Adiponectin (15–36) stimulates steroidogenic acute regulatory (StAR) protein expression and cortisol production in human adrenocortical cells: Role of AMPK and MAPK kinase pathways

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A B S T R A C T

Adiponectin is an abundantly circulating adipokine, orchestrating its effects through two 7-transmembrane receptors (AdipoR1 and AdipoR2). Steroidogenesis is regulated by a variety of neuropeptides and adipokines. Earlier studies have reported adipokine mediated steroid production. A key rate-limiting step in steroidogenesis is cholesterol transportation across the mitochondrial membrane by steroidogenic acute regulatory protein (StAR). Several signalling pathways regulate StAR expression. The actions of adiponectin and its role in human adrenocortical steroid biosynthesis are not fully understood. The aim of this study was to investigate the effects of adiponectin on StAR protein expression, steroidogenic genes, and cortisol production and to dissect the signalling cascades involved in the activation of StAR expression. Using qRT-PCR, Western blot analysis and ELISA, we have demonstrated that stimulation of human adrenocortical H295R cells with adiponectin results in increased cortisol secretion. This effect is accompanied by increased expression of key steroidogenic pathway genes including StAR protein expression via ERK1/2 and AMPK-dependent pathways. This has implications for our understanding of adiponectin receptor activation and peripheral steroidogenesis. Finally, our study aims to emphasise the key role of adipokines in the integration of metabolic activity and energy balance partly via the regulation of adrenal steroid production.

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1. Introduction

Adiponectin, a recently discovered adipokine circulates abundantly in human plasma [1]. In contrast to other adipokines, adiponectin levels are inversely correlated with body mass index, percentage body fat and visceral fat; adiponectin is also significantly reduced in obesity-related disease states such as type 2 diabetes [1]. Adiponectin binds and activates two transmembrane adiponectin receptors (ADIPOR1 and 2), which share a 66% homology in their amino acid sequence [1].

In non-obese healthy men, serum adiponectin exhibits ultradian and circadian variations preceding or matching closely to that of cortisol, suggesting a potential feedback loop between adiponectin and cortisol secretions [2]. Interestingly, adiponectin receptors are expressed in the human adrenal gland [3]; however the functional relevance of the ADIPORs within the adrenal gland is currently not known. Adiponectin also increases IGF-1 induced progesterone secretion in chicken ovary [4] and in rat ovarian granulosa cells [5], suggesting that adiponectin plays a significant role in the biosynthesis of steroid hormones.

Previous studies from Bornstein’s group have shown how certain adipokines regulate steroidogenesis in an adrenal cell-line [6]. Steroidogenesis involves transcription and activation of various enzymes required for this successive breakdown of cholesterol. The first key mediator of steroidogenesis is the 30 kDa steroidogenic acute regulatory (StAR) protein [7]. StAR is abundantly expressed in the steroid-producing cells, required for the transportation of cholesterol across the mitochondrial membrane (an obligatory step for steroid production) [8]. Reduction in StAR expression is the only known cause of the steroid-deficiency disease, familial lipid adrenal hyperplasia [9].

A number of proteins are known to stimulate or inhibit StAR expression, including ACTH, insulin, neuropeptide-Y orexin, TGF-alpha, TGF-B and angiotensin [10–12] through the action of multiple signalling pathways [11,13]. These signalling pathways include AMPK, AKT and various MAPK cascades including those mediated by ERK1/2 and p38. This variation may reflect the effects of different agonistic sites within the full length adiponectin molecule either at each of the two ADIPORs or possibly utilising even further receptor complexity.
2. Materials and methods

2.1. Biochemical reagents and inhibitors

Insulin, transferrin, selenium and Ultraserum-G (PALL Life Sciences, Cergy, France), growth supplement for H295R cells, were obtained from Sigma-Aldrich Co. Ltd. (Gillingham, Dorset, UK). For mediating up-regulation of the first mediator of cholesterol metabolism, the steroidogenic acute regulatory (StAR) protein. This study increases our understanding of the agonistic properties of the adiponectin molecule and may represent a potentially important therapeutic peptide domain.

2.2. Cell culture

H295R human adrenocortical cells were cultured in H295R complete media containing DMEM/F12 (1:1) supplemented with 2% Ultraserum G (Biosepra, Villeneuve-la-Garenne, France) and ITS (Discovery Labware, Bedford, MA), in six-well plates for 24 h after reaching confluence.

2.3. RT-PCR

Total RNA was extracted using the Qiagen RNeasy Mini Kit and reverse-transcribed into cDNA as previously described [14]. Adiponectin receptor(s) and steroidogenic gene expression was measured by RT-PCR, using 1 μg RNA and random primers as reverse transcription primers. A control reaction which omitted reverse transcriptase was included to check for the presence of genomic DNA. Steroidogenic gene, ADIPOR1 and ADIPOR2 expressions were amplified using a Hybrid Thermal Cycler in a 50 μl reaction medium containing 1 unit of Taq polymerase (Fermentas, York, UK), 20 pmol of each sense and anti-sense primer and dNTP (10 mmol/l each), using the following cycling conditions: 94 °C for 1 min, then 38 cycles of 94 °C for 60 s, 60 °C for 45 s, and 72 °C for 30 s, followed by a 10 min extension at 72 °C. The sequences for the sense and anti-sense primers are shown in Table 1. PCR products were stained with ethidium bromide and visualised by electrophoresis through 1.5% agarose gels. Sequencing of the PCR products confirmed the sequence identities.

2.4. Quantification of mRNA

The concentrations of target mRNAs were measured by reverse transcription followed by real-time PCR performed on a Roche Light Cycler system (Roche Molecular Biochemicals, Mannheim, Germany). Table 1 describes the primers used for this study. cDNAs were carried out using 2.5 μl cDNA in a 5.5 μl PCR SYBR Green-1 Light Cycler Master Mix (Biogene, Cambridgeshire, UK) and 1 μl sense and anti-sense primers. For studies involving steroidogenic gene expression H295R cells were incubated with adiponectin (100 nmol/l) for 4 h. A series of three dilutions for each cDNA was used to ensure linear amplification. Protocol conditions consisted of denaturation of 95 °C for 60 s, followed by 40 cycles of 94 °C for 1 s, 60 °C for 8 s, and 72 °C for 15 s, followed by a 10 min extension at 72 °C. The sequences of the genes of interest were standardised against the housekeeping gene GAPDH. Negative controls for all the reactions included preparations lacking cDNA or RNA-lacking reverse transcriptase in place of the cDNA. The relative mRNA levels were expressed as a ratio using “Delta-delta method” for comparing relative expression results between treatments in real-time PCR [15].

2.5. Western blotting

Protein lysates were prepared by adding equal amounts of Laemmli buffer (5 M urea, 0.17 M SDS, 0.4 M dithiothreitol, and 50 mM Tris–HCl, pH 8.0) to each well, and samples were denatured by sonication and boiling. Samples were separated by SDS-PAGE (10% resolving gel) and transferred to polyvinylidene difluoride (PVDF) membrane was purchased from Amersham Biosciences; all of the primers were obtained from TAGN (Newcastle, UK). Following inhibitors were obtained from Bio-Rad Laboratories Ltd. (Hertfordshire, UK). Polyvinylidene difluoride (PVDF) membrane was purchased from Amersham Biosciences; all of the primers were obtained from TAGN (Newcastle, UK). Camptothecin was obtained from Calbiochem (Darmstadt, Germany). For experiments involving StAR protein the primary antibody for adiponectin receptors 1 and 2 (Alpha Diagnostics Intl. Inc, San Antonio, USA) at 1:1500 dilution and β-actin (Abcam plc, Cambridge, UK) at 1:25,000 was used as control for loading made up in Tris buffered saline (TBS)-0.1% Tween (TBST), and 5% BSA overnight at 4 °C. For experiments involving StAR protein the primary antibody StAR (Abcam plc, Cambridge, UK) was used at a 1:7000 dilution and β-actin (Abcam plc, Cambridge, UK) at 1:25,000 dilution in Tris buffered saline (TBS)-0.1% Tween (TBST), and 5% BSA overnight at 4 °C. All the membranes were washed, incubated with a secondary anti-rabbit (ADIPOR1 and ADIPOR2), anti-rabbit (StAR), anti-mouse (β-actin, Abcam plc, Cambridge, UK) horseradish

Table 1

<table>
<thead>
<tr>
<th>Name</th>
<th>Sense Sence</th>
<th>Anti Sence</th>
<th>Product size (bp)</th>
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<tr>
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<td>5′-CCGACCCTCCCTCTCTCT-3′</td>
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<tr>
<td>ADIPOR2</td>
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<td>5′-GCCATCGTCTGACACTC-3′</td>
<td>108</td>
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peroxidase-conjugated antibody (1:2000) for 1 h at room temperature, and washed for 60 min with TBST. Antibody complexes were visualised using an ECL Plus chemiluminescence detection kit (GE Healthcare UK Ltd, Buckinghamshire, UK). The densities were measured using a scanning densitometer coupled to a Scion Image scanning software (Scion Corporation, Frederick, MD, USA).

2.6. Cortisol measurements

For cortisol release experiments H295R human adrenocortical cells were cultured in H295R complete media containing DMEM/F12 (1:1) supplemented with 2% Ultroser G (Biosepra, Villeneuve-la-Garenne, France) and ITS (Discovery Labware, Bedford, MA), in six-well plates until the cells were about 80–85% confluence. Then cells were serum starved overnight in media containing DMEM/F12 (1:1) supplemented with ITS and no Ultroser G for overnight. Following overnight starvation the medium was replaced with a 2 ml fresh medium containing adiponectin (100 nmol/l) or (angiotensin 100 nmol/l) and cultured for 24 h, unless otherwise indicated. At the end of the incubation period, the supernatant was taken and snap frozen immediately and stored at −80°C until the cortisol measurements were done. Cortisol measurements were done using the ELISA kit according to the manufacturer’s instructions (ALPCO Cortisol EIA kit, Paris, France).

2.7. Cell-Titre Glo proliferation assay for analysis of adiponectin-mediated proliferation of H295R cells

H295R cells were seeded on a 96-well plate at 1 × 10^4 cells per well in quadruplet and allowed to grow for 24 h followed with serum starvation overnight. Prior to treatment with 100 nM adiponectin for 24 h at 37 °C/5% CO2, the cells were allowed to grow in serum containing media for 24 h followed with serum starvation overnight. The Cell-Titre Glo assay (Promega, Southampton, UK) was performed according to the manufacturer’s instructions. Following incubation, proliferation was quantified via bioluminescence using a luminometer. Cells stimulated with vehicle and non-seeded wells containing media alone were included as a control.

2.8. Statistical analysis

Data are shown as the means±SE from 4 different experiments. One-way ANOVA was used for multiple group comparisons followed by a Tukey’s test for post hoc analysis. To analyse measurements of the intensity of immunoreactive staining, a scanning densitometer was used (Scion Image; Scion, Frederick, MD, USA). The values were considered significant if the differences in the means between the control and treated groups were greater than the P-value <0.05 (*P<0.05; **P<0.01; ***P<0.001).

3. Results

3.1. Adiponectin receptors are expressed in H295R cells

The expression of adiponectin receptors in human adrenocortical H295R cells was analysed by RT-PCR and Western blot analysis. Fig. 1A and B shows clear mRNA and protein (Fig. 1C and D) expressions of both adiponectin receptors (ADIPOR1 and ADIPOR2) in the H295R adrenal cell-line, concomitant with that observed for the native human adrenal tissue [17]. Fig. 1A shows a representative ethidium bromide-stained gel showing a 125 bp PCR product for the ADIPOR1 and a 108 bp PCR product for the ADIPOR2. Bands were excised, purified and sequenced to confirm their identity. Analysis of protein by Western blotting revealed a band of 42 kDa for ADIPOR1 and 35 kDa for ADIPOR2 in both H295R cells and adipose tissue which was used as positive control. This suggests that this pluripotent cell model of adrenal steroidogenesis is also representative of an adiponectin-sensitive model of the adrenal tissue.

3.2. Adiponectin causes cortisol secretion from H295R cells

To assess the role of adiponectin on cortisol production, H295R cells were incubated with adiponectin (100 nM) for 6 h, 12 h, 24 h and 36 h with an ANG II control (100 nM; 24 h). ELISA analysis of the H295R cell media (Fig. 2A) showed that a 24 hour stimulation of (100 nM) adiponectin maximally increased cortisol secretion to 165%±10% (p<0.01). This significant functional outcome was comparable to (100 nM) angiotensin (200%±8%; p<0.01), a potent standard for assessing steroidogenesis in this adrenal cell model [16]. Fig. 2C shows dose-dependent effects of adiponectin on cortisol secretion. Maximal response occurs between 10 nM and 100 nM treatments.

3.3. Effects of adiponectin on steroid regulatory gene expression in H295R cells

Quantitative determination of effects of adiponectin (100 nM) on steroid regulatory genes measured at 4 h following stimulation resulted in a significant up-regulation of StAR (165.3%±8; p<0.01), CYP11A (341.2%±11; p<0.001), 3βHSD (57.3%±4.6; p<0.05), CYP17 (70.3%±10; p<0.01) and CYP11B1 (185.6%±12; p<0.01). Fig. 2B shows the changes in mRNA levels of steroidogenic regulatory enzyme genes following adiponectin treatment.

3.4. Effects of adiponectin on StAR protein expression

The genetic expression data above suggests a clear steroidogenic pathway for the observed effects of adiponectin on cortisol secretion. To confirm this at the protein level, we used Western blotting to measure the effects of adiponectin on the expression of the StAR protein following stimulation for 4 h and angiotensin (100 nM) was used as a

![Fig. 1](image-url). The detection of ADIPOR1 and ADIPOR2 genes and protein in H295R cells. The A) and B) are representative results showing ethidium bromide-stained electrophoresis gel of PCR products from reverse transcribed RNA using primers for ADIPOR1 (125 bp) and ADIPOR2 (108 bp). C) and D) are representative Western blot analysis for ADIPOR1 and ADIPOR2 protein expression in H295R cells. A) Human subcutaneous adipose tissue (AD) lane 1, H295R cells (lane 2), and negative control lane 3. B) Human subcutaneous adipose tissue (AD) lane 1, H295R cells (lane 2), and negative control (lane 3). C) ADIPOR1 1) H295R cells, 2) human subcutaneous adipose tissue lysates, 3) HEK293-ADIPOR1 clone cell lysates, and 4) Laemmli buffer. D) ADIPOR2 1) H295R cells, 2) human subcutaneous adipose tissue lysates, 3) HEK293-ADIPOR2 clone cell lysates, and 4) Laemmli buffer.
positive control. Both adiponectin (6.8±2.3 fold; p<0.001) and angiotensin induced significant increases in the expression of StAR protein. Similar to the cortisol secretion data, this response was comparable with angiotensin effects (8.9±0.9 fold; p<0.001). Fig. 3A shows a representative Western blot demonstrating changes in StAR protein expression following adiponectin treatment. These increases in StAR protein expression were dose-dependent (Fig. 3B). A concentration range of 1–200 nM was used for this study showing a maximal expression at a concentration of 100 nM. Interestingly, at a higher concentration the StAR protein expression recedes towards basal levels. Fig. 3B shows a representative Western blot showing dose dependent effects of adiponectin on StAR protein expression.

3.5. Adiponectin signals through the AMPK, AKT, and ERK1/2 MAPK pathways, but not p38 in H295R cells

The downstream effects of adiponectin agonists are known to be mediated by two membrane bound receptors ADIPOR1 and ADIPOR2.
These have been implicated in the activation of AMPK, AKT and several members of the MAPK family in a number of tissues and cellular models [18–20] and the N-terminal region of adiponectin has been shown to activate several pathways including ERK1/2 activation [21] and the COX2 expression [22]. Activation of some of these signalling cascades is crucial for the survival of adrenal cells. We employed Western blot analysis to test whether adiponectin-stimulation of H295R cells induced activation of AMPK, AKT, ERK1/2 and p38 MAPK. Time-dependent studies showed adiponectin activated the AMPK, AKT and ERK1/2 MAPK pathways (Fig. 4A and B). However there was no activation of p38 MAPK either in a time- (Fig. 4C) or concentration-dependent manner (Supplementary Fig. 1). 100 nM adiponectin-stimulated ERK1/2 activation peaked (5-fold) after 10 min and remained significantly higher than the basal levels even after 60 min (2.5 fold, Fig. 4A). Activation of AMPK was slightly delayed peaking at about 20 min (2-fold) and remained significantly higher levels than basal even at 60 min.

### 3.6. The effects of AMPKi and ERKi on adiponectin-mediated StAR protein expression and cortisol secretion

It is known that adiponectin leads to the activation of ERK1/2 and AMPK through one or both of the ADIPORs. To assess the involvement of the above mentioned key signalling molecule involvement in adiponectin mediated StAR protein expression, we compared the relative StAR expression in response to adiponectin stimulation in the presence and absence of U0126 (MEK1/2/ERK1/2 inhibition) and compound-C (AMPK inhibition) in our experimental design. The data in Fig. 5A and B showed that both ERKi (from 4.5 ± 0.36-fold to 2.3 ± 0.20-fold over inhibitor alone) and AMPKi (from 3.4 ± 0.36-fold to 2.3 ± 0.20-fold inhibitor alone) led to a partial reduction of StAR protein expression in response to adiponectin. For cortisol secretion studies, U0126 (1 and 10 μM) alone showed a slight elevation peaking at about 20 min (2-fold) and remained significantly higher levels than basal stimulation for 60 min.

### 3.7. Adiponectin is not proliferative and has a small effect on apoptosis

Earlier studies from Ehrhart-Bornstein [23] reported a detachment of H295R cells following stimulation with adiponectin. To eliminate the possibility of induction of apoptosis in H295R by adiponectin, we studied the effects of adiponectin on proliferation and apoptosis. Stimulation of H295R cells with 100 nM adiponectin showed no significant changes in H295R cell proliferation after a 24 hour stimulation (Fig. 6A). However DNA fragmentation assay (marker of apoptosis) assessing the effects of adiponectin on apoptosis at 24 h showed a slight increase in apoptosis of H295R cells but was not significant. Camptothecin (used as positive control for the study) showed a significant increase in apoptosis (350%) compared to the control stimulation. This data is interesting as we observed a significant, transient increase on AKT activation in response to adiponectin (Fig. 4D) which peaked at about 10 min (2-fold) and returned to near basal by 20 min.

### 4. Discussion

Adiponectin is a recently discovered adipokine, circulating in the plasma in the range of 3 to 30 μg/ml (100 nM–1 μM) concentration in

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**Fig. 4.** Time dependent effects of adiponectin, on ERK1/2, p38, AMPK and AKT protein phosphorylation in H295R cells. Western blot analysis of protein extracts prepared from H295R cells following stimulation with 100 nM adiponectin for various time points were analysed for ERK1/2, p38, AMPK and AKT phosphorylation. The antibody against p-ERK1/2 and ERK1/2 recognised bands with apparent molecular weights of 42 and 44 kDa. p-p38 and p38 recognised bands with apparent molecular weights of 38 kDa. P-AMPK and AMPK recognised bands with apparent molecular weights of 63 kDa. p-AKT and AKT recognised bands with apparent molecular weights of 60 kDa. Densitometric analysis of phosphorylated proteins was normalised to total protein content. The figure depicts the representative experiment; the relative intensity was measured by densitometry using a Scion image software from 3 independent experiments. Data points are mean ± SE, where basal was considered as one for each experiment, and fold increase over basal is shown. Significance was determined by one-way ANOVA followed with Tukey test for post hoc analysis against control cells (***P < 0.01; **P < 0.05).
various multimeric forms. Adipokines reach the adrenal glands in an endocrine manner at concentrations sufficient to induce adrenal steroidogenesis. Also intra-adrenal adipose tissue depots in direct contact with steroid-producing adrenocortical cells could act as local sources of adipokines. This close cellular proximity forms the prerequisite for paracrine interactions and indicates a direct stimulation of steroidogenesis by adipokines [23]. Adipokines can stimulate aldosterone synthesis and secretion in an endocrine or paracrine manner with implications for hyperaldosteronism and consequently hypertension in obesity [6]. The functional importance of adiponectin receptor activation in the adrenal gland is not well studied.

In this current study, we demonstrate for the first time the expression of ADIPOR1 and ADIPOR2 mRNA and protein in human adrenocortical H295R cells. More importantly, we show stimulation of H295R cells with adiponectin results in increased expression of many steroidogenic genes, STAR protein expression, cortisol secretion and the signalling cascades involved in the regulation of STAR protein expression.

The regulation of the adrenal steroidogenic machinery is a complex process that involves the interaction of diverse hormones and multiple signalling pathways that coordinate the cooperation and interaction of transcriptional machinery as well as a number of post-transcriptional mechanisms that govern mRNA and protein expression [24]. Although ACTH and angiotensin are the major regulators of adrenal steroid production, recent evidences point out the importance of adipokines in fine tuning the regulation of steroidogenesis process [25–27].

Our findings on investigating the effects of adiponectin stimulation in H295R cells showed a significant increase in cortisol production, together with increases in mRNA levels of key steroidogenic genes including STAR.
CYP11A, 3β-HSD, CYP17 and CYP11B1. It is now well documented that StAR protein expression is the first rate limiting step in the steroid biosynthesis in the adrenal gland, as StAR protein promotes the transfer of cholesterol across the mitochondrial membrane for steroid biosynthesis to take place [24,28,29]. Therefore we performed comprehensive analysis of effects of adiponectin and the key signalling events involved in the regulation of StAR protein expression upon stimulation with adiponectin. Stimulation of H295R cells with adiponectin resulted in a significant increase in StAR protein expression. The bell-shaped effect of dose-dependent adiponectin on StAR expression was particularly interesting due to the inverse relationship between circulating adiponectin levels and obesity. Adiponectin concentrations similar to those of endogenous adiponectin found in obese people resulted in a higher StAR expression, suggesting that in obese people ADIPOR activation might play a role in activating the adrenal cells to produce more cortisol.

Induction of CAMP and the CAMP-dependent signalling mechanisms is undoubtedly the principal pathway regulating StAR expression and steroid biosynthesis [30–32]. Activation of two key MAPKs (ERK1/2 and p38) and AMPK is also involved in the regulation of StAR expression and steroid production. However, the precise mechanism involved is poorly understood as a consequence of conflicting reports demonstrating stimulation, inhibition, or no effect in different steroidogenic cells [33–36].

We have shown that stimulation with adiponectin resulted in a significant increase in ERK1/2 and AMPK activation in H295R cells. To elucidate the involvement of ERK1/2 and AMPK in adiponectin induced StAR expression, MEK/ERK1/2 inhibitor U0126 and the AMPK inhibitor compound-C were used. These inhibitors have been widely used for blocking relevant signalling pathways in other steroidogenic cells [11,37]. Inhibition of both ERK1/2 and AMPK pathways resulted in partial but significant reductions in adiponectin-induced StAR expression suggesting that these two major pathways may be involved in regulating StAR expression in H295R cells. Angiotensin (used as positive control throughout the study) also showed significant increase in StAR expression, however when adiponectin was co-incubated with angiotensin there was no additive/synergistic effect observed. This data shows that adiponectin-induced cortisol secretion involves both AMPK and ERK1/2 dependent pathways. However, the combined effects of adiponectin with either inhibitors did not completely reduce the secretion of cortisol to basal levels. This may reflect that there exists a small effect of an AMPK and ERK1/2-independent mechanism for adiponectin-induced cortisol secretion.

Whilst a previous study suggested that cortisol secretion was not observed in a screen of potential mineralocorticoid-releasing factors including “adiponectin” [23], the study does not mention which form of adiponectin was used in their studies, and they reported possible cellular necrosis with 100 nM adiponectin treatment. Our studies with the same concentration of adiponectin (100 nM) did not show any cell detachment/necrosis nor showed any significant induction of apoptosis or reduction in cell viability.

Altogether our data implicates that the ADIPORs are clearly expressed in a pluripotent H295R adrenal cell model. We show that stimulation of H295R cells with adiponectin results in increased cortisol secretion. This effect is accompanied by the increased gene expression of several key members of the steroidogenic pathway including the expression of StAR protein via ERK1/2 and AMPK-dependent pathways. This has implications for our understanding of adiponectin receptor activation and peripheral steroidogenesis. Furthermore adiponectin might play an important role in the pathophysiology of adrenal secretions in obese people.

Supplementary materials related to this article can be found online at doi:10.1016/j.bbamcr.2011.02.010.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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