmol/l)</td>
<td>2.0 (1.5–2.3)</td>
<td>0.9 (0.7–1.4)</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Progesterone (nmol/l)</td>
<td>348.0 (305.0–387.0)</td>
<td>304.5 (211.0–322.0)</td>
<td>NS</td>
</tr>
<tr>
<td>E₂ (pmol/l)</td>
<td>353.5 (287.0–471.0)</td>
<td>174.5 (129.0–264.0)</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Androstenedione (nmol/l)</td>
<td>1.6 (1.3–2.1)</td>
<td>2.2 (1.7–2.3)</td>
<td>NS</td>
</tr>
<tr>
<td>17-Hydroxyprogesterone (nmol/l)</td>
<td>2.5 (2.1–2.8)</td>
<td>2.0 (1.2–2.3)</td>
<td>NS</td>
</tr>
<tr>
<td>Testosterone (nmol/l)</td>
<td>1.7 (1.5–2.1)</td>
<td>0.8 (0.6–0.9)</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Androstenedione (nmol/l)</td>
<td>15.6 (14.2–16.8)</td>
<td>6.3 (5.0–8.2)</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>DHEA-S (μmol/l)</td>
<td>5.9 (5.4–6.6)</td>
<td>4.5 (4.0–5.3)</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>SHBG (nmol/l)</td>
<td>31.5 (26.7–35.2)</td>
<td>59.0 (47.7–66.0)</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>FAI</td>
<td>19.5 (14.5–21.2)</td>
<td>3.9 (3.3–6.1)</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Leptin (ng/ml)</td>
<td>24.9 (19.7–29.0)</td>
<td>24.1 (19.3–28.8)</td>
<td>NS</td>
</tr>
<tr>
<td>Adiponectin (μg/ml)</td>
<td>5.7 (4.5–7.5)</td>
<td>6.3 (5.5–9.5)</td>
<td>NS</td>
</tr>
<tr>
<td>Adipolin (pg/ml)</td>
<td>416.3 (322.4–568.4)</td>
<td>805.2 (570.2–961.4)</td>
<td>&lt;0.05</td>
</tr>
</tbody>
</table>

NS, not significant.
confirmed gene identity. The CV of β-actin mRNA expression (quantification cycle \(C_{q}\)) in all samples tested was 3.24%. Therefore, normalization against β-actin alone meets the standards of the MIQE guidelines (Bustin et al. 2009). Real-time RT-PCR analysis corrected over β-actin showed a significant decrease in adipolin mRNA and protein concentrations in subcutaneous \(P<0.05\) adipose tissue of PCOS compared with control subjects (Fig. 1A and B). However, no significant difference in adipolin mRNA and protein concentrations was observed when comparing mRNA and protein concentrations in omental adipose tissue of PCOS to control subjects (Fig. 1A and B). Western blot analysis of protein extracts from subcutaneous and omental adipose tissue demonstrates that the antibody against adipolin and the antibody against β-actin recognized bands with apparent molecular weights of 32 and 42 kDa respectively.

**Correlation of adipolin with covariates**

Spearman’s rank analysis showed that serum adipolin concentrations were significantly negatively correlated with BMI, WHR, and glucose \(P<0.05\). However, when subjected to multiple regression analysis, none of these variables were predictive of serum adipolin concentrations \(P>0.05\). Also, subcutaneous adipose tissue adipolin mRNA expression and protein concentrations were only significantly negatively correlated with glucose \(P<0.05\). In addition, we did find a non-significant positive correlation between serum adipolin and subcutaneous adipose tissue adipolin mRNA expression and protein levels \(R=0.202, P=0.135; R=0.185, P=0.181\) respectively. No significant correlations were found with omental adipose tissue adipolin mRNA expression and protein concentrations \(P>0.05\).

**Concentration-dependent effects of d-glucose and metformin on adipolin protein production and secretion into conditioned media in control human subcutaneous adipose tissue explants**

Given that serum adipolin concentrations, subcutaneous adipose tissue adipolin mRNA expression, and protein concentrations were only significantly negatively correlated with glucose, we examined the effects of d-glucose and metformin *ex vivo*. Adipolin protein production was significantly decreased by d-glucose in control human subcutaneous adipose tissue explants (Fig. 2; \*\(P<0.05\) and \**P<0.01\)). In order to account for the increase in osmolarity of the incubation medium attributable to d-glucose as a confounding factor, we undertook experiments using similar concentrations of d-mannitol as a control. We found no significant difference on adipolin protein production in control human subcutaneous adipose tissue explants (data not shown). Furthermore, metformin significantly increased adipolin protein production in control human subcutaneous adipose tissue explants (Fig. 3A; \*\(P<0.05\)). This effect was attenuated by the AMPK inhibitor (Compound C; 10 μM; Fig. 3B; \*\(P<0.05\)). Similar results were obtained with respect to adipolin secretion into conditioned media (data not shown).

**Discussion**

This is the first study in humans to demonstrate that serum and subcutaneous adipose tissue adipolin mRNA
expression and protein concentrations are significantly lower in women with PCOS. We also found significant negative correlations between serum adipolin and BMI, WHR, and glucose; subcutaneous adipose tissue adipolin was only negatively correlated with glucose. It is unlikely that either BMI or WHR is responsible for these findings, as both groups were matched for these variables. Unfortunately, because of technical limitations in adipose tissue procurement, we could not obtain sufficient quantities of sample/patient to perform stromal vascular separation in adipose tissue depots and thus could not account for a possible adipocyte hypertrophy-related side effect. Nonetheless, it is clear that subcutaneous adipose tissue from our women with PCOS, compared to controls, express less adipolin. Thus, given adipolin’s insulin-sensitizing effects, we tentatively hypothesize that the lower circulating and presence of 5 mmol/l glucose on adipolin protein production in control human subcutaneous adipose tissue explants at 24 h was assessed by western blotting. Densitometric analysis of adipolin immune complexes having normalized to β-actin revealed that protein concentrations of adipolin were significantly decreased by glucose (20 and 40 mmol/l) in control human subcutaneous adipose tissue explants. Data are expressed as % difference of median of basal (B). Each experiment was carried out with six different control subjects in three replicates. Group comparison by Friedman’s ANOVA and post hoc Dunn’s test, *P<0.05 and **P<0.05. AU, arbitrary units.

In order to elucidate the regulation of adipolin, given that serum adipolin concentrations, subcutaneous adipose tissue adipolin mRNA expression, and protein concentrations were only significantly negatively correlated with glucose, we studied the effects of glucose on adipolin protein production and secretion in human subcutaneous adipose tissue explants. We found that glucose caused a significant reduction in adipolin protein production and secretion in human subcutaneous adipose tissue explants. Of relevance, Sun et al. (2010) had reported that adiponectin (the foremost adipokine of the CTRP
superfamily) protein production in adipocytes was also attenuated by glucose.

The Biguanide insulin sensitizer, metformin, is commonly utilized in the management of women with PCOS. Metformin is able to rectify the endocrine and metabolic abnormalities in women with PCOS (Nardo & Rai 2001). Metformin achieves this by optimizing hepatic glucose metabolism by enhancing the AMPK signaling pathway and thus suppressing hepatic gluconeogenesis (Johnson et al. 1993, Perriello et al. 1994, Inzucchi et al. 1998). Interestingly, adiponectin, similar to metformin, has glucose-lowering effects, also through modulating hepatic gluconeogenesis (Wei et al. 1998). Interestingly, adiponectin production in adipocytes was also attenuated by glucose.

In our study, a limitation may relate to the number of subjects studied and hence we would advise caution with regard to our findings; further studies with a larger number of study subjects are needed to reaffirm our results. However, obtaining BMI/WHR-matched and menstrual cycle-synchronized blood and adipose tissue samples from two adipose tissue depots impeded subject recruitment. Nevertheless, our observations are highly consistent and significant and raise interesting questions on the mechanisms regulating adipolin production. Furthermore, we used a commercially available ELISA kit and an antibody (western blotting) that was not able to determine uncleaved and cleaved/globular forms of adipolin separately. Unraveling the interactions of uncleaved and cleaved/globular forms of adipolin would be important in advancing our knowledge of adipolin biology. Future studies need to address this point. Finally, given ethical and logistical constraints, our study was designed to investigate only PCOS and control subjects. It would be of interest to perform similar experiments in men and patients with type 2 diabetes mellitus.

Research into adipolin biology is still in its infancy. Our work has yielded important novel data on the metabolic aspects of adipolin biology and will hopefully stimulate further research into the newly described family of adiponectin paralogs. The discovery of a definitive receptor for adipolin would be crucial in paving the way on how this novel adipokine affects various functions of the human body in both health and disease, in particular, with respect to the metabolic syndrome and its associated complications such as type 2 diabetes.
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