HHS Public Access

Author manuscript

Diabetologia. Author manuscript; available in PMC 2015 September 01.

Published in final edited form as:

Diabetologia. 2013 September ; 56(9): 1999–2009. doi:10.1007/s00125-013-2971-4.

Deficiency of APPL1 in mice impairs glucose-stimulated insulin secretion through inhibition of pancreatic beta cell mitochondrial function

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Duality of interest The authors declare that there is no duality of interest associated with this manuscript.

Contribution statement CW and LQD were responsible for designing the experiments. XL, KM, LL, SW, YZ, MZ, JR, ZX, DS, WJZ were responsible for acquisition of data, and analysis and interpretation of data. CW, LQD and WJ analysed and interpreted data. CW drafted the manuscript. All authors critically revised and approved the final version of the manuscript.

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Abstract

Aims/hypothesis—Adaptor protein, phosphotyrosine interaction, pleckstrin homology domain and leucine zipper containing 1 (APPL1) is an adapter protein that positively mediates adiponectin signalling. Deficiency of APPL1 in the target tissues of insulin induces insulin resistance. We therefore aimed, in the present study, to determine its role in regulating pancreatic beta cell function.

Methods—A hyperglycaemic clamp test was performed to determine insulin secretion in APPL1 knockout (KO) mice. Glucose- and adiponectin-induced insulin release was measured in islets from APPL1 KO mice or INS-1(832/13) cells with either APPL1 knockdown or overproduction. RT-PCR and western blotting were conducted to analyse gene expression and protein abundance. Oxygen consumption rate (OCR), ATP production and mitochondrial membrane potential were assayed to evaluate mitochondrial function.

Results—APPL1 is highly expressed in pancreatic islets, but its levels are decreased in mice fed a high-fat diet and db/db mice compared with controls. Deletion of the *Appl1* gene leads to impairment of both the first and second phases of insulin secretion during hyperglycaemic clamp tests. In addition, glucose-stimulated insulin secretion (GSIS) is significantly decreased in islets

from APPL1 KO mice. Conversely, overproduction of APPL1 leads to an increase in GSIS in beta cells. In addition, expression levels of several genes involved in insulin production, mitochondrial biogenesis and mitochondrial OCR, ATP production and mitochondrial membrane potential are reduced significantly in APPL1-knockdown beta cells. Moreover, suppression or overexproduction of APPL1 inhibits or stimulates adiponectin-potentiated GSIS in beta cells, respectively.

Conclusions/interpretation—Our study demonstrates the roles of APPL1 in regulating GSIS and mitochondrial function in pancreatic beta cells, which implicates APPL1 as a therapeutic target in the treatment of type 2 diabetes.

Keywords

Adiponectin; APPL1; Beta cells; Insulin; Mitochondrial function

Introduction

Pancreatic beta cell failure to compensate peripheral insulin needs is a hallmark of type 2 diabetes [1], and obesity-induced insulin resistance is one of the key factors attributed to the increase in peripheral insulin requests [2]. However, the mechanisms underlying obesity-induced beta cell dysfunction are not fully understood. Identification of key signal molecules involved in regulating beta cell function is thus essential to underpin the development of new pharmacological approaches to the treatment of type 2 diabetes.

Adaptor protein, phosphotyrosine interaction, pleckstrin homology (PH) domain and leucine zipper containing 1 (APPL1) protein, an adapter protein containing a PH domain, phosphotyrosine-binding (PTB) domain and leucine zipper motif [3], is ubiquitously expressed in mouse tissues including heart, ovary, brain, liver, pancreas and skeletal muscle [4]. It interacts with several membrane receptors (e.g. the adiponectin receptor and follicle-stimulating hormone receptor) and signal proteins (e.g. Akt and Rab5) to mediate the respective signalling transductions [5–7]. APPL1 also plays critical roles in regulating cell cycle arrest [8], cell survival [9], neuritogenesis [10] and insulin sensitisation [5, 11, 12].

An accumulating body of evidence has shown that APPL1 is influential in mediating the insulin-sensitising role of adiponectin in several target tissues of insulin [4]. In pancreatic beta cells, adiponectin treatment induces a reduction in NEFA-induced cell death [13], suggesting a protective role of adiponectin in regulating beta cell function. However, it remains controversial whether adiponectin can regulate glucose-stimulated insulin secretion (GSIS) [14–17]. As pancreatic beta cells possess insulin and adiponectin signalling machinery [14, 18] and APPL1 is critical for mediating signal transduction in both pathways [5, 11], it is important to understand the role of APPL1 in regulating beta cell function.

Cheng et al have recently reported that deletion of the *Appl1* gene leads to a reduction in first-phase insulin secretion in APPL1-deleted islets [19]. However, the mechanism underlying the action of APPL1 still remains largely unknown. In the present study, we report that APPL1 is critical for both the first and second phases of insulin secretion in response to glucose stimulation. Impairments of beta cell mitochondrial structure and

function contribute significantly to the impairment of the GSIS phenotype observed in APPL1 knockout (KO) mice. The results reveal novel roles and mechanisms for APPL1 in regulating beta cell functions.

Methods

Mouse pancreatic islet isolation

Four-week-old wild-type (WT) C57BL/6 male mice (Shanghai Slaccas Company, Shanghai, People's Republic of China) were fed either a high-fat diet (HFD; 60% fat) or normal chow for 3 months. The db/db mice, obtained from Shanghai Slaccas Company, were fed with a chow diet. Breeding of APPL1 KO mice was achieved using the gene trap technique (see electronic supplementary material [ESM] Fig. 1a), and the chimeras were crossed with C57BL/6 mice for six generations. Genotyping was performed by PCR analysis using primers that recognised the β-geo cassette (forward, 5′-TTCAACATCAGCCGCTACA G-3′; reverse, 5′-CTCGTCCTGCAGTTCATTCA-3′; ESM Fig. 1). The mice were housed at 23°C±1°C in a 12 h light– dark cycle with free access to food and water. All procedures involving the care and use of animals were carried out in accordance with Shanghai Jiao Tong University Guidelines for the care and use of laboratory animals. Pancreatic islets were isolated from male mice at 10–12 weeks of age and prepared as previously described [20].

Animal studies

All in vivo experiments were performed with male mice, and littermate controls were used throughout this study. Glucose tolerance tests (GTTs) and insulin secretion tests were performed after 12 h of fasting with mice at 9–10 weeks old. Glucose (1.5 g/kg for GTTs and 3 g/kg for insulin secretion tests) was injected intraperitoneally. Blood samples were taken from the tail vein. Glucose levels were measured using a glucose monitor. Insulin and glucagon levels were measured using ELISA kits (insulin: Mercodia, Uppsala, Sweden; glucagon: Millipore, St Charles, MO, USA). Hyperglycaemic clamp studies were performed with mice aged 10–12 weeks. After a 6 h fast, conscious mice were primed and variably infused with 20% glucose to maintain their plasma glucose levels. Blood samples were collected to measure glucose and insulin concentrations. The average glucose infusion rate (GIR) was calculated as the average GIR during the whole period of the clamp.

Human tissue collection

Human tissues (liver, pancreas and adipose tissue) were collected from patients undergoing resection of benign focal hepatic lesions or benign pancreatic pathology at the Department of General Surgery (Shanghai Jiao Tong University Affiliated Sixth People's Hospital, People's Republic of China). Human muscle specimens were obtained from the quadriceps femoris muscles of healthy volunteers by muscle biopsy. The protocol was approved by the Ethics Committee of Shanghai Jiao Tong University, following the principles of the Declaration of Helsinki. All volunteers provided informed consent.

Generation of recombinant adenoviruses

Recombinant adenoviruses for the overproduction of APPL1 (Ad-APPL1) and enhanced green fluorescent protein (Ad-EGFP) were generated using the Gateway system (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. Briefly, cDNAs encoding full-length APPL1 or EGFP were amplified from pcDNA3.1-Myc-His (+)-APPL1 [5] or pIRES2-EGFP (Invitrogen), respectively. The APPL1-targeting recombinant adenoviruses containing the short hairpin (sh)RNA, shAPPL1 (Ad-shAPPL1), or a scrambled (Ad-scrambled) sequence were constructed using the pDC316-EGFP vector (GeneChem Management, Montreal, QC, Canada) and pBHG lox ΔE1, 3Cre (Microbix, Toronto, ON, Canada) according to the manufacturer's instructions. Multiplicity of infection (MOI) of the generated adenoviruses was determined by an endpoint dilution assay [21].

Cell culture and adenovirus-mediated gene silencing or overexpression

INS-1(832/13) cells (a kind gift from Dr Y. Liu, the Institute for Nutritional Sciences, Shanghai Institute for Biological Sciences, Chinese Academy of Sciences) were maintained in RPMI 1640 (Sigma, St Louis, MO, USA) [22]. The cells were infected with 10 MOI Ad-shAPPL1 and Ad-scrambled, or 2 MOI Ad-APPL1 and Ad-EGFP. After 16–18 h, the virus-containing medium was removed, and the cells were cultured for an additional 24 h before analysis. Adenovirus-infected INS-1(832/13) cells under these conditions did not have significant effects on cellular viability (ESM Fig. 2) as visualised by fluorescence microscopy after staining with Hoechst 33324 (Sigma) and propidium iodide (Sigma).

Measurement of insulin content and secretion

Infected cells or isolated mouse islets were pre-incubated in KRB solution (ESM Materials and methods) with 2.8 mmol/l glucose for 2 h (for a 2 h experiment), and then incubated in KRB (for a 2 h study) or RPMI 1640 (for a 24 h study) containing different concentrations of glucose in the presence or absence of 10 mmol/l α-ketoisocaproate (α-KIC; Sigma), 10 mmol/l succinic acid methyl ester (SAME; Sigma), 20 mmol/l KCl and 2.5 mg/l full-length adiponectin (R&D Systems, Minneapolis, MN, USA) for 2 h or 24 h. The insulin secreted into the supernatant fraction and the insulin content were measured with ELISA analysis (Mercodia).

Histological and electron microscopic studies

Immunofluorescence staining was performed using a previously described protocol [23] with specific antibodies (ESM Materials and methods). Electron microscopic study was carried out as described by Koyanagi et al [24]. Sections were observed using a Philips CM 120 Transmission Electron Microscope (Philips, Eindhoven, the Netherlands).

Measurement of oxygen consumption, ATP levels, mitochondrial membrane potential and reactive oxygen species (ROS) production

An Extracellular Flux Analyzer XF24 (Seahorse Bioscience, North Billerica, MA, USA) was used to determine the cellular oxygen consumption rate (OCR) in the cells. $NaHCO_3$-free KRB was used as the assay medium. Following pre-incubation in 2.8 mmol/l glucose for 2 h, the OCR was assayed in 2.8 mmol/l glucose, and then 16.7 mmol/l glucose with or

without drugs: 1 μmol/l oligomycin, 2 μmol/l carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (FCCP), 1 μmol/l antimycin A and rotenone (Seahorse Bioscience). The amount of ATP in the cells was determined using the CellTiter-Glo Luminescent Cell Viability Assay (Promega, Madison, WI, USA) as previously described [25]. In brief, cells were seeded in 96-well tissue culture microplates at 2.5×10^4 cells/well or 20 islets in glucose-free KRB in an incubator at 37°C before the ATP assay. The intensity of luminescence was measured using a Synergy HT microplate reader (BioTek Instruments, Winooski, VT, USA). For measurement of mitochondrial membrane potential (MMP) or ROS, a group of 20–30 islets were loaded with JC-1 (Beyotime, Jiangsu, China) or with DCFH-DA (Sigma) in KRB as reported by Yano et al [26]. Fluorescence from the mitochondrial matrix or from ROS generation was monitored using the Synergy HT microplate reader (BioTek Instruments).

Western immunoblotting and quantitative real-time PC

Protein was extracted from cell or tissue samples with RIPA buffer containing protease inhibitors and its expression was analysed by western blotting (ESM Materials and methods). Quantitative real-time PCR was performed using the ABI 7500 Fast Real-Time PCR System (Applied Biosystems, Foster City, CA, USA) (ESM Materials and methods). Primers for each target gene examined are shown in ESM Table 1.

Statistical analysis

The data shown are the mean±SEM of a given number of independent experiments. The statistical significance of differences was determined using either the Student's *t* test or ANOVA followed by Bonferroni's multiple comparison post hoc tests. A value of $p<0.05$ was considered statistically significant.

Results

APPL1 protein levels in pancreatic islets are decreased under diabetic conditions in vivo

To determine APPL1 tissue distributions, we detected its protein levels in different tissues isolated from human volunteers, C57BL/6 mice and Sprague Dawley rats using the APPL1 antibody. APPL1 is abundantly produced in most tissues including pancreas, fat, liver, muscle and isolated islets (Fig. 1a). To identify the type of pancreatic cell within which APPL1 was located, mouse pancreas sections were co-immunostained with antibodies for APPL1 and insulin or glucagon. Our data indicated that APPL1 was exclusively produced in the pancreatic islets, co-localising significantly with insulin (Fig. 1b) and to a lesser extent with glucagon (Fig. 1b).

Next, we investigated the correlation between the abundance of APPL1 and insulin sensitivity in vivo. APPL1 protein levels were dramatically decreased in islets isolated from db/db and HFD-fed mice compared with their controls (data not shown), which is consistent with a recent report by Cheng et al [19]. In addition, the APPL1 content of the INS-1(832/13) cells was significantly downregulated in the presence of high levels of glucose (20 mmol/l; $p<0.05$; Fig. 2a) or palmitate (1 mmol/l; Fig. 2b). Collectively, these

data suggest that APPL1 levels in pancreatic beta cells are decreased under gluco(lipo)toxic conditions, indicating a role of APPL1 in regulating pancreatic endocrine function.

GSIS is impaired in APPL1 KO mice and APPL1 downregulated INS-1(832/13) cells

Deletion of Appl1 gene expression was confirmed by western blot analysis in islets from WT and APPL1 KO mice (ESM Fig. 1b). No significant changes in body weight (Fig. 3a), fasting glucose (Fig. 3b) or fasting insulin level (Fig. 3c) were detected between APPL1 KO mice and their control littermates. Fasting glucagon levels tended to increase in the KO mice (p=0.07; Fig. 3d). On the other hand, APPL1 KO mice exhibited impaired glucose tolerance (Fig. 3e, f) and reduced GSIS (Fig. 3g) at the age of 12 weeks.

To understand the role of APPL1 in regulating beta cell function, a hyperglycaemic clamp test was performed. Plasma glucose levels were maintained at 16–18 mmol/l for both WT and APPL1 KO mice during the clamp assay (Fig. 4a). APPL1 KO mice exhibited significant decreases in GIR and average GIR compared with their WT littermates (Fig. 4b, c). Analysis of plasma insulin levels at multiple time points during the clamp test indicated significant impairments of insulin secretion in APPL1 KO mice during both the first (0–5 min; p<0.01) and second (5–90 min; p<0.05) phases of secretion, based on the area under the insulin curve during the two periods (Fig. 4d, e). The insulin content of the pancreas remained the same between WT and APPL1 KO mice (data not shown).

To further demonstrate that altered APPL1 production is sufficient to regulate GSIS, we isolated islets from WT and APPL1 KO mice and measured ex vivo insulin secretion. Under basal glucose (2.8 mmol/l) conditions, the islets isolated from WT and KO mice secreted similar amounts of insulin (Fig. 4f). Consistent with the clamp data (Fig. 4d, e), GSIS was completely abolished in the islets from APPL1 KO mice under 20 mmol/l glucose conditions (Fig. 4f).

Similar results were observed in INS-1(832/13) cells, where APPL1 content was significantly knocked down (64.03%) with the adenovirus-mediated *Appl1* shRNA approach (Fig. 5a and ESM Fig. 3a). Insulin release under glucose stimulation (20 mmol/l) was significantly reduced in the APPL1 knockdown cells compared with that in the scrambled controls (Fig. 5c). The effect of APPL1 on GSIS was continued when the cells were cultured for an extended period of time (Fig. 5d). Moreover, total insulin production (release and content) at the end of the 24 h period of glucose stimulation was also significantly decreased in APPL1 knockdown cells compared with that in the control cells (data not shown). Conversely, overproduction of APPL1 in INS-1(832/13) cells caused by infecting the cells with adenovirus-mediated APPL1 greatly enhanced GSIS (Fig. 5b, e and ESM Fig. 3b). Together, these data suggest that APPL1 plays an essential role in regulating GSIS in pancreatic beta cells.

Characterisation of APPL1-regulated insulin secretion in INS-1(832/13) cells

To investigate the molecular mechanisms underlying APPL1-regulated GSIS, we investigated whether proximal and distal mitochondrial events were altered in the APPL1 knockdown beta cells. α-KIC (a transamination product of leucine) and SAME are

mitochondrial fuels that provide anaplerotic inputs. As expected, treatment of INS-1(832/13) cells with a combination of α-KIC and SAME led to an increase in insulin secretion, which was comparable to the levels of insulin secreted under glucose stimulation conditions (Fig. 5f). Interestingly, both glucose- and α-KIC/SAME-stimulated insulin secretion was significantly reduced in APPL1 knockdown cells (Fig. 5f), suggesting that a mechanism underlying APPL1-regulated insulin secretion could lie downstream of glycolysis. We then tested the effect of distal mitochondrial steps on insulin secretion using KCl, which depolarises the beta cell membrane by bypassing the ATP-dependent potassium channel. As shown in Fig. 5g, treatment of APPL1 knockdown INS-1(832/13) cells with KCl partially rescued the effect of APPL1 knockdown on GSIS.

Knockdown of APPL1 in beta cells induces mitochondrial dysfunction

Next, we investigated the possibility that APPL1 manipulates mitochondrial biogenesis and insulin levels in beta cells. We first detected the expression of several genes encoding molecules involved in mitochondrial biogenesis and the respiratory chain by real-time PCR. Among these, *Tfam* and *Pgc-1α* were significantly downregulated in APPL1 knockdown INS-1(832/13) cells compared with their scrambled controls (Fig. 6), indicating that APPL1 is responsible for maintaining the expression of several key genes involved in mitochondrial biogenesis. In addition, the mRNA levels of *Pdx1*, *Neurod1* and *Ins1* were significantly reduced in the APPL1 knockdown cells (Fig. 6), suggesting that APPL1 is also involved in regulating insulin synthesis and production.

We then examined the effect of APPL1 on mitochondrial function. Under basal conditions (2.8 mmol/l glucose), the mitochondrial OCR was similar between the APPL1 knockdown cells and the scrambled controls. However, the glucose-stimulated (16.7 mmol/l) OCR was significantly reduced in the knockdown cells compared with the scrambled controls (Fig. 7a, b). In addition, the maximal mitochondrial respiration capacity measured by the addition of FCCP, an uncoupling agent, was significantly decreased in the knockdown cells (Fig. 7a, c). Consistent with this observation, high glucose-induced ATP production and MMP were significantly decreased in APPL1 knockdown INS-1(832/13) cells and/or APPL1 KO islets (Fig. 7d, e and ESM Fig. 4a). Furthermore, beta cells from the KO mice contained swollen mitochondria with disordered cristae (Fig. 7f) and lower mitochondrial numbers (p=0.057; Fig. 7g). No significant difference could be detected in glucose-induced ROS production between WT and APPL1 KO islets (data not shown). These data demonstrate that APPL1 plays a critical role in regulating mitochondrial OCR, MMP and ATP production and mitochondrial structure, which may contribute to APPL1-regulated GSIS.

The role of adiponectin in potentiating GSIS depends on APPL1

It is well known that APPL1 plays a critical role in regulating adiponectin signalling in insulin target tissues. We therefore tested the role of APPL1 in mediating the effects of adiponectin on insulin secretion in INS-1(832/13) cells. Treatment of adiponectin potentiated GSIS (Fig. 8a). Overproduction of APPL1 further promoted the effects of glucose and adiponectin on insulin secretion (Fig. 8a). In contrast, knockdown of APPL1 production abolished both glucose- and adiponectin-induced insulin secretion (Fig. 8b). Together, our data indicate that adiponectin could potentiate GSIS in pancreatic beta cells

and that APPL1 is essential for the action of adiponectin in regulating insulin secretion. Since APPL1 mediates adiponectin signalling from adiponectin receptors to downstream molecules such as AMP activated protein kinase (AMPK) in insulin target tissues, we next tested whether APPL1 is essential for adiponectin-induced activation of AMPK in beta cells. As shown in Fig. 8c, adiponectin induced phosphorylation of AMPK in INS-1(832/13) cells. Downregulation of APPL1 led to a significant reduction in AMPK phosphorylation in response to adiponectin stimulation (Fig. 8c), suggesting that the adiponectin–APPL1–AMPK axis is a common pathway to mediating adiponectin signalling in cells.

Discussion

In the present study, we have provided evidence demonstrating that APPL1 regulates GSIS from pancreatic beta cells both in vivo and in vitro. APPL1 is specifically produced in pancreatic islets but not in the exocrine area (Fig. 1). The levels of this protein decrease under insulin resistance (Fig. 2). Deletion of the *Appl1* gene in mice leads to impaired glucose tolerance and reduced GSIS. Our study revealed novel molecular mechanisms underlying APPL1-regulated pancreatic beta cell functions: (1) APPL1 plays a critical role in regulating both the first and second phases of GSIS; and (2) APPL1 manipulates beta cell mitochondrial structure and function, which contributes to the beta cell dysfunction observed in APPL1 KO mice.

Downregulation of APPL1 could be a mechanism underlying the development of insulin resistance and type 2 diabetes. It has been reported that APPL1 plays an important role in regulating insulin sensitivity in insulin's target tissues by enhancing glucose transporter-4 translocation, glucose uptake, glycogen synthesis and inhibition of gluconeogenesis [5, 11, 27, 28]. In hepatic tissue, its content is reduced under conditions of insulin resistance [29]. Genetic studies indicated that single-nucleotide polymorphisms within the *APPL1* gene have been linked to obesity [30]. In the islets in animal models of diabetes (db/db mice and HFD mice), the APPL1 protein content is significantly decreased compared with controls [19]. In this study, we have provided further evidence that APPL1 is downregulated under high levels of glucose and lipid in INS-1(832/13) cells (Fig. 2). The correlation between reduced pancreatic APPL1 content and insulin resistance suggests that APPL1 might play a key role in regulating beta cell function and insulin sensitivity in vivo.

A fundamental characteristic of pancreatic beta cells is their capacity to synthesise and secrete insulin in response to changes in circulating glucose and other nutrients. The failure of GSIS observed in APPL1 KO mice and knockdown beta cells indicates the role of APPL1 in controlling insulin production and secretion (Figs 4, 5 and 6). In addition, our ex vivo studies suggest that impaired insulin release in APPL1 KO mice was intrinsic to the pancreatic islets (Fig. 4f) rather than secondary to an alteration in insulin sensitivity in the peripheral tissues of APPL1 KO mice. While this paper was being written, Cheng et al reported that APPL1 KO mice are insulin resistant and exhibit impaired GSIS in the first phase of insulin secretion [19], findings that are consistent with some of the results outlined in this study.

Compared with Cheng et al's study, however, we have reported several new findings. First, we observed a significant decrease in insulin release in both the first and second phases of insulin secretion in APPL1 KO mice (Fig. 4d, e) as well as changes in *Vamp2* and *Snap25* gene expression in APPL1 knockdown INS-1(832/13) cells (data not shown). The differences between our study and Cheng et al's may result from the use of different methods and model systems, in which we performed hyperglycaemic clamp tests with whole animals, whereas Cheng et al used an islet perfusion approach at a cellular level.

Second, we revealed for the first time that APPL1 regulates beta cell function by manipulating mitochondrial function. Mitochondria generate metabolic coupling factors required for insulin biosynthesis and secretion in pancreatic beta cells in response to glucose stimulation [31]. These factors, derived from glucose oxidation in the mitochondria, act as signal molecules to promote insulin biosynthesis [31] and enhance intracellular ATP/ADP ratios, which subsequently result in the closure of ATP-sensitive potassium ion channels and the increase of intracellular calcium ion concentrations; this in turn stimulates insulin release. We have shown that a defect in GSIS with APPL1 silencing of beta cells is probably the consequence of glucose-driven mitochondrial activity dysfunction (Figs 6, 7 and ESM Fig. 4). The following lines of evidence support this conclusion. First, the defect in GSIS could not be rescued by two mitochondrial fuels, α-KIC and SAME (Fig. 5f), which have been shown to increase insulin secretion by providing tricarboxylic acid cycle intermediates [32]. Second, the mRNA levels of *Tfam* and *Pgc-1α* were significantly reduced in APPL1 knockdown beta cell lines (Fig. 6). Mitochondrial transcription factor A (TFAM) is a mitochondrial transcription factor that is essential for mammalian mitochondrial biogenesis. Ablation of TFAM in mice results in embryonic lethality due to severe respiratory chain deficiency [33]. Pancreatic beta cell-specific disruption of TFAM resulted in diabetes at a young age and deficient oxidative phosphorylation [34]. Peroxisome proliferator-activated receptor-γ coactivator-1α (PGC-1α) is critical for mitochondrial biogenesis and oxidative metabolism [35]. Whole-body PGC-1α-null mice had lower ATP levels and lower metabolic efficiency [36, 37]. Finally, we demonstrated that glucose-induced OCR, ATP production and MMP were blunted in APPL1 knockdown beta cells and/or APPL1 KO islets (Fig. 7a–e and ESM Fig. 4).

In addition, we found that mitochondria in the beta cells of APPL1 KO mice were swollen, with disordered cristae and reduced numbers compared with normal mitochondrial morphology in WT controls (Fig. 7f). The key role of beta cell mitochondria is underlined by the development of diabetes in families harbouring mutations in mtDNA in humans [38] and TFAM pancreatic beta cell-specific deletion in mice, as mentioned above [34]. It is well accepted that mitochondrial activation is essential for glucose-induced insulin exocytosis. Production of ATP from mitochondria is necessary for the membrane-dependent increase in calcium ions, the main trigger for exocytosis of insulin granules in readily released pools. The linkage of mitochondrial metabolism to second-phase insulin secretion has been established by a study using beta cells with mitochondrial DNA deletion [39], in which the cells fail to respond to glucose stimulation while still secreting insulin in response to membrane depolarisation by KCl [39]. Mitochondrial metabolism-derived intermediates, such as glutamate and long-chain acyl-CoA derivatives, might act as signalling molecules

for amplifying pathways responding to insulin secretion [40]. It is currently unclear how APPL1 manipulates mitochondrial function in pancreatic beta cells. APPL1 is mainly localised in the cytosol, with small proportions in nuclear/mitochondrial fractions and light microsomal fractions in adipocytes [28]. Insulin stimulation increases its transport to microsomes, plasma membranes and the nuclear/mitochondrial fraction [28]. It will be interesting to test whether or not subcellular translocalisation of APPL1 contributes to its function.

Furthermore, we demonstrated a role of APPL1 in regulating the effect of adiponectin on GSIS in beta cells. Consistent with a report by Wijesekara et al [16], we showed that adiponectin potentiates GSIS in INS-1(832/13) cells (Fig. 8a). In addition, we have shown that an overproduction or silencing of APPL1 in INS-1(832/13) cells results in stimulation and inhibition of adiponectin-induced GSIS, respectively (Fig. 8a, b). It is known that APPL1 plays an important role in mediating adiponectin signalling in the peripheral tissues [4, 5]. Both adiponectin and metformin can regulate the subcellular translocation of APPL1 in muscle cells [41]. Adiponectin has been reported to regulate mitochondrial function in skeletal muscles through activating PCG-1α and enhancing mitochondrial biogenesis [42–44]. However, the augmentation of GSIS by adiponectin was accompanied by a lowering of glucose-induced ATP production rather than an enhanced OCR (ESM Fig. 4). Further studies are needed to explore the mechanisms underlying the roles of adiponectin on GSIS regulation.

Finally, we found that adiponectin treatment induced AMPK phosphorylation in beta cells (Fig. 8c), which is similar to the reports by Huypens et al and Staiger et al [14, 15]. This stimulation seems to occur via an APPL1-dependent mechanism since a decrease in APPL1 production resulted in a reduction in adiponectin-stimulated AMPK phosphorylation (Fig. 8c) that was proportional to the degree of APPL1 knockdown. Interestingly, this partial reduction of APPL1 content actually caused a complete abolition of GSIS (Fig. 8b). The role of AMPK in regulating beta cell function remains controversial [45, 46]. Evidence using genetic approaches favours the notion that activation of AMPK in beta cells is associated with impairment of GSIS in vitro. Overproduction of the active form of AMPK has been reported to suppress GSIS in vitro [46–48]. On the other hand, deletion of AMPK catalytic subunits in beta cells enhances GSIS in isolated islets [48]. Taken together, these data imply that the APPL1-mediated effects of adiponectin on GSIS may occur via an alternative pathway to APPL1-AMPK signalling. Further studies should be performed to explore the possibilities here.

In summary, our study has demonstrated that APPL1 plays a vital role in regulating GSIS and maintaining mitochondrial functions in beta cells. The finding that APPL1 links GSIS and mitochondria highlights the importance of this adaptor protein in the development of insulin resistance and type 2 diabetes.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We thank Pengying Li and Kelei Dong (Department of Biochemistry and Molecular Biology, Shanghai Medical College of Fudan University, China) for technical help.

Funding This work was supported by grants from the National Natural Science Foundation (30971121) (to CW), Shanghai Committee of Science and Technology (11140900900) (to CW), and National Institute of Health grant R01 DK080344 (to LQD).

Abbreviations

Ad	Adenovirus
AMPK	AMP activated protein kinase
APPL1	Adaptor protein, phosphotyrosine interaction, pleckstrin homology domain and leucine zipper containing 1
EGFP	Enhanced green fluorescent protein
FCCP	Carbonylcyanide p-trifluoromethoxyphenylhydrazone
GIR	Glucose infusion rate
GSIS	Glucose-stimulated insulin secretion
GTT	Glucose tolerance test
HFD	High-fat diet
α-KIC	α-Ketoisocaproate
KO	Knockout
MMP	Mitochondrial membrane potential
MOI	Multiplicity of infection
OCR	Oxygen consumption rate
PGC-1α	Peroxisome proliferator-activated receptor-γ coactivator-1α
PH	Pleckstrin homology
ROS	Reactive oxygen species
SAME	Succinic acid methyl ester
sh	Short hairpin
TFAM	Mitochondrial transcription factor A
WT	Wild-type

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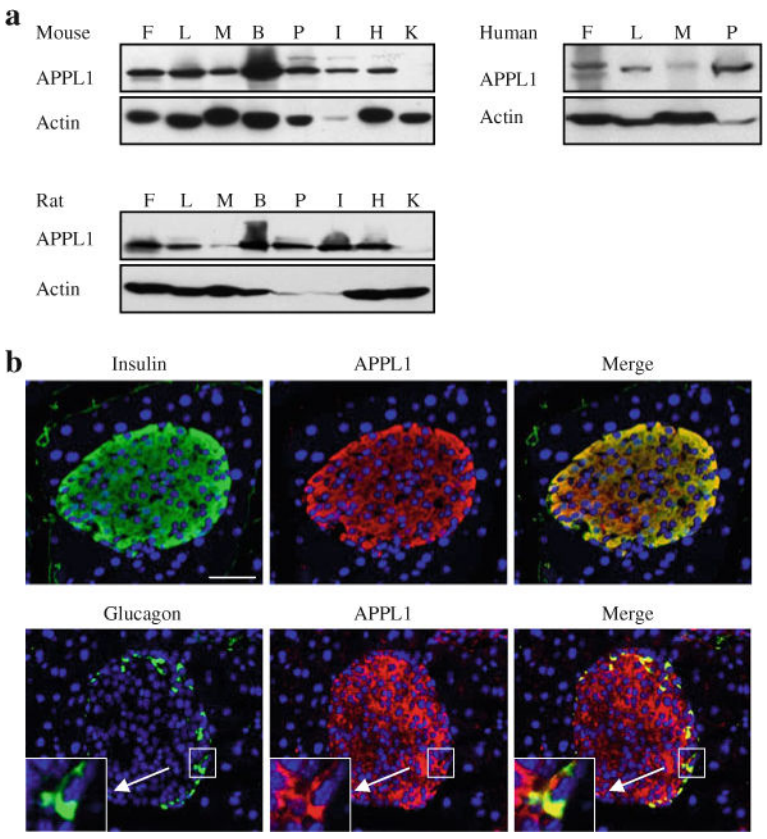

Fig. 1.

APPL1 is highly expressed in pancreatic islets. **(a)** APPL1 protein level in mouse, rat and human tissues. F, fat; L, liver; M, muscle; B, brain; P, pancreas; I, islets; H, heart; K, kidney. The data are representative of three independent experiments. **(b)** Co-localisation of APPL1 with insulin in mouse islets. Representative micrographs of mouse pancreatic sections from male mice dual-stained for insulin or glucagon in green and APPL1 in red (scale bar, 50 μm). The inserts are higher magnification images of the areas indicated

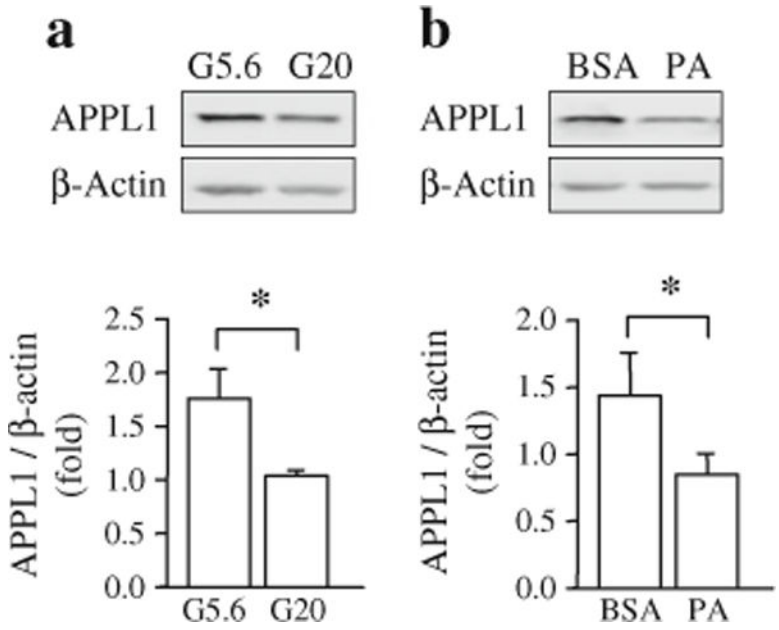

Fig. 2.

APPL1 levels decrease in the presence of high levels of glucose and palmitate (PA). NS-1(832/13) cells were treated with **(a)** 5.6 mmol/l glucose (G5.6), 20 mmol/l (G20) or **(b)** 0.5% BSA in the presence or absence of 1 mmol/l PA. After 48 h, APPL1 levels were detected by western blotting. Quantification of APPL1 levels was normalised to β-actin. $^{*}p<0.05$ between indicated groups, $n=5–7$

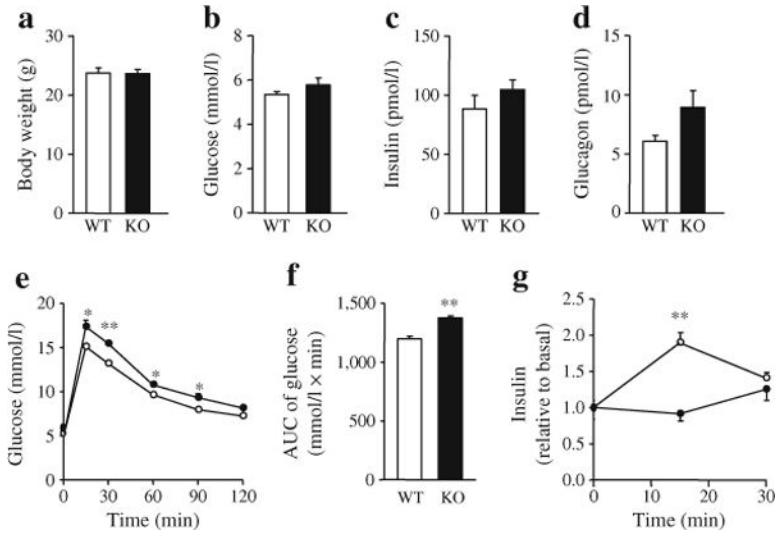

Fig. 3.

APPL1 deficiency results in impaired glucose tolerance. **(a)** Body weight, **(b)** fasting blood glucose, **(c)** fasting insulin levels, **(d)** fasting glucagon levels, **(e)** GTT curve, **(f)** AUC of GTT, and **(g)** insulin secretion test from male WT and APPL1 KO mice at age 9–10 weeks. $^{*}p<0.05$, $^{**}p<0.01$ compared with WT, n=4–5. White symbols, WT mice; black symbols, KO mice

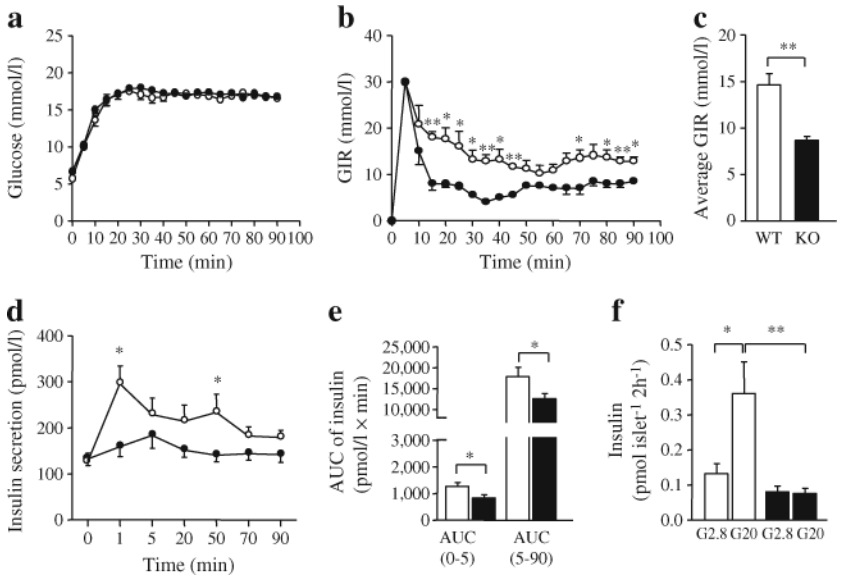

Fig. 4.

APPL1 deficiency reduced insulin secretion. Hyperglycaemic clamp tests were conducted in male WTand APPL1 KO mice at age 10–12 weeks. **(a)** Blood glucose level, **(b)** GIR, and **(c)** average GIR during clamps. **(d)** Insulin levels and **(e)** AUC of insulin levels during clamps. $^{*}p<0.05$, $^{**}p<0.01$ compared with KO or indicated groups, n=7–8. **(f)** Insulin release from islets isolated from WT and KO mice incubated at either 2.8 mmol/l glucose (G2.8) or 20 mmol/l glucose (G20) for 2 h. $^{*}p<0.05$, $^{**}p<0.01$ between the groups indicated, n=12. White symbols, WT mice; black symbols, KO mice

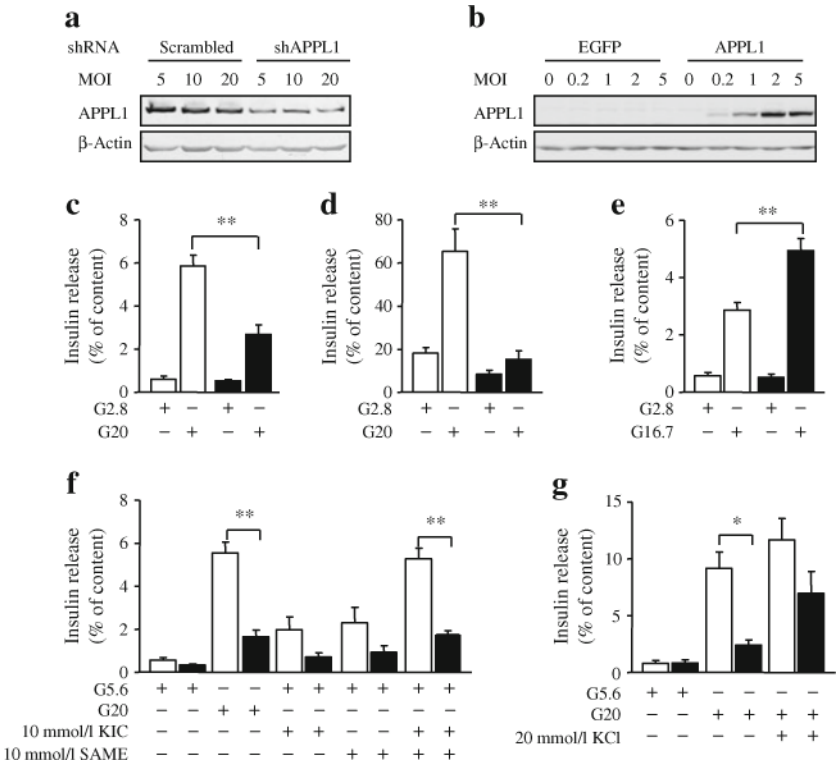

Fig. 5.

Effect of APPL1 on insulin release from INS-1(832/13) cells. INS-1(832/13) cells infected with either adenovirus encoding scrambled shRNA and *Appl1* shRNA (shAPPL1) **(a, c, d, f, g)**, or EGFP and APPL1 **(b, e)**. At 40–42 h post-infection, the cells were either investigated for APPL1 content **(a, b)**, or treated with a different concentration of glucose in the presence or absence of the indicated stimuli for another 2 h **(c, e, f, g)** or 24 h **(d)**. $^{*}p<0.05$, $^{**}p<0.01$ between the groups indicated, $n=4–6$. White bars, scrambled or EGFP; black bars, shAPPL1 or APPL1. G2.8, 2.8 mmol/l glucose; G5.6, 5.6 mmol/l glucose; G16.7, 16.7 mmol/l glucose; G20, 20 mmol/l glucose; KIC, alpha-ketoisocaproate; MOI, multiplicity of infection; SAME, succinic acid methyl ester

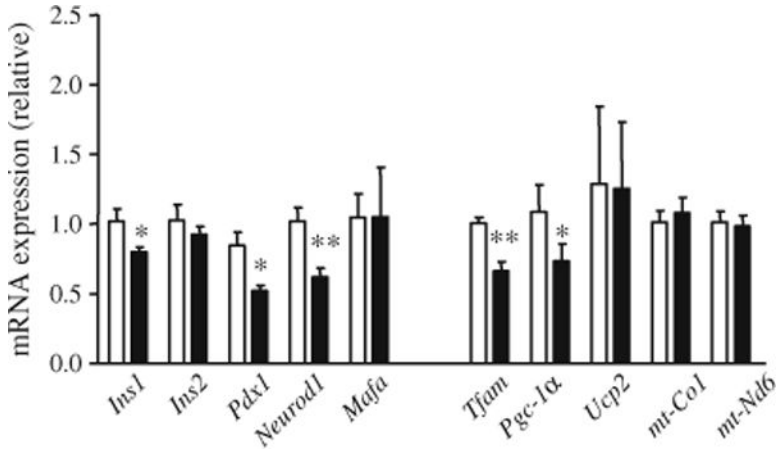

Fig. 6.

Effect of APPL1 on gene expression. INS-1(832/13) cells were infected with adenovirus encoding scrambled shRNA or *Appl1* shRNA (shAPPL1). At 42 h post-infection, real-time PCR analysis was performed and mRNA expression was normalised with respect to Gapdh. $^{*}p<0.05$, $^{**}p<0.01$ compared with scrambled, $n=5$. White bars, scrambled; black bars, shAPPL1

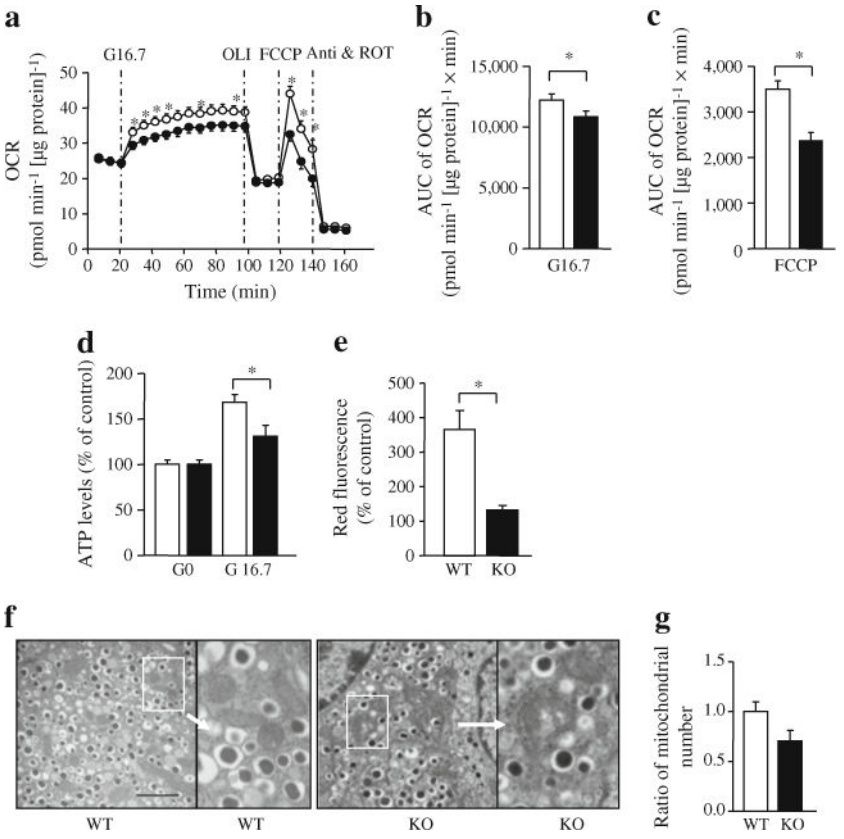

Fig. 7.

Downregulation of APPL1 lowers mitochondrial function in beta cells. INS-1(832/13) cells were infected with adenovirus encoding scrambled shRNA or *Appl1* shRNA (shAPPL1) **(a–d). (a)** OCR measured using an Extracellular Flux Analyzer XF24. After three assay cycles at 2.8 mmol/l glucose, the INS-1(832/13) cells were exposed to 16.7 mmol/l glucose (G16.7) and 1 µmol/l oligomycin (OLI), 2 µmol/l FCCP and a mixture of 1 µmol/l antimycin A (Anti) and rotenone (ROT). AUC of OCR in the presence of G16.7 **(b)** and FCCP **(c). (d)** Intracellular ATP levels in INS-1(832/13) cells in presence or absence of 16.7 mmol/l glucose for 30 min. **(e)** MMP of islets from WT and APPL1 KO male mice in the presence of 20 mmol/l glucose for 30 min. $^{*}p<0.05$ compared with shAPPL1 or between the groups indicated, $n=5–6$. **(f)** Electron micrographs of islets from 12-week-old WT mice and KO mice, with magnified inserts. Mitochondria in beta cells from KO mice were swollen, with disordered cristae, compared with those from WT controls; scale bar 2 µm. **(g)** Mitochondrial number in the beta cells in WT and KO electron microscopic sections. White symbols, scrambled or WT; black symbols, APPL1 shRNA or KO

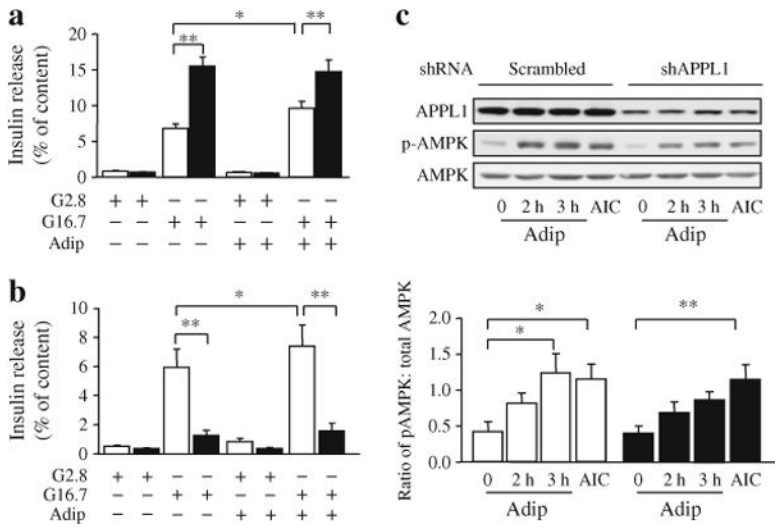

Fig. 8.

Effects of APPL1 on adiponectin- or glucose-stimulated insulin secretion and AMPK phosphorylation. **(a)** INS-1(832/13) cells were infected with adenovirus encoding EGFP as a control or encoding APPL1. **(b, c)** INS-1(832/13) cells were infected with adenovirus encoding scrambled shRNA or *Appl1* shRNA (shAPPL1). At 40–42 h post-infection, the cells were assayed for insulin release in the presence or absence of 2.5 μg/ml adiponectin (Adip) in either 2.8 mmol/l glucose (G2.8) or 16.7 mmol/l glucose (G16.7) **(a, b)**, or assayed for APPL1, p-AMPK and AMPK content **(c)**. Quantification of AMPK phosphorylation levels was normalised to total AMPK **(c)**. $^{*}p<0.05$, $^{**}p<0.01$ between the groups indicated, n=5–8. White bars, scrambled or EGFP; black bars, shAPPL1 or APPL1. AIC, 5-aminoimidazole-4-carboxamide riboside