

Adiponectin induces insulin secretion in vitro and in vivo at a low glucose concentration

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Abstract

Aims/hypothesis A decrease in plasma adiponectin levels has been shown to contribute to the development of diabetes. However, it remains uncertain whether adiponectin plays a role in the regulation of insulin secretion. In this study, we investigated whether adiponectin may be involved in the regulation of insulin secretion in vivo and in vitro.

Methods The effect of adiponectin on insulin secretion was measured in vitro and in vivo, along with the effects of adiponectin on ATP generation, membrane potentials, Ca^{2+} currents, cytosolic calcium concentration and state of 5'-AMP-activated protein kinase (AMPK). In addition, insulin granule transport was measured by membrane capacitance and total internal reflection fluorescence (TIRF) analysis.

Results Adiponectin significantly stimulated insulin secretion from pancreatic islets to approximately 2.3-fold the baseline value in the presence of a glucose concentration of 5.6 mmol/l. Although adiponectin had no effect on ATP generation, membrane potentials, Ca^{2+} currents, cytosolic calcium concentrations or activation status of AMPK, it caused a significant increase of membrane capacitance to approximately 2.3-fold the baseline value. TIRF analysis revealed that adiponectin induced a significant increase in the number of fusion events in mouse pancreatic beta cells under 5.6 mmol/l glucose loading, without affecting the status of previously docked granules. Moreover, intravenous injection of adiponectin significantly increased insulin secretion to approximately 1.6-fold of baseline in C57BL/6 mice.

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Conclusions/interpretation The above results indicate that adiponectin induces insulin secretion in vitro and in vivo.

Keywords Adiponectin · Beta cell · Capacitance · Fusion events · Insulin granules · Insulin secretion · Islet

Abbreviations

AICAR	5-amino-imidazole-4-carboxamide riboside
AMPK	5'-AMP-activated protein kinase
[Ca ²⁺] _c	cytosolic Ca ²⁺ concentration
GFP	green fluorescent protein
LPS	lipopolysaccharide
TIRF	total internal reflection fluorescence

Introduction

The adipocyte-derived hormone adiponectin (also known as Acrp30, GBP28 or AdipoQ) [1–4] has been shown to play important roles in the regulation of glucose and lipid metabolism. Plasma adiponectin levels are reduced in obese and insulin-resistant humans and in animal models [2, 5, 6]. Adiponectin improves insulin sensitivity in muscle and liver by enhancing fatty acid oxidation via activation of 5'-AMP-activated protein kinase (AMPK) [7, 8] and peroxisome proliferator-activated receptor α [6, 9].

We previously demonstrated that adiponectin-deficient (*Adipo*^{-/-}) mice exhibit insulin resistance [10], but that after glucose loading the plasma insulin levels tended to be lower in *Adipo*^{-/-} mice than in wild-type mice, suggesting that adiponectin may induce insulin secretion [10]. The adiponectin receptors ADIPOR1 and ADIPOR2 have recently been cloned [11] and identified in human and rat pancreatic beta cells [12]. Expression of the adiponectin receptors by INS-1 cells (a clonal rat beta cell line) has been found to increase following exposure to oleic acid, an unsaturated NEFA. Moreover, a previous study has suggested that adiponectin also exerts anti-apoptotic actions and that this protective function of adiponectin might serve to counteract autoimmune- and lipotoxicity-induced beta cell destruction [13]. However, it is still uncertain whether adiponectin plays a role in the regulation of insulin secretion. In this study we investigated whether adiponectin might be involved in the regulation of insulin secretion in vitro and in vivo.

Methods

Animals C57BL/6 mice were obtained from CLEA Japan (Tokyo, Japan). Male C57BL/6 mice (10 to 16 weeks old) were housed under a 12 h light–dark cycle and given free

access to food. The animal care and experimental procedures were approved by the Animal Care Committee of the University of Tokyo.

RNA preparation and real-time quantitative PCR Total RNA was extracted from isolated islets using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. cDNA synthesis was performed using the SuperScript Preamplification System (Invitrogen), followed by TaqMan quantitative PCR (Applied Biosystems, Foster City, CA, USA; 50°C for 2 min and 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min) with an ABI Prism 7000 PCR instrument (Applied Biosystems) to amplify samples of the *Adipor1*, *Adipor2* and β -actin genes. The sequences of the primers and probes are described elsewhere [11].

Insulin secretion by islets Islets were isolated from 10- to 16-week-old C57BL/6 mice as described elsewhere [14]. In brief, after clamping the common bile duct at a point close to the duodenal outlet, 2.5 ml KRB (129 mmol/l NaCl, 4.8 mmol/l KCl, 1.2 mmol/l MgSO₄, 1.2 mmol/l KH₂PO₄, 2.5 mmol/l CaCl₂, 5 mmol/l NaHCO₃ and 10 mmol/l HEPES; pH 7.4) containing 4 mg/ml collagenase (Sigma, St Louis, MO, USA) was injected into the duct. Insulin release by the pancreatic islets was measured by static incubation with KRB containing 0.2% (wt/vol.) bovine serum albumin [15]. In the static incubation, batches of ten freshly isolated islets were preincubated at 37°C for 30 min in KRB containing 2.8 mmol/l glucose. The preincubation solutions were replaced with KRB containing test agents and the batches of islets were incubated at 37°C for 60 min. The insulin released in the supernatant fractions was then measured by radioimmunoassay (Biotrak; GE Healthcare, Chalfont St Giles, UK).

Generation of recombinant adiponectin Bacterially expressed murine full-length adiponectin was generated as described previously [7, 11]. The endotoxin content of the purified protein was determined using a Limulus Amebocyte Lysate Assay (Bio Whittaker, Walkersville, MD, USA). Mammalian adiponectin was purchased from Alexis Biochemicals (San Diego, CA, USA).

Glucose oxidation and fatty acid oxidation Glucose oxidation in the islets was evaluated by measuring the ¹⁴CO₂ generation from D-[6-¹⁴C]glucose [16]. Batches of ten freshly isolated islets were incubated at 37°C for 60 min in KRB containing 29.6 kBq D-[6-¹⁴C]glucose and 5.6 or 22.2 mmol/l glucose with and without 10 μ g/ml adiponectin. For fatty acid oxidation, batches of ten freshly isolated islets were incubated at 37°C for 60 min in KRB containing 25.9 kBq [U-¹⁴C]palmitic acid, 1 mmol/l carnitine, and 5.6 or

22.2 mmol/l glucose with and without 10 µg/ml adiponectin. The $^{14}\text{CO}_2$ produced was volatilised by adding HCl, captured with Solvable (Packard Instrument Company, Meriden, CT, USA) and measured by liquid scintillation counting.

ATP and cAMP content The ATP and cAMP contents of the islets were determined as described previously [17]. Briefly, batches of ten islets were incubated at 37°C for 60 min in KRB containing 5.6 mmol/l glucose with and without 10 µg/ml adiponectin. Incubation was stopped by the addition of ice-cold HClO_4 and the islets were homogenised by sonication. The lysates were neutralised by the addition of NaOH. The ATP content and cAMP content of the supernatant fraction were measured using a bioluminescent assay kit (Sigma) and an enzyme-linked immunoassay kit (GE Healthcare), respectively.

Electrical recordings Electrophysiological experiments on the islets were performed using cells in situ in intact pancreatic islets. The islets were washed extensively in collagenase-free solution and then maintained in a short-term tissue culture (<24 h) in RPMI 1640 containing 10% (vol./vol.) fetal calf serum supplemented with 100 µg/ml streptomycin and 100 IU/ml penicillin.

To establish the whole-cell mode, the amphotericin B-perforated patch-clamp technique was used. The membrane potential and the current of superficial cells in the intact pancreatic islets were recorded using an EPC-9 patch-clamp amplifier (HEKA Electronics, Lambrecht/Pfalz, Germany). The beta cells were identified by the lack of inward Na^+ currents in the presence of a depolarising pulse from -70 to 0 mV (5 ms in duration) [18]. The capacitance measurements were performed using software-based lock-in software (Pulse version 8.11; HEKA Electronics). The changes in cell capacitance were estimated by the Lindau–Neher technique [19, 20] by implementing the ‘Sine + DC’ feature of the lock-in module. The amplitude of the sine wave was 20 mV and the frequency was set at 1250 Hz. Patch pipettes (tip resistance 5–7 MΩ when filled with the pipette solution) were pulled from borosilicate tubing. The capacitance measurements were performed at 32–34°C. Capacitance was measured again 2 min 30 s after treatment with or without adiponectin. The standard extracellular medium consisted of 120 mmol/l NaCl, 20 mmol/l tetraethylammonium-Cl, 3.6 mmol/l KCl, 2 mmol/l NaHCO_3 , 0.5 mmol/l NaH_2PO_4 , 0.5 mmol/l MgSO_4 , 5 mmol/l HEPES (at pH 7.4), 2.6 mmol/l CaCl_2 and 5 mmol/l D-glucose. The pipette solution for the perforated patch was composed of 76 mmol/l Cs_2SO_4 , 10 mmol/l NaCl, 10 mmol/l KCl, 1 mmol/l MgCl_2 and 5 mmol/l HEPES (at pH 7.35).

The cytosolic Ca^{2+} concentration ($[\text{Ca}^{2+}]_c$) was measured using fura-2 by exciting its fluorescence in a dual-wavelength ratiometric mode at 340 and 380 nm. The

emission wavelength was filtered at 500 nm. $[\text{Ca}^{2+}]_c$ was expressed as the 340:380 nm ratio.

Western blot analysis Mouse islets were homogenised in ice-cold buffer A (25 mmol/l Tris-HCl; pH 7.4, 10 mmol/l sodium orthovanadate, 10 mmol/l sodium pyrophosphate, 100 mmol/l sodium fluoride, 10 mmol/l EDTA, 10 mmol/l EGTA and 1 mmol/l phenylmethylsulfonyl fluoride). Samples were separated on polyacrylamide gels and transferred to a nitrocellulose membrane (Schleicher & Schuell, Dassel, Germany). Rabbit anti-phospho-AMPKα (Thr-172) antibody and rabbit AMPKα antibody were purchased from Cell Signaling (Beverly, MA, USA).

Total internal reflection fluorescence microscopy We performed total internal reflection fluorescence (TIRF) microscopy using an inverted microscope (IX70; Olympus, Tokyo, Japan) with a high-aperture objective lens (Apo 100× OHR, Na 1.65; Olympus) as described previously [21, 22]. Mouse pancreatic islets were isolated by collagenase digestion. Isolated islets were dissociated into single cells by incubation in Ca^{2+} -free KRB containing 1 mmol/l EGTA and the beta cells were cultured on high-refractive-index glass (Olympus) coated with fibronectin (Koken, Tokyo, Japan). To label the insulin-containing granules, pancreatic beta cells were infected with recombinant adenovirus Adex1CA insulin green fluorescent protein (GFP) as described previously [22]. The experiments were performed 2 days after infection. To observe GFP, we used a 488 nm laser line for excitation. The procedure used to monitor the motion of GFP-labelled insulin granules in pancreatic beta cells by TIRF microscopy has been described elsewhere [21]. Infected cells on a glass coverslip (Olympus) were mounted in an open chamber and incubated for 30 min at 37°C in KRB and 0.3% (wt/vol.) bovine serum albumin in the presence of 2.8 mmol/l glucose. The cells were then transferred to a thermostatically controlled stage (37°C) and stimulated with 10 µg/ml adiponectin in the presence of 5.6 mmol/l glucose in the chamber. Diodomethane sulphur immersion oil (Cargille Laboratories, Cedar Grove, NJ, USA) was used to establish contact between the objective lens and the coverslip. The space constant for the exponential decay of the evanescent field was approximately 43 nm.

Image acquisition and analysis Images were acquired every 300 ms with a cooled charge-coupled-device camera (DV887DCSBV; Andor Technology, South Windsor, CT, USA) operated with Metamorph 6.2 software (Universal Imaging, Downingtown, PA, USA). Most analyses, including tracking (single projection of difference images) and area calculations, were performed with the Metamorph software. To analyse the data, fusion events were selected

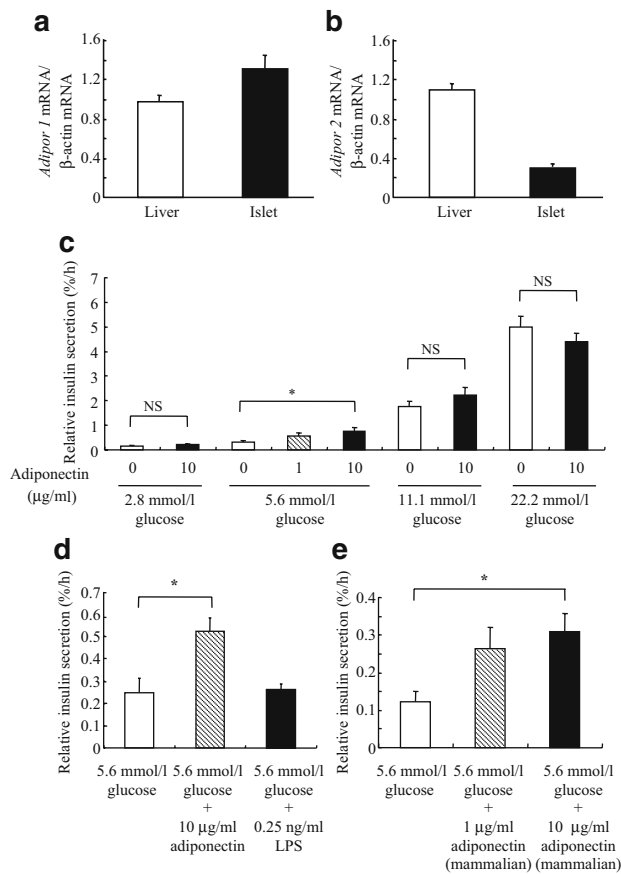


Fig. 1 Adiponectin stimulates insulin secretion from islets. TaqMan RT-PCR of *Adipor1* ($n=3$) (a) and *Adipor2* ($n=3$) (b) in mouse pancreatic islets. c Islets were incubated in KRB containing the indicated concentrations of glucose with or without adiponectin ($n=9-20$). d Islets were incubated with glucose and with adiponectin or LPS ($n=3$). e Islets were incubated with glucose with or without mammalian-derived adiponectin ($n=3$). Values are means \pm SEM. * $p<0.05$

manually and the average fluorescence intensity of the individual granules in a $1\times 1\ \mu\text{m}$ square placed over the granule centre was calculated. The number of fusion events was counted manually while looping approximately 15,000 time-lapsed frames. The sequences were exported as single TIRF files and further processed using Adobe Photoshop 6.0 software or converted into Quick Time movies.

Insulin secretion study in vivo Four days before the insulin secretion study, a catheter consisting of a silicone part (Phicon Tube; Fuji Systems, Tokyo, Japan) and a polyethylene part (PE-50; BD Biosciences, Franklin Lakes, NJ, USA) was inserted into the right jugular vein of animals under general anaesthesia with sodium pentobarbital, to administer infusions. The studies were performed on the mice under conscious, unstressed conditions after 24 h fast. D-Glucose (0.2 g/kg) was injected intravenously through the catheter, either alone or with 0.6 mg/kg adiponectin. Blood samples were collected into a heparinised tube before and 10 and

20 min after the injection. After immediate centrifugation, the plasma was separated and stored at $-20\ ^\circ\text{C}$ until analysed.

Insulin levels were determined by radioimmunoassay. The insulin to glucose ratio was calculated using the formulas: (10 min insulin level–fasting insulin level)/(10 min blood glucose level–fasting blood glucose) or (20 min insulin level–fasting insulin level)/(20 min blood glucose level–fasting blood glucose level). The adiponectin levels were determined with a mouse adiponectin ELISA kit (Otsuka, Tokyo, Japan). All data were obtained from six independent experiments.

Statistical analysis The statistical significance of differences between groups was determined using Student's *t* test for unpaired comparisons, the Welch test, Dunnett's *t* test or Steel's *z* test. A *p* value of <0.05 was regarded as significant.

Results

Adiponectin stimulates insulin secretion from mouse pancreatic islets at low glucose concentration We first confirmed the expression of *Adipor1* and *Adipor2* in mouse pancreatic islets (Fig. 1a,b), obtaining results that were essentially consistent with those previously reported [12]. We next examined the effect of adiponectin on glucose-induced insulin secretion from isolated islets during a 60 min static incubation (Fig. 1c). Adiponectin significantly stimulated insulin secretion to 2.3-fold the baseline value at a 5.6 mmol/l glucose concentration ($p=0.012$; Fig. 1c). However, adiponectin did not significantly increase insulin secretion in the presence of 11.1 or 22.2 mmol/l glucose. Since high concentrations of lipopolysaccharide (LPS) have been reported to stimulate insulin secretion from islets [23], we investigated the effect of LPS on insulin secretion from islets at 5.6 mmol/l glucose. When mouse pancreatic islets were treated with LPS at the same quantity as was present in recombinant adiponectin derived from *Escherichia coli*, no effect of LPS was found on insulin secretion (Fig. 1d). In addition, 10 $\mu\text{g/ml}$ mammalian-derived adiponectin also stimulated insulin secretion from mouse pancreatic islets ($p=0.039$; Fig. 1e). These results suggest that adiponectin acts directly on beta cells to increase insulin secretion.

Adiponectin stimulates insulin secretion without causing ATP generation, palmitic acid oxidation or cAMP generation in islets To examine the effect of adiponectin on ATP generation, we measured glucose-induced changes in D-[6- ^{14}C] glucose oxidation and the ATP content of islets (Table 1). D-[6- ^{14}C]Glucose oxidation to $^{14}\text{CO}_2$ in the control islets increased 5.5-fold when the extracellular glucose concentration was raised from 5.6 to 22.2 mmol/l. Adiponectin at a concentration of 10 $\mu\text{g/ml}$ did not affect D-[6- ^{14}C]glucose oxidation in the presence of 5.6 mmol/l of glucose

compared with control. In the presence of 22.2 mmol/l glucose, adiponectin also failed to significantly change D-[6-¹⁴C]glucose oxidation compared with control islets incubated at the same glucose concentration. Next, we measured the ATP content of islets to directly monitor the efficiency of mitochondrial ATP synthesis. The results showed that adiponectin at a concentration of 10 µg/ml did not affect ATP content in the presence of 5.6 mmol/l glucose (Table 1). The above findings indicate that adiponectin has no stimulatory effect on ATP generation from mitochondrial glucose metabolism. Increase in cAMP content potentiates glucose-stimulated insulin secretion through activation of cAMP-dependent protein kinase [24]. Adiponectin also failed to significantly change the cAMP content as compared with control islets (Table 1). Although adiponectin has been reported to reduce elevated fatty acid levels in muscle by oxidising fatty acids [7–9], no significant change in the oxidation of palmitic acid was observed in pancreas islets treated with adiponectin as compared with control at 5.6 or 22.2 mmol/l of glucose (Table 1).

Adiponectin stimulates insulin secretion without causing membrane depolarisation, closure of K_{ATP}^+ channels or Ca^{2+} entry into the cytosol We measured the membrane potentials of pancreatic beta cells to determine whether adiponectin caused membrane depolarisation. In the presence of 5.6 mmol/l glucose, adiponectin at a concentration of 10 µg/ml had no effect on the membrane potentials (Fig. 2a), but membrane of pancreatic beta cells depolarised when the extracellular glucose level was raised from 5.6 to 22.2 mmol/l (Fig. 2a). Figure 2b shows the current–voltage relationship for Ca^{2+} currents. When the membrane potential was held at –70 mV, Ca^{2+} currents elicited by 200 ms depolarising voltage-clamp pulses from –40 to 40 mV were applied in the presence of 5.6 mmol/l glucose. Adiponectin at a concentration of 10 µg/ml did not change the current–voltage curve via Ca^{2+} influx into the cytosol (Fig. 2b). To investigate the effect of adiponectin on the increase in the $[Ca^{2+}]_c$, we monitored the fluorescence of fura-2/acetoxymethyl ester excited at 340 and 380 nm in perifused islets

(Fig. 2c). The 340:380 nm fluorescence ratio increased in islets when the glucose concentration was raised from 5.6 to 22.2 mmol/l, but adiponectin had no effect on the $[Ca^{2+}]_c$ (Fig. 2c). To clarify whether adiponectin stimulates insulin secretion without causing Ca^{2+} influx, we next investigated the effects of adiponectin on mouse pancreatic islets in the presence of the L-type Ca^{2+} -channel blocker, nitrendipine. Nitrendipine at a concentration of 5 µmol/l is known to inhibit Ca^{2+} influx [25]. Nevertheless, insulin secretion was markedly diminished at 22.2 mmol/l glucose in the presence of nitrendipine, whereas adiponectin significantly stimulated insulin secretion at 5.6 mmol/l glucose ($p=0.043$; Fig. 2d). Interestingly, adiponectin also significantly stimulated insulin secretion at 22.2 mmol/l glucose concentration in the presence of nitrendipine ($p=0.047$) (Fig. 2d). These findings suggest that adiponectin is able to stimulate insulin secretion when the influx of Ca^{2+} through voltage-dependent Ca^{2+} channels is blocked.

AMPK is not involved in adiponectin-stimulated insulin secretion Adiponectin has been previously reported to improve insulin sensitivity in muscle and liver by enhancing fatty acid oxidation via the activation of AMPK [7, 8], which has been found to increase glucose transport by stimulating the translocation of GLUT4 [26, 27]. To elucidate whether AMPK might be involved in adiponectin-stimulated insulin secretion, we examined the effect of adiponectin on phosphorylation of AMPK in mouse pancreatic islets. However, adiponectin did not affect the phosphorylation of AMPK at 5.6 mmol/l glucose (Fig. 3a). The AMPK activator 5-amino-imidazole-4-carboxamide riboside (AICAR), on the other hand, significantly increased the phosphorylation of AMPK at 5.6 mmol/l glucose ($p=0.00083$; Fig. 3b). However, AICAR significantly decreased insulin secretion from mouse pancreatic islets ($p=0.024$; Fig. 3c) [28]. These results suggest that AMPK is not involved in adiponectin-stimulated insulin secretion at low glucose concentrations.

Adiponectin stimulates insulin release via induction of increased fusion events at a low glucose concentration We

Table 1 Adiponectin stimulates insulin secretion without causing ATP generation, palmitic acid oxidation or cAMP generation in islets

	D-[6- ¹⁴ C]Glucose oxidation (pmol h ⁻¹ islet ⁻¹)		ATP content (pmol/islet)	[U- ¹⁴ C]Palmitic acid oxidation (fmol h ⁻¹ islet ⁻¹)		cAMP content (fmol/islet)
Glucose (mmol/l)	5.6	22.2	5.6	5.6	22.2	5.6
Control	1.92±0.27	10.6±1.1 ^b	1.16±0.09	2.98±0.20	2.41±0.07 ^a	49±7
<i>n</i>	4	4	5	4	4	5
Adiponectin	2.37±0.22	11.7±1.8 ^b	1.29±0.08	3.23±0.23	2.59±0.10 ^a	62±10
<i>n</i>	4	4	5	4	4	5

Values are means±SEM.

^a $p<0.05$, ^b $p<0.01$ compared with the 5.6 mmol/l glucose group

then examined the capacitance of mouse pancreatic beta cells. The technique used is characterised by high time resolution and thus can detect dynamic changes in the cell surface area that reflect exocytosis. Figure 4a shows the capacitance of mouse pancreatic beta cells. Interestingly, adiponectin at a concentration of 10 $\mu\text{g/ml}$ significantly increased the capacitance of mouse pancreatic beta cells to 2.3-fold above baseline ($p=0.0091$; Fig. 4a), indicating that adiponectin may stimulate insulin granule exocytosis in the absence of any changes in membrane depolarisation, Ca^{2+} currents or $[\text{Ca}^{2+}]_i$.

To elucidate the mechanism by which adiponectin increased the exocytosis of insulin-containing granules, we used TIRF microscopy to monitor the real-time docking and fusion process of single insulin granules labelled with a GFP-tagged insulin near the plasma membrane in mouse pancreatic beta cells. Interestingly, adiponectin significantly increased the number of fusion events by newcomers (40.5 ± 4.1 per $200 \mu\text{m}^2$ in 0–30 min control beta cells vs 72.3 ± 6.5 per $200 \mu\text{m}^2$ in 0–30 min adiponectin-treated beta cells, $p < 0.0005$, $n=45$ for control and $n=52$ for adiponectin-treated cells; Fig. 4b,c and Electronic supplementary material [ESM], video clips), suggesting that adiponectin may accelerate transport of insulin granules from a cytoplasmic pool to the plasma membrane.

Adiponectin stimulates insulin secretion in vivo To elucidate the physiological roles of adiponectin in insulin secretion in vivo, we studied the effect of adiponectin on insulin secretion in C57BL/6 mice after intravenous transcatheter injection of adiponectin and a low concentration of glucose. We tried to adjust the blood glucose level to approximately 5.6 mmol/l. Figure 5a shows the plasma adiponectin level at 10 and 20 min after the intravenous injection of glucose and saline or adiponectin. The plasma adiponectin levels increased significantly to 1.8 fold of baseline following the injection of adiponectin, while the increased blood glucose levels at 10 and 20 min after the injection were not significantly different between the two groups (Fig. 5b). The blood glucose level and insulin level at the starting point were 3.7 ± 0.4 mmol/l and 36.2 ± 5.0 pmol/l, respectively. No significant differences in the increased plasma insulin levels at 10 min after administration were observed between the adiponectin and the saline groups (Fig. 5c). However, the increase in plasma insulin levels at 20 min after the intravenous injection of glucose was approximately 1.6-fold greater in the adiponectin group ($n=19$) than in the saline group ($n=21$; $p=0.048$; Fig. 5c). We evaluated the changes in ratio of the increment of plasma insulin level to that of blood glucose level. At 20 min after glucose loading the ratio in the adiponectin group was approximately sevenfold higher than that in the saline group ($p=0.0088$) (Fig. 5d). These results suggest that adiponectin stimulates insulin secretion in vivo.

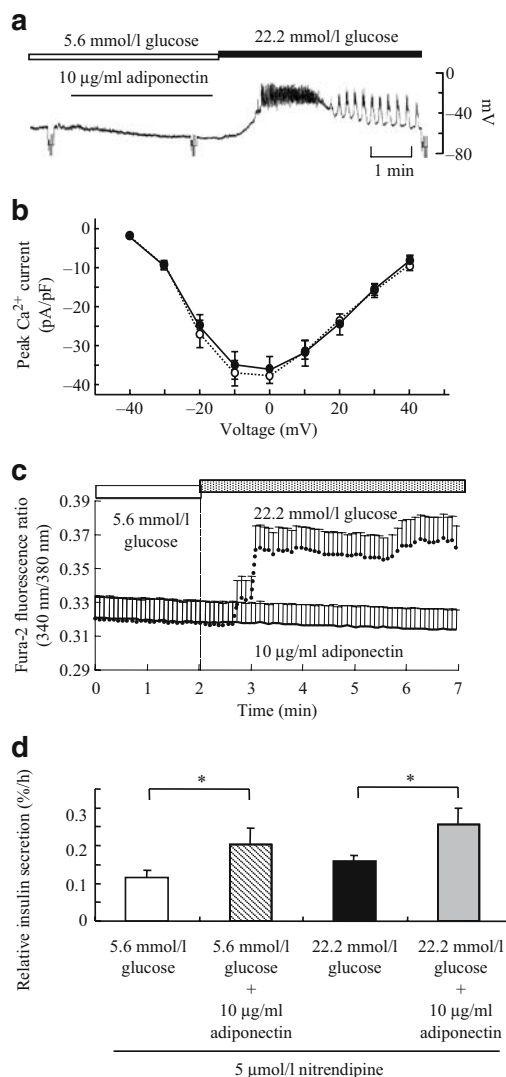


Fig. 2 Adiponectin stimulates insulin secretion without causing membrane depolarisation, closure of K_{ATP}^+ channels or Ca^{2+} entry into the cytosol. **a** Membrane potentials in response to the addition of adiponectin and increasing concentrations of glucose (5.6–22.2 mmol/l) were recorded under the conventional whole-cell mode. Results are representative of three independent experiments. **b** The current–voltage relationships of beta cells treated with (black circles, $n=3$) or without (white circles, $n=3$) adiponectin (10 $\mu\text{g/ml}$). **c** Cytosolic calcium concentrations in response to the addition of adiponectin and increasing concentrations of glucose were measured using fura-2. Mean values are shown ($n=5$). **d** Islets were incubated in KRB containing the indicated concentrations of glucose and 5 $\mu\text{mol/l}$ nitrendipine with or without 10 $\mu\text{g/ml}$ adiponectin ($n=18$ –23). Values are means \pm SEM. * $p < 0.05$

Discussion

In this study we have shown that adiponectin stimulates insulin secretion in vitro and in vivo. In addition, our results also indicate that adiponectin stimulates insulin secretion without causing ATP generation, closure of K_{ATP}^+ channels, Ca^{2+} entry into the cytosol or activation of AMPK.

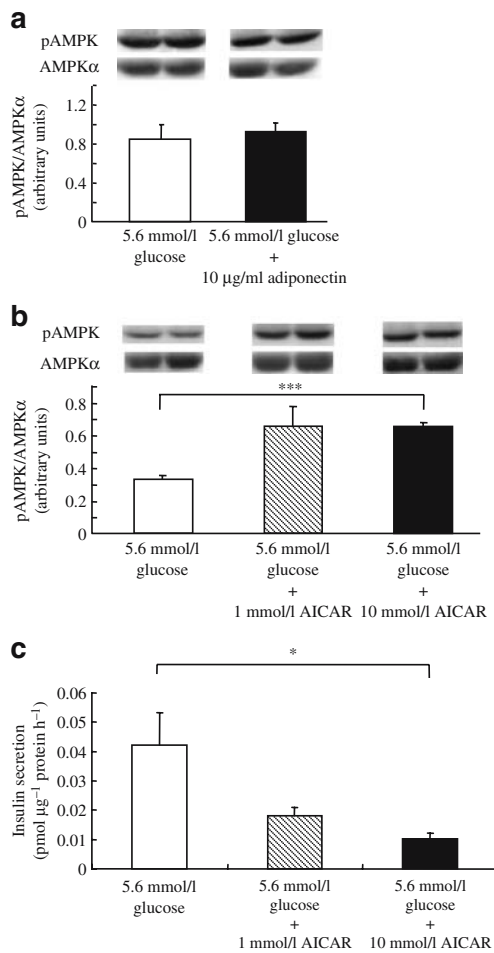


Fig. 3 Adiponectin stimulates insulin secretion without causing activation of AMPK. **a, b** Western blots of phosphorylated AMPK and AMPK α in mouse pancreatic islets ($n=3$, each group). Lysates from 30 islets from each group were subjected to western blot analysis. **c** Isolated islets were incubated with AICAR at 5.6 mmol/l glucose. Values are means \pm SEM. * $p<0.05$, *** $p<0.001$

Adiponectin has previously been reported to increase fatty acid oxidation via activation of AMPK [7, 8], which has been found to increase glucose transport by stimulating the translocation of GLUT4 to the sarcolemma in heart [26] and skeletal muscle [27]. However, adiponectin did not affect the phosphorylation of AMPK in pancreatic islets at 5.6 mmol/l glucose. Since AMPK in pancreatic beta cells is activated by low glucose concentrations [28], adiponectin may be unable to further activate AMPK at this low glucose concentration. By contrast, although the AMPK activator AICAR activated AMPK at this low glucose concentration, AICAR also decreased insulin secretion under these conditions. These results indicate that AMPK is not involved in adiponectin-stimulated insulin secretion at a low glucose concentration.

This study has shown that adiponectin stimulated insulin secretion without causing Ca²⁺ entry into the cytosol. The following cascade is generally accepted to be involved in

glucose-induced insulin secretion. When glucose is metabolised in the cytosol and mitochondria, ATP is generated and promotes the closure of ATP-sensitive potassium channels, thereby depolarising the plasma membrane potential. Depolarisation of the plasma membrane leads to activation of voltage-dependent Ca²⁺ channels, Ca²⁺ entry into the cytosol and a rise in [Ca²⁺]_c, which is thought to finally trigger the exocytosis of insulin-containing granules [29–31]. However, a Ca²⁺-independent pathway has also been proposed. Thus, Komatsu et al. have suggested that both Ca²⁺-dependent and Ca²⁺-independent augmentation occurs via a pathway dependent on glucose metabolism [32, 33]. In this context, we confirmed that adiponectin is able to stimulate insulin secretion when the influx of Ca²⁺ through voltage-dependent Ca²⁺ channels is blocked by nitrendipine; in other words, adiponectin stimulates insulin

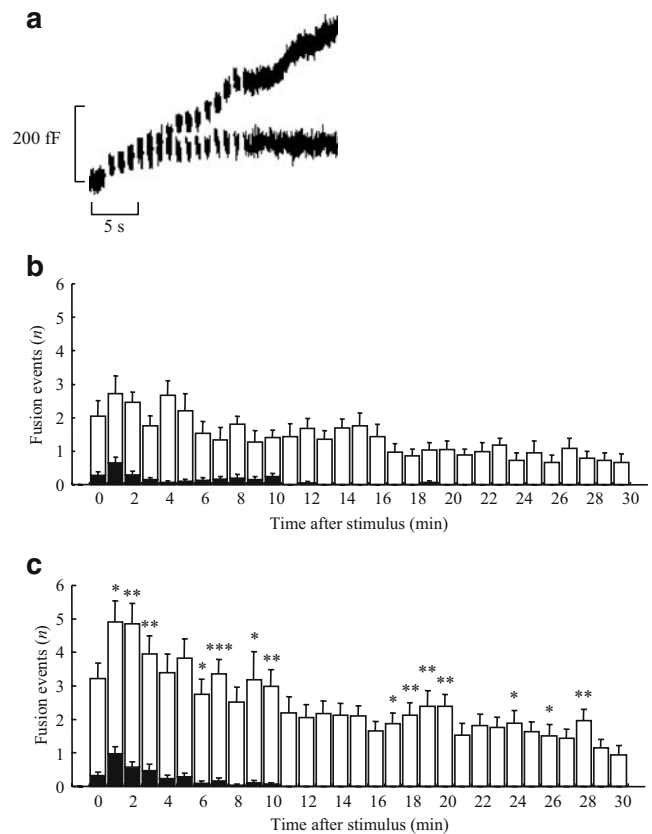


Fig. 4 Adiponectin stimulates insulin release via induction of increased fusion events. **a** The membrane capacitance of mouse beta cells exposed to 5.6 mmol/l glucose alone (lower curve) and 5.6 mmol/l glucose + 10 μ g/ml adiponectin (upper curve) cumulatively increased upon repetitive application of depolarising pulses and then plateaued (glucose alone). The traces shown are representative of three experiments. **b, c** TIRF images were acquired every 300 ms after the addition of adiponectin. The fusion events per 200 μ m² were counted manually. Histograms of the number of fusion events in 5.6 mmol/l glucose in the absence (**b**) ($n=45$) and presence (**c**) ($n=52$) of adiponectin (10 μ g/ml). Black bars, previously docked granules; white bars, newly docked granules. Values are mean \pm SEM. * $p<0.05$, ** $p<0.01$, *** $p<0.005$

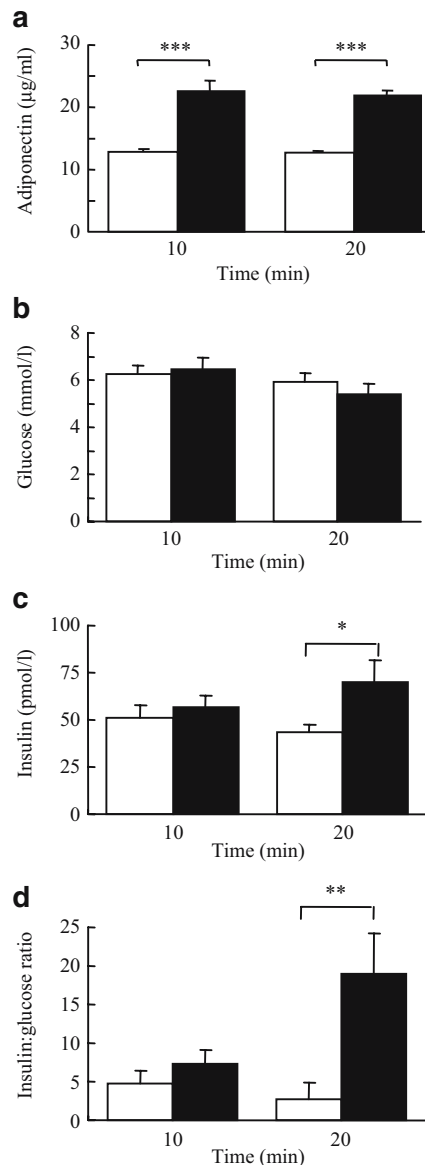


Fig. 5 Adiponectin stimulates insulin secretion in vivo. **a** Plasma adiponectin level after intravenous injection of glucose and saline (white bars, $n=8-21$) or adiponectin (black bars, $n=8-19$). **b** Blood glucose and **(c)** plasma insulin levels after the intravenous injections. **d** Ratio of the increment in plasma insulin level to that of blood glucose level after the intravenous injections. Values are means \pm SEM. * $p<0.05$, ** $p<0.01$, *** $p<0.005$

secretion without requiring influx of Ca^{2+} through voltage-dependent Ca^{2+} channels. It may do this through these augmentation pathways triggered by glucose metabolism [33], namely by replacing part of the Ca^{2+} -dependent/ Ca^{2+} -independent augmentation on newly docked granules. In this regard, it should be noted that adiponectin was able to stimulate insulin secretion at 22.2 mmol/l glucose when the influx of Ca^{2+} was blocked by nitrendipine (Fig. 2d). The observation that this stimulation of insulin secretion by adiponectin is relatively small as compared with that of glucose itself under physiological conditions (Fig. 1c) may

reflect the relatively small role of the Ca^{2+} -independent augmentation pathway, as suggested by Sato et al. [34].

As another possible mechanism, adiponectin may affect remodelling of the cytoskeleton. The cytoskeleton plays a critical role in glucose-stimulated intracellular trafficking and the exocytosis of insulin-containing granules at the plasma membrane of beta cells. In fact, a previous report showed direct evidence of a dynamic interaction between target membrane soluble *N*-ethylmaleimide-sensitive factor attachment protein receptor protein and F-actin during glucose-stimulated insulin secretion in MIN6 cells [35]. It was recently reported that calpain plays a role in facilitating the actin reorganisation required for glucose-stimulated insulin secretion [36]. Another study has suggested that kinesin I plays an important role in the microfilament-dependent movements of insulin containing granules [37]. Adiponectin may cause direct activation of calpain or kinesin I, to influence remodelling of the cytoskeleton.

Conflicting results on the effects of adiponectin on insulin secretory function in vitro have been reported so far. A previous study showed that while adiponectin decreased glucose/forskolin-induced insulin secretion, it reversed NEFA-induced inhibition of insulin secretion [13]. Another group reported that adiponectin had no effect on either basal or stimulated insulin secretion from human islets [38]. Winzell et al. [39] reported that adiponectin decreases insulin secretion from the pancreatic islets of insulin-resistant mice at a 2.8 mmol/l glucose, but stimulates insulin secretion at 16.7 mmol/l glucose. However, there have been no reports on the effects of adiponectin on insulin secretion in vivo. This study is the first study to demonstrate that adiponectin stimulates insulin secretion not only in vitro, but also in vivo.

In conclusion, we have described a novel effect of adiponectin, namely stimulation of insulin secretion, in addition to its known insulin-sensitising and anti-atherogenic effects. Consequently, adiponectin receptor agonists may be useful in the activation of adiponectin signalling as a new therapeutic strategy for treating diabetes and atherogenic diseases.

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

References

1. Scherer PE, Williams S, Fogliano M, Baldini G, Lodish HF (1995) A novel serum protein similar to C1q, produced exclusively in adipocytes. *J Biol Chem* 270:26746–26749
2. Hu E, Liang P, Spiegelman BM (1996) AdipoQ is a novel adipose-specific gene dysregulated in obesity. *J Biol Chem* 271: 10697–10703

3. Maeda K, Okubo K, Shimomura I, Funahashi T, Matsuzawa Y, Matsubara K (1996) cDNA cloning and expression of a novel adipose specific collagen-like factor, apM1 (AdiPose Most abundant Gene transcript 1). *Biochem Biophys Res Commun* 221:286–296
4. Nakano Y, Tobe T, Choi-Miura NH, Mazda T, Tomita M (1996) Isolation and characterization of GBP28, a novel gelatin-binding protein purified from human plasma. *J Biochem (Tokyo)* 120: 803–812
5. Arita Y, Kihara S, Ouchi N et al (1999) Paradoxical decrease of an adipose-specific protein, adiponectin, in obesity. *Biochem Biophys Res Commun* 257:79–83
6. Yamauchi T, Kamon J, Waki H et al (2001) The fat-derived hormone adiponectin reverses insulin resistance associated with both lipotrophy and obesity. *Nat Med* 7:941–946
7. Yamauchi T, Kamon J, Minokoshi Y et al (2002) Adiponectin stimulates glucose utilization and fatty-acid oxidation by activating AMP-activated protein kinase. *Nat Med* 8:1288–1295
8. Tomas E, Tsao TS, Saha AK et al (2002) Enhanced muscle fat oxidation and glucose transport by ACRP30 globular domain: acetyl-CoA carboxylase inhibition and AMP-activated protein kinase activation. *Proc Natl Acad Sci U S A* 99:16309–16313
9. Fruebis J, Tsao TS, Javorschi S et al (2001) Proteolytic cleavage product of 30-kDa adipocyte complement-related protein increases fatty acid oxidation in muscle and causes weight loss in mice. *Proc Natl Acad Sci U S A* 98:2005–2010
10. Kubota N, Terauchi Y, Yamauchi T et al (2002) Disruption of adiponectin causes insulin resistance and neointimal formation. *J Biol Chem* 277:25863–25866
11. Yamauchi T, Kamon J, Ito Y et al (2003) Cloning of adiponectin receptors that mediate antidiabetic metabolic effects. *Nature* 423:762–769
12. Kharroubi I, Rasschaert J, Eizirik DL, Cnop M (2003) Expression of adiponectin receptors in pancreatic beta cells. *Biochem Biophys Res Commun* 312:1118–1122
13. Rakatzi I, Mueller H, Ritzeler O, Tennagels N, Eckel J (2004) Adiponectin counteracts cytokine- and fatty acid-induced apoptosis in the pancreatic beta-cell line INS-1. *Diabetologia* 47:249–258
14. Lacy PE, Malaisse WJ (1973) Microtubules and beta cell secretion. *Recent Prog Horm Res* 29:199–228
15. Eto K, Tsubamoto Y, Terauchi Y et al (1999) Role of NADH shuttle system in glucose-induced activation of mitochondrial metabolism and insulin secretion. *Science* 283:981–985
16. Schuit F, De Vos A, Farfari S et al (1997) Metabolic fate of glucose in purified islet cells. Glucose-regulated anaplerosis in beta cells. *J Biol Chem* 272:18572–18579
17. Malaisse WJ, Sener A (1987) Glucose-induced changes in cytosolic ATP content in pancreatic islets. *Biochim Biophys Acta* 927:190–195
18. Gopel S, Zhang Q, Eliasson L et al (2004) Capacitance measurements of exocytosis in mouse pancreatic alpha-, beta-, and delta-cells within intact islets of Langerhans. *J Physiol* 556:711–726
19. Gillis KD (1995) Single-channel recording. Plenum, New York, pp 155–197
20. Kanno T, Ma X, Barg S et al (2004) Large dense-core vesicle exocytosis in pancreatic beta-cells monitored by capacitance measurements. *Methods* 33:302–311
21. Ohara-Imaizumi M, Nakamichi Y, Tanaka T, Ishida H, Nagamatsu S (2002) Imaging exocytosis of single insulin secretory granules with evanescent wave microscopy: distinct behavior of granule motion in biphasic insulin release. *J Biol Chem* 277: 3805–3808
22. Ohara-Imaizumi M, Nishiwaki C, Kikuta T, Nagai S, Nakamichi Y, Nagamatsu S (2004) TIRF imaging of docking and fusion of single insulin granule motion in primary rat pancreatic beta-cells: different behaviour of granule motion between normal and Goto-Kakizaki diabetic rat beta-cells. *Biochem J* 381:13–18
23. Vives-Pi M, Somoza N, Fernandez-Alvarez J et al (2003) Evidence of expression of endotoxin receptors CD14, toll-like receptors TLR4 and TLR2 and associated molecule MD-2 and of sensitivity to endotoxin (LPS) in islet beta cells. *Clin Exp Immunol* 133:208–218
24. Eddlestone GT, Oldham SB, Lipson LG, Premdas FH, Beigelman PM (1985) Electrical activity, cAMP concentration, and insulin release in mouse islets of Langerhans. *Am J Physiol* 248:C145–C153
25. Ikeda Y, Iguchi H, Nakata M et al (2005) Identification of N-arachidonylglycine, U18666A, and 4-androstene-3, 17-dione as novel insulin secretagogues. *Biochem Biophys Res Commun* 333:778–786
26. Russell RR 3rd, Bergeron R, Shulman GI, Young LH (1999) Translocation of myocardial GLUT4 and increased glucose uptake through activation of AMPK by AICAR. *Am J Physiol* 277: H643–H649
27. Kurth-Kraczek EJ, Hirshman MF, Goodyear LJ, Winder WW (1999) 5' AMP-activated protein kinase activation causes GLUT4 translocation in skeletal muscle. *Diabetes* 48:1667–1671
28. da Silva Xavier G, Leclerc I, Varadi A, Tsuboi T, Moule SK, Rutter GA (2003) Role for AMP-activated protein kinase in glucose-stimulated insulin secretion and preproinsulin gene expression. *Biochem J* 371:761–774
29. Ashcroft FM, Proks P, Smith PA, Ammala C, Bokvist K, Rorsman P (1994) Stimulus-secretion coupling in pancreatic beta cells. *J Cell Biochem* 55:54–65
30. Dukes ID, Philipson LH (1996) K⁺ channels: generating excitement in pancreatic beta-cells. *Diabetes* 45:845–853
31. Wollheim CB, Lang J, Regazzi R (1996) The exocytotic process of insulin secretion and its regulation by Ca²⁺ and G-proteins. *Diabetes Rev* 4:276–297
32. Komatsu M, Schermerhorn T, Aizawa T, Sharp GW (1995) Glucose stimulation of insulin release in the absence of extracellular Ca²⁺ and in the absence of any increase in intracellular Ca²⁺ in rat pancreatic islets. *Proc Natl Acad Sci U S A*. 92:10728–10732
33. Komatsu M, Noda M, Sharp GW (1998) Nutrient augmentation of Ca²⁺-dependent and Ca²⁺-independent pathways in stimulus-coupling to insulin secretion can be distinguished by their guanosine triphosphate requirements: studies on rat pancreatic islets. *Endocrinology* 139:1172–1183
34. Sato Y, Nenquin M, Henquin JC (1998) Relative contribution of Ca²⁺-dependent and Ca²⁺-independent mechanisms to the regulation of insulin secretion by glucose. *FEBS Lett* 421:115–119
35. Thurmond DC, Gonelle-Gispert C, Furukawa M, Halban PA, Pessin JE (2003) Glucose-stimulated insulin secretion is coupled to the interaction of actin with the t-SNARE (target membrane soluble N-ethylmaleimide-sensitive factor attachment protein receptor protein) complex. *Mol Endocrinol* 17:732–742
36. Turner MD, Fulcher FK, Jones CV et al (2007) Calpain facilitates actin reorganization during glucose-stimulated insulin secretion. *Biochem Biophys Res Commun* 352:650–655
37. Varadi A, Tsuboi T, Johnson-Cadwell LI, Allan VJ, Rutter GA (2003) Kinesin I and cytoplasmic dynein orchestrate glucose-stimulated insulin-containing vesicle movements in clonal MIN6 beta-cells. *Biochem Biophys Res Commun* 311:272–282
38. Staiger K, Stefan N, Staiger H et al (2005) Adiponectin is functionally active in human islets but does not affect insulin secretory function or beta-cell lipoptosis. *J Clin Endocrinol Metab* 90:6707–6713
39. Winzell MS, Nogueiras R, Dieguez C, Ahren B (2004) Dual action of adiponectin on insulin secretion in insulin-resistant mice. *Biochem Biophys Res Commun* 321:154–160