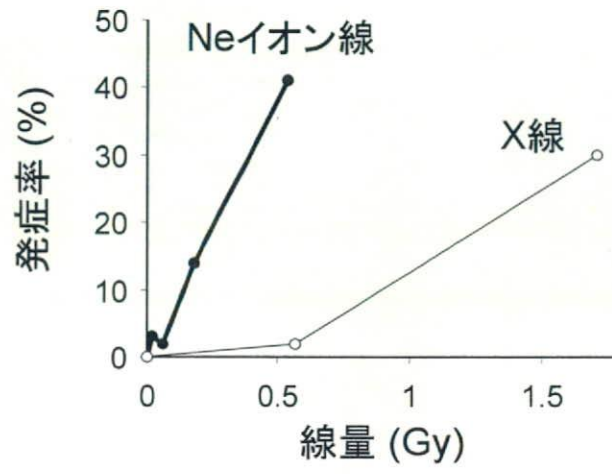


図1. (今岡)

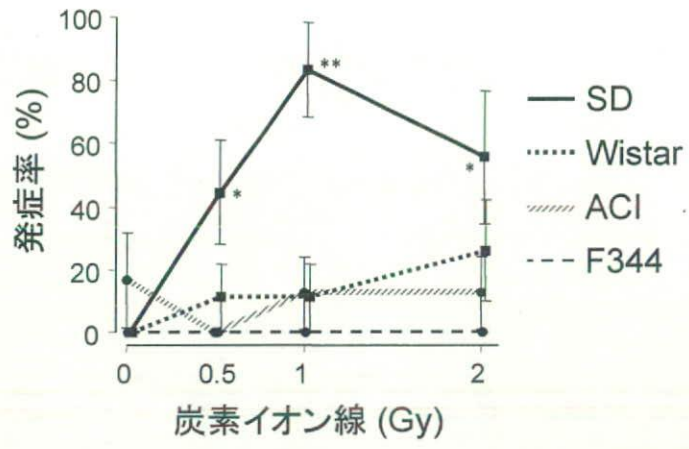
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図2. (今岡)

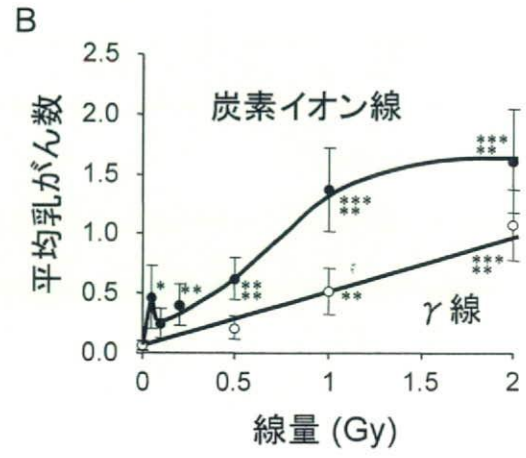
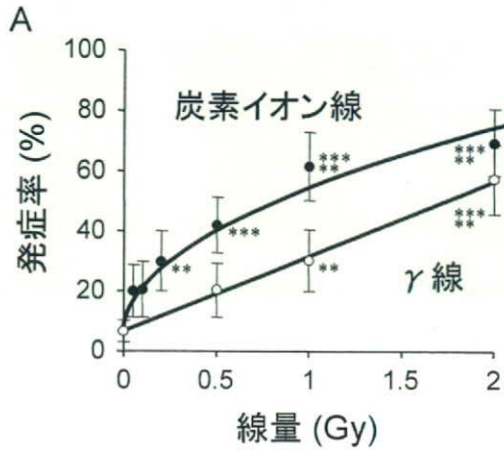
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図3. (今岡)

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- 図1. 6週齢ラットに7.36 GeV/u ネオンイオン線 (0.2 cm 厚鉛散乱体を通過後の推定エネルギー6.6 GeV/u) を照射した後の、51週齢までの乳がん発生率における線量効果関係の系統差。文献(3)より作成。
- 図2. 8週齢ラットに290 MeV/u 炭素イオン線 (SOBP 内) を照射した後の、50週齢までの乳がん発生率における線量効果関係の系統差。* $P < 0.05$, ** $P < 0.01$ vs. 0 Gy。文献(11)より作成。
- 図3. 7週齢ラットに290 MeV/u 炭素イオン線 (SOBP 内) を照射した後の、50週齢までの乳がん発生率(A)ならびに個体当たり平均乳癌数(B)の線量効果関係。* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$, ***** $P < 0.00001$ vs. 0 Gy。文献(11)より作成。

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

M. Okamoto · M. Ohara-Imaizumi · N. Kubota ·
S. Hashimoto · K. Eto · T. Kanno · T. Kubota ·
M. Wakui · R. Nagai · M. Noda · S. Nagamatsu ·
T. Kadowaki

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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.

M. Okamoto and M. Ohara-Imaizumi contributed equally to this study.

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M. Okamoto · N. Kubota · S. Hashimoto · K. Eto · T. Kubota ·
T. Kadowaki (✉)
Department of Metabolic Diseases, Graduate School of Medicine,
University of Tokyo,
Hongo 7-3-1, Bunkyo-ku,
Tokyo 113-8655, Japan
e-mail: kadowaki-3im@h.u-tokyo.ac.jp

M. Ohara-Imaizumi · S. Nagamatsu
Department of Biochemistry,
Kyorin University School of Medicine,
Tokyo, Japan

N. Kubota · T. Kubota · T. Kadowaki
Division of Applied Nutrition,
National Institute of Health and Nutrition,
Tokyo, Japan

T. Kanno · M. Wakui
Department of Physiology,
Hirosaki University School of Medicine,
Hirosaki, Japan

R. Nagai
Department of Cardiovascular Medicine,
Graduate School of Medicine, University of Tokyo,
Tokyo, Japan

M. Noda
Department of Diabetes and Metabolic Medicine,
International Medical Center of Japan,
Tokyo, Japan

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

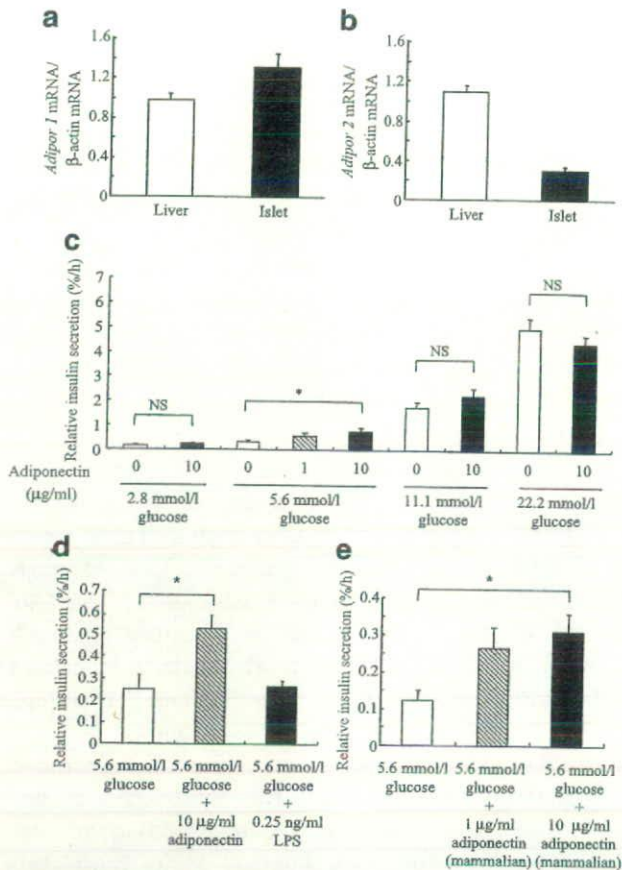


Fig. 1 Adiponectin stimulates insulin secretion from islets. TaqMan RT-PCR of *Adipor1* ($n=3$) (a) and *Adipor2* ($n=3$) (b) in mouse pancreatic islets. c Islets were incubated in KRB containing the indicated concentrations of glucose with or without adiponectin ($n=9-20$). d Islets were incubated with 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°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 perfused 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 $[\text{Ca}^{2+}]_c$.

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.

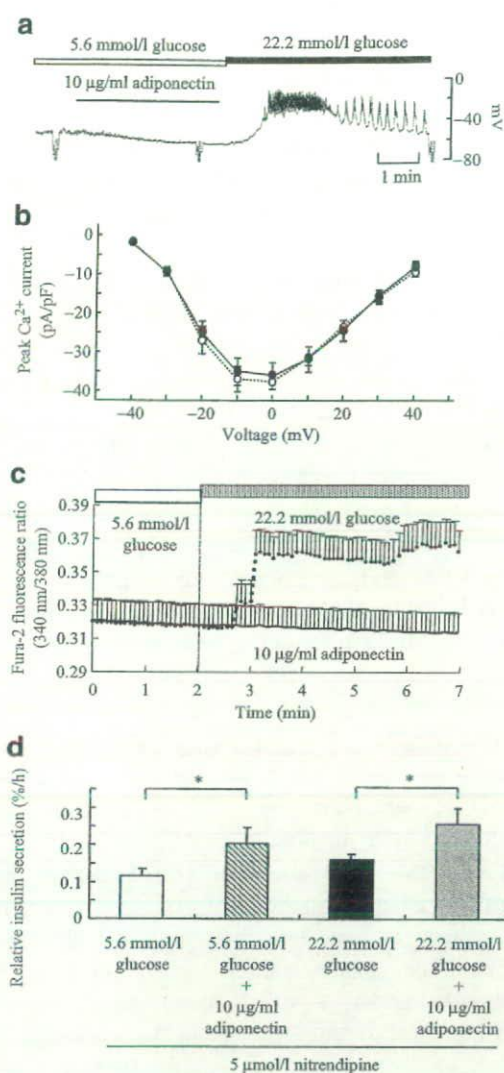


Fig. 2 Adiponectin stimulates insulin secretion without causing membrane depolarisation, closure of K_{ATP}^+ channels or Ca^{2+} entry into the cytosol. **a** Membrane potentials in response to the addition of adiponectin and increasing concentrations of glucose (5.6–22.2 mmol/l) were recorded under 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.

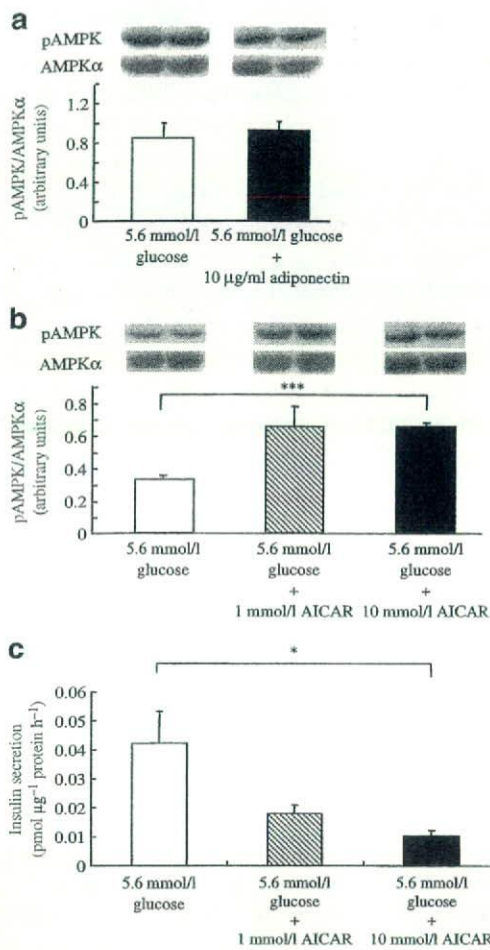


Fig. 3 Adiponectin stimulates insulin secretion without causing activation of AMPK. **a, b** Western blots of phosphorylated AMPK and AMPK α in mouse pancreatic islets ($n=3$, each group). Lysates from 30 islets from each group were subjected to western blot analysis. **c** Isolated islets were incubated 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 Ca $^{2+}$ 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 Ca $^{2+}$ channels, Ca $^{2+}$ entry into the cytosol and a rise in [Ca $^{2+}$] $_c$, which is thought to finally trigger the exocytosis of insulin-containing granules [29–31]. However, a Ca $^{2+}$ -independent pathway has also been proposed. Thus, Komatsu et al. have suggested that both Ca $^{2+}$ -dependent and Ca $^{2+}$ -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 Ca $^{2+}$ through voltage-dependent Ca $^{2+}$ channels is blocked by nifedipine; in other words, adiponectin stimulates insulin

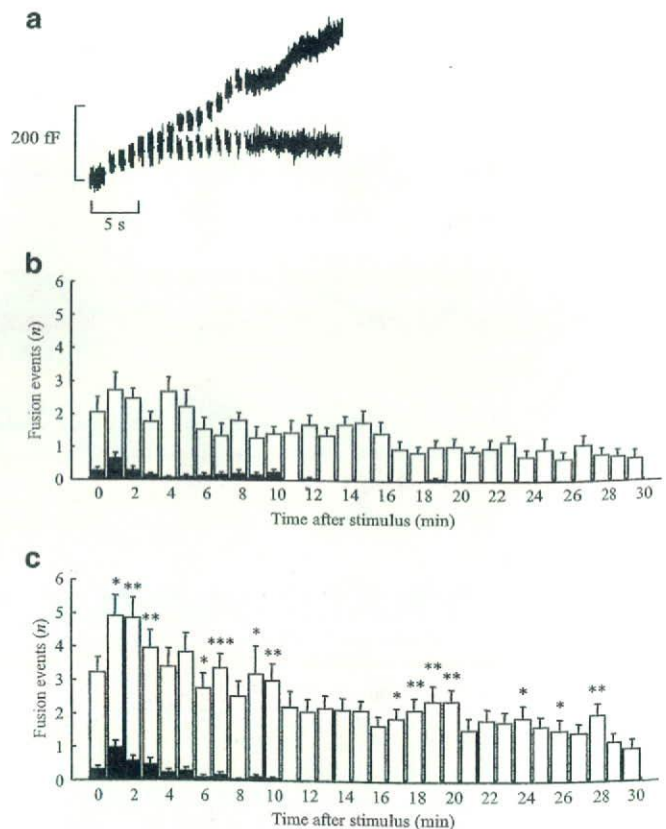


Fig. 4 Adiponectin stimulates insulin release via induction of increased fusion events. **a** The membrane capacitance of mouse beta cells exposed to 5.6 mmol/l glucose alone (lower curve) and 5.6 mmol/l glucose + 10 μ g/ml adiponectin (upper curve) cumulatively increased upon repetitive application 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 m 2 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. * $p<0.05$, ** $p<0.01$, *** $p<0.005$

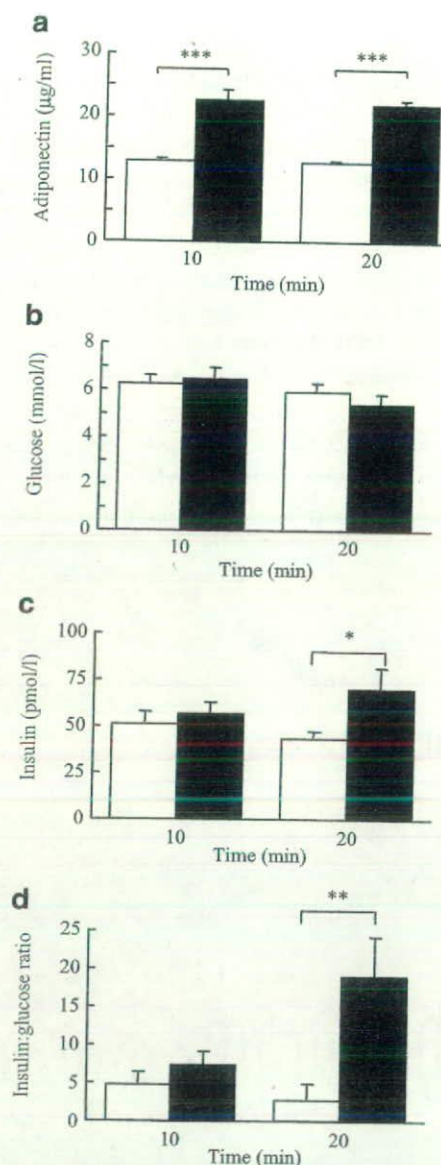


Fig. 5 Adiponectin stimulates insulin secretion in vivo. **a** Plasma adiponectin level after intravenous injection of glucose and saline (white bars, $n=8-21$) or adiponectin (black bars, $n=8-19$). **b** Blood glucose and **(c)** plasma insulin levels after the intravenous injections. **d** Ratio of 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.

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Molecular Mechanism of Moderate Insulin Resistance in Adiponectin-Knockout Mice

WATARU YANO*, NAOTO KUBOTA*^{*,**,*}, SHINSUKE ITOH*, TETSUYA KUBOTA*^{*,***,****},
MOTOHARU AWAZAWA*, MASAO MOROI^{****}, KAORU SUGI^{****}, ISEKI TAKAMOTO*^{*,***,****},
HITOMI OGATA[#], KUMPEI TOKUYAMA[#], TETSUO NODA^{##}, YASUO TERAUCHI^{###}, KOHJIRO UEKI*
AND TAKASHI KADOWAKI*^{*,***,****}

*Department of Diabetes and Metabolic Disease, Graduate School of Medicine, University of Tokyo, Tokyo, Japan

**Translational Systems Biology and Medicine Initiative, University of Tokyo, Tokyo, Japan

***Division of Applied Nutrition, National Institute of Health and Nutrition, Tokyo, Japan

****Division of Cardiovascular Medicine, Toho University, Ohashi Hospital, Tokyo, Japan

#Graduate School of Comprehensive Human Sciences, University of Tsukuba, Tsukuba, Japan

##Department of Cell Biology, Japanese Foundation for Cancer Research-Cancer Institute, Tokyo, Japan

###Department of Endocrinology and Metabolism, Yokohama City University Graduate School of Medicine, Yokohama, Japan

Abstract. Adiponectin has been proposed to act as an antidiabetic adipokine, suppressing gluconeogenesis and stimulating fatty acid oxidation in the liver and skeletal muscle. Although adiponectin-knockout (*adipo(-/-)*) mice are known to exhibit insulin resistance, the degrees of insulin resistance and glucose intolerance are unexpectedly only moderate. In this study, the *adipo(-/-)* mice showed hepatic, but not muscle, insulin resistance. Insulin-stimulated phosphorylation of IRS-1 and IRS-2 was impaired, the IRS-2 protein level was decreased, and insulin-stimulated phosphorylation of Akt was decreased in the liver of the *adipo(-/-)* mice. However, the triglyceride content in the liver was not increased in these mice, despite the decrease in the PPAR α expression involved in lipid combustion, since the expressions of lipogenic genes such as SREBP-1 and SCD-1 were decreased in association with the increased leptin sensitivity. Consistent with this, the down-regulation of SREBP-1 and SCD-1 observed in the *adipo(-/-)* mice was no longer observed, and the hepatic triglyceride content was significantly increased in the adiponectin leptin double-knockout (*adipo(-/-)ob/ob*) mice. On the other hand, the triglyceride content in the skeletal muscle was significantly decreased in the *adipo(-/-)* mice, probably due to up-regulated AMPK activity associated with the increased leptin sensitivity. In fact, these phenotypes in the skeletal muscle of these mice were no longer observed in the *adipo(-/-)ob/ob* mice. In conclusion, *adipo(-/-)* mice showed impaired insulin signaling in the liver to cause hepatic insulin resistance, however, no increase in the triglyceride content was observed in either the liver or the skeletal muscle, presumably on account of the increased leptin sensitivity.

Key words: Adiponectin, Insulin resistance, Adiponectin-knockout mice, Euglycemic-hyperinsulinemic clamp.

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ADIPONECTIN (also known as Acrp30) [1–4] is a hormone secreted by adipocytes that acts as a major an-

tidabetic adipokine. Plasma adiponectin levels are decreased in obesity, insulin resistance and type 2 diabetes mellitus [1–4]. Decreased adiponectin has been implicated in the development of insulin resistance in obesity, which has been shown to be reversed by replenishment of adiponectin [5–7]. This insulin-sensitizing effect of adiponectin seems to be mediated by the inhibition of gluconeogenesis in the liver and stimulation of fatty acid oxidation via activation of AMP-activated protein kinase (AMPK) and peroxi-

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Correspondence to: Takashi KADOWAKI, M.D., Ph.D. Department of Metabolic Diseases, Graduate School of Medicine, University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113-8655, Japan.

Abbreviations: EGP, Endogenous Glucose Production; Rd Rate of disappearance

some proliferator-activated receptor (PPAR) alpha in the liver and skeletal muscle [8–12]. Thus, adiponectin ameliorates insulin resistance in both the liver and skeletal muscle.

Adiponectin-knockout (adipo(-/-)) mice have been described mainly by four groups. We reported that adipo(-/-) mice showed insulin resistance [13], indicating that adiponectin acts as an insulin-sensitizing hormone *in vivo*. Maeda *et al.* reported that adipo(-/-) mice fed a normal diet failed to show insulin resistance and glucose intolerance [14]. Ma *et al.* described the absence of insulin resistance and unexpectedly, increased fatty acid oxidation, in the skeletal muscle of adipo(-/-) mice [15]. Nawrocki *et al.* described that adipo(-/-) mice exhibited hepatic, but not muscle, insulin resistance, and increased endogenous glucose production (EGP), with absence of any change in the rate of disappearance (Rd) of glucose during the euglycemic-hyperinsulinemic clamp study [16]. However, the degrees of insulin resistance and glucose intolerance were unexpectedly moderate in these adipo(-/-) mice [13, 15, 16]. We recently reported increased leptin sensitivity in adipo(-/-) mice [17]. Leptin is known to decrease the expressions of lipogenic genes and also the triglyceride content in the liver [18, 19] and, it has also been shown to activate AMPK in the skeletal muscle [20]. In fact, muscle AMPK activity was shown to be increased in the adipo(-/-) mice [17]. In this study, we investigated the molecular mechanisms of the insulin resistance observed in the adipo(-/-) and adiponectin/leptin double-knockout (adipo(-/-)ob/ob) mice.

Materials and methods

Animals

Mice lacking adiponectin were generated as described previously [13, 21]. Adiponectin/leptin double-knockout mice and ob/ob mice were generated by intercrossing of adipo(+/-)ob/+ mice [21]. The mice were allowed free access to water and ordinary laboratory diet. All experiments in this study were conducted on littermate male mice. The animal care and procedures of the experiments were approved by the Animal Care Committee of the University of Tokyo.

Euglycemic-hyperinsulinemic clamp study

A clamp study was carried out as described previously [21]. A catheter was inserted into the jugular vein 2–3 days prior to the clamp study. After overnight food deprivation, insulin was injected constitutively by intravenous infusion at 4 mU/kg/min, and 50% glucose solution enriched to 20% with 6,6-d₂ glucose as tracer was injected to maintain the blood glucose at about 120 mg/dl under conscious and unstressed conditions. Blood was sampled via tail tip bleeds at 90, 105 and 120 min to determine the rate of glucose disappearance (Rd) and endogenous glucose production (EGP).

RNA preparation and analysis

Tissue samples were homogenized to isolate RNA with ISOGEN reagent (Wako, Japan) and analyzed by northern blotting and real-time quantitative PCR. Northern blotting for PPARalpha and SREBP-1 mRNA was carried out as described previously [6, 19]. For real-time quantitative PCR, the ABI 7900 sequence detection system (Applied Biosystems, CA, USA) was used. The RNA sample was processed with TURBO DNase (Ambion, TX, USA) before reverse transcription to synthesize cDNA. 36B4 mRNA was used as the internal control. The primer sets for PEPCK and G6Pase were purchased from Applied Biosystems. The sequences of the primer sets for SREBP-1c, PPARalpha and 36B4 were as follows [22]; SREBP-1c: forward primer, ATCGGCGCGGAAGCTGTCGGGGTAGCGTC; reverse primer, TGAGCTGGAGCATGTCTCAA; probe, FAM-ACCACGGAGCCATGGATTGCACATT-TAMRA. PPARalpha: forward primer, CAACGGCGTCGAAGACAAA; reverse primer, GACGGTCTCCACGGACATG; probe, FAM-CAGAGGTCCGATTCTTCCACTGCTGC-TAMRA. 36B4: forward primer, TGCCACACTCCATCATCAATG; reverse primer, CCGCAAATGCAGATGATC; probe, FAM-CCCACTTACTGAAAAGGTCAAGCCTTCCTG-TAMRA.

Measurement of the tissue triglyceride content

Tissue homogenate was extracted with 2:1 (vol/vol) chloroform/methanol, and the triglyceride content was determined as described previously [22]. In brief, chloroform/methanol was added to the homogenate and shaken for 15 min. After centrifugation at 14,000

rpm for 10 min, the organic layer was collected. This extraction step was repeated three times. The collected sample was dried and resuspended in 1% Triton X-100/ethanol, and measured using L-type Wako (Wako, Japan).

Immunoprecipitation and western blotting

Immunoprecipitation and western blot analyses were carried out as described previously [23]. Tissue lysate was immunoprecipitated with anti-IRS-1 antibody or anti-IRS-2 antibody (Upstate, VA, USA) and blotted with an anti-phospho-tyrosine (anti-pY) antibody (Upstate, VA, USA) to assess the degree of phosphorylation of IRS-1 or IRS-2. For the western blot analyses, antibodies against Akt, phospho-Akt, AMPK and phospho-AMPK (Cell Signaling Technology, MA, USA) were used.

Statistics

All values were expressed as means \pm SEM. The statistical significances of differences were calculated using the *t*-test.

Results

The euglycemic-hyperinsulinemic clamp study revealed hepatic insulin resistance in the *adipo(-/-)* mice

We carried out the euglycemic-hyperinsulinemic clamp study using the tracer technique in the wild-type and *adipo(-/-)* mice. Significant decrease of the GIR was observed in the *adipo(-/-)* mice as compared with that in the wild-type mice (Fig. 1A), indicating that the *adipo(-/-)* mice indeed exhibited insulin resistance, as previously reported [13]. *Adipo(-/-)* mice showed similar Rd to the wild-type mice, but significantly increased EGP. (Fig. 1B, C). The expressions of PEPCK (Fig. 1D) and G6Pase (Fig. 1E), which are involved in gluconeogenesis, were up-regulated during the euglycemic-hyperinsulinemic clamp study in the *adipo(-/-)* mice, indicating the hepatic insulin resistance in the *adipo(-/-)* mice.

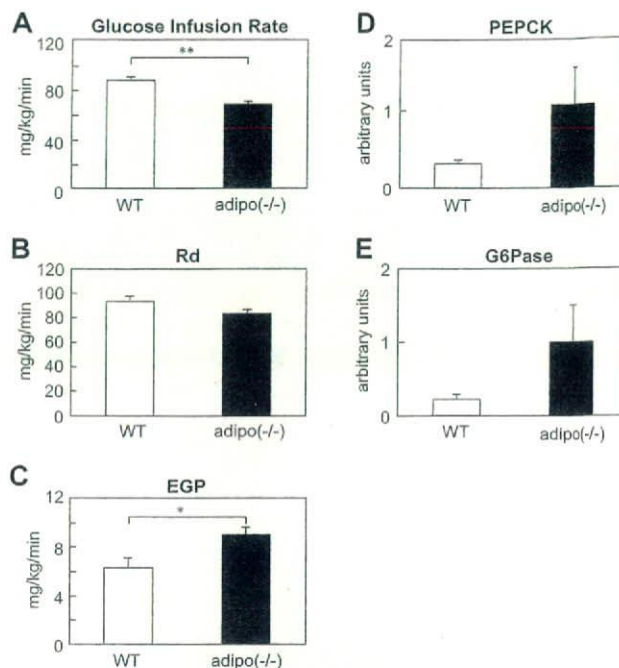


Fig. 1. The euglycemic-hyperinsulinemic clamp study revealed hepatic insulin resistance in the *adipo(-/-)* mice. (A-E) Glucose infusion rate (GIR) (A), rate of glucose disappearance (Rd) (B), endogenous glucose production (EGP) (C), PEPCK (D) and G6Pase (E) mRNA levels in the liver during the euglycemic-hyperinsulinemic clamp study. All values are expressed as means \pm SEM of data ($n = 7$) obtained from the analysis of wild-type (open bars) and *adipo(-/-)* mice (closed bars). * $p < 0.05$, ** $p < 0.01$.

Impaired insulin signaling in the liver of the *adipo(-/-)* mice

Insulin signaling was investigated in the liver and the skeletal muscle of the *adipo(-/-)* mice. Insulin-stimulated tyrosine phosphorylation of IRS-1 was significantly decreased and that of IRS-2 was markedly decreased in the liver of the *adipo(-/-)* mice as compared with that in the liver of the wild-type mice (Fig. 2A). The protein level of IRS-2 was significantly decreased, while that of IRS-1 was not altered (Fig. 2A). Insulin-stimulated phosphorylation of Akt was also significantly reduced in the *adipo(-/-)* mice (Fig. 2B). In the skeletal muscle, the insulin-stimulated tyrosine phosphorylation level of IRS-1 was similar in the wild-type and *adipo(-/-)* mice (Fig. 2C). The insulin-stimulated phosphorylation level of Akt was also similar in the two genotypes (Fig. 2D). These data indicate impairment of hepatic insulin signaling in the *adipo(-/-)* mice.

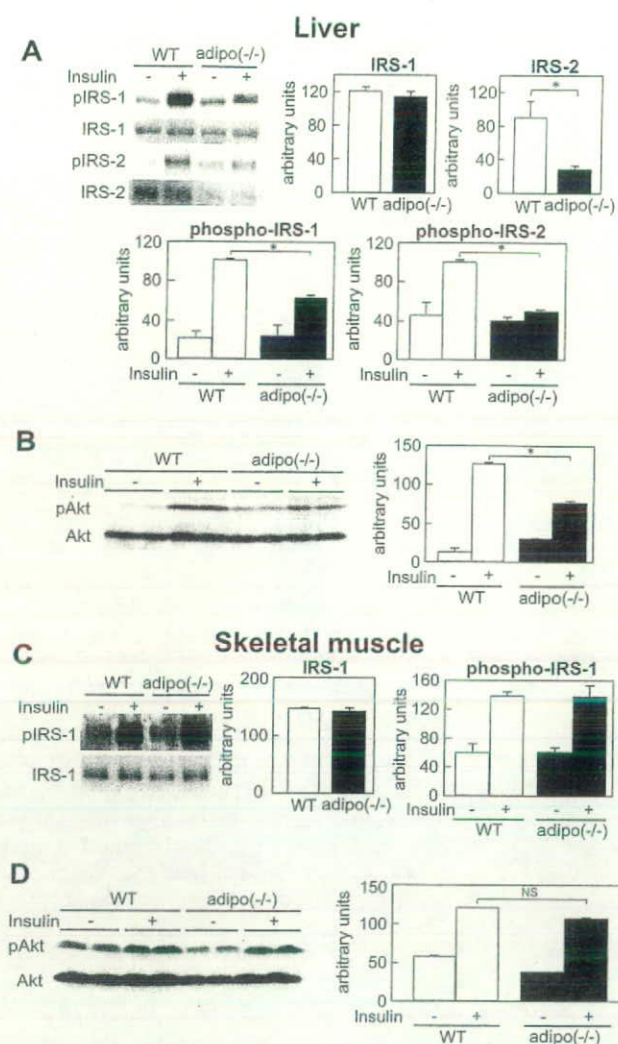


Fig. 2. Impaired insulin signaling in the liver of the *adipo(-/-)* mice.

(A) Insulin-stimulated tyrosine phosphorylation of IRS-1 and IRS-2 in the liver. Quantification of the protein levels (*upper, right*) and phosphorylation levels of IRS-1 and IRS-2 (*lower*). (B) Insulin-stimulated phosphorylation of Akt in the liver. (C) Insulin-stimulated tyrosine phosphorylation of IRS-1 in the skeletal muscle. Quantification of the protein level (*middle*) and phosphorylation level of IRS-1 (*right*). (D) Insulin-stimulated phosphorylation of Akt in the skeletal muscle. All values are expressed as means \pm SEM of data ($n = 4$) obtained from the analysis of wild-type (*open bars*) and *adipo(-/-)* mice (*closed bars*). * $p < 0.05$.

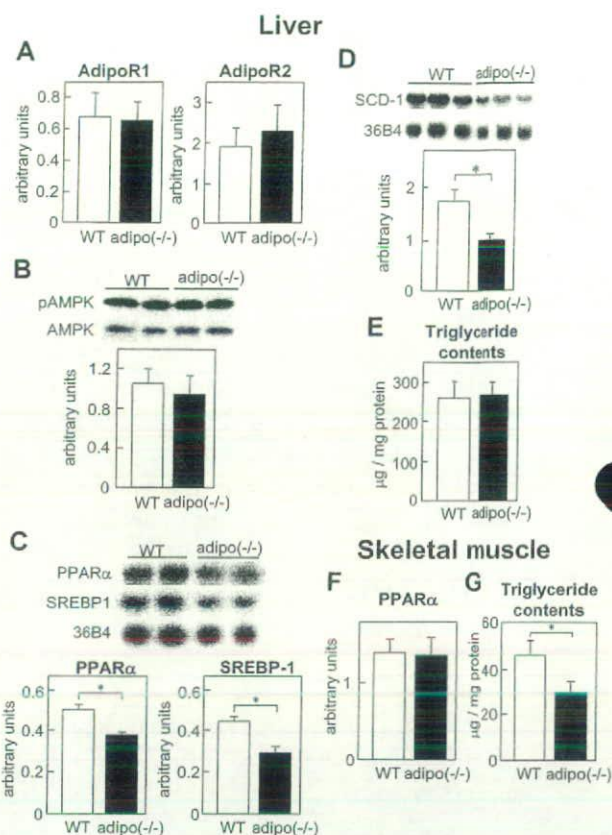


Fig. 3. Hepatic triglyceride content was not elevated and the expressions of lipogenic genes were down-regulated in the *adipo(-/-)* mice.

(A) AdipoR1 and AdipoR2 mRNA expression levels in the liver ($n = 3$). (B) AMPK phosphorylation in the liver ($n = 8-10$). (C-D) The mRNA levels of PPAR α , SREBP-1 (C) and SCD-1 (D) in the liver ($n = 4-5$). (E) Hepatic triglyceride content ($n = 11-14$). (F) PPAR α expression level in the skeletal muscle ($n = 5$). Triglyceride content (G) in the skeletal muscle ($n = 5$). All values are expressed as means \pm SEM of data obtained from the analysis of wild-type (*open bars*) and *adipo(-/-)* mice (*closed bars*). * $p < 0.05$.

*Hepatic triglyceride content was not elevated and the expressions of lipogenic genes were down-regulated in the *adipo(-/-)* mice*

We next investigated the lipid metabolism in the liver of these mice. No significant differences in the expression levels of the adiponectin receptors AdipoR1 and AdipoR2 [24] were observed between the wild-type and *adipo(-/-)* mice (Fig. 3A). While the degree of AMPK phosphorylation remained unchanged (Fig. 3B), significant down-regulation of PPAR α

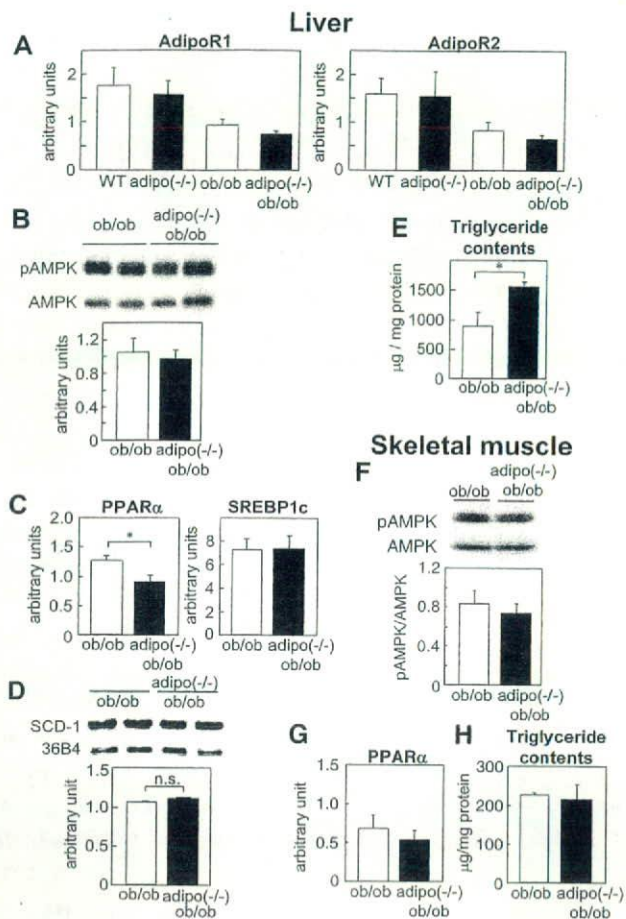


Fig. 4. The reduced expressions of the lipogenic genes in the *adipo(-/-)* mice were no longer seen in the *adipo(-/-)ob/ob* mice with a leptin-deficient background. (A) AdipoR1 and AdipoR2 mRNA expression levels in the liver ($n = 3-6$). (B) AMPK phosphorylation in the liver ($n = 5$). (C-D) mRNA levels of PPAR α , SREBP-1c (C) and SCD-1 (D) in the liver ($n = 4-5$). (E) Hepatic triglyceride content ($n = 5$). (F) AMPK phosphorylation in the skeletal muscle ($n = 5$). (G) PPAR α expression level in the skeletal muscle ($n = 5$). (H) Triglyceride content in the skeletal muscle ($n = 5$). All values are expressed as means \pm SEM of data obtained from the analysis of *ob/ob* (*open bars*) and *adipo(-/-)ob/ob* mice (*closed bars*). * $p < 0.05$.

was observed in the *adipo(-/-)* mice as compared with that in the wild-type mice (Fig. 3C), suggesting that fatty acid oxidation may be reduced in the liver of the *adipo(-/-)* mice. The expressions of lipogenic genes such as SREBP-1 (Fig. 3C) and SCD-1 (Fig. 3D) were also significantly down-regulated in the *adipo(-/-)* mice, and the hepatic triglyceride content was not elevated in the *adipo(-/-)* mice (Fig. 3E). This unexpect-

ed down-regulation of lipogenic genes, which may be explained by the increased leptin sensitivity seen in the *adipo(-/-)* mice [17], might have prevented the elevation of the triglyceride content in the liver of the *adipo(-/-)* mice. In the skeletal muscle, we previously reported that the phosphorylation of AMPK was increased in *adipo(-/-)* mice, presumably due to increased leptin sensitivity [17]. Consistent with this, although the expression of PPAR α was similar in the wild-type and *adipo(-/-)* mice (Fig. 3F), the muscle triglyceride content was significantly decreased in the *adipo(-/-)* mice (Fig. 3G).

*The reduced expressions of the lipogenic genes observed in the *adipo(-/-)* mice no longer seen in the *adipo(-/-)ob/ob* mice with a leptin-deficient background*

To evaluate the existence of the aforementioned compensatory mechanism in the *adipo(-/-)* mice, we generated *adipo(-/-)ob/ob* mice. The expression levels of AdipoR1 and AdipoR2 were similar in the liver of the *ob/ob* and *adipo(-/-)ob/ob* mice. Comparison of the wild-type with *ob/ob* mice, and of the *adipo(-/-)* with *adipo(-/-)ob/ob* mice demonstrated a tendency towards reduced expression levels of the adiponectin receptors in the leptin-deficient background (Fig. 4A). Significant decrease of PPAR α expression was observed in the liver of the *adipo(-/-)ob/ob* mice (Fig. 4C), as in the liver of the *adipo(-/-)* mice (Fig. 3C). The expression levels of SREBP-1c (Fig. 4C) and SCD-1 (Fig. 4D) were not altered in the liver of the *adipo(-/-)ob/ob* mice, unlike in the liver of the *adipo(-/-)* mice, indicating that the compensatory mechanism in the *adipo(-/-)* mice was no longer operative in the *adipo(-/-)ob/ob* mice. In fact, the hepatic triglyceride content was significantly increased in the *adipo(-/-)ob/ob* mice (Fig. 4E). These data suggest that the leptin pathway might have contributed to the reduced expressions of the lipogenic genes and absence of elevation of the hepatic triglyceride content in the *adipo(-/-)* mice. In the skeletal muscle, increased phosphorylation of AMPK in the *adipo(-/-)* was no longer observed in the *adipo(-/-)ob/ob* mice (Fig. 4F). The expression level of PPAR α (Fig. 4G) and the triglyceride content (Fig. 4H) were also unaltered in the skeletal muscle of *adipo(-/-)ob/ob* mice.

Discussion

In the present study, we investigated the molecular mechanisms of the insulin resistance in *adipo(-/-)* mice. *Adipo(-/-)* mice showed hepatic, but not muscle, insulin resistance. Insulin-stimulated tyrosine phosphorylation of IRS-1 and IRS-2 was impaired in the liver of the *adipo(-/-)* mice, despite the absence of any change in the hepatic triglyceride content. One of the underlying mechanisms responsible for this may be the increased phosphorylation of the serine/threonine residue of IRS-1 in the liver of these mice, which is currently under investigation. Moreover, the IRS-2 protein level was also significantly decreased in the *adipo(-/-)* mice; as a result, insulin-stimulated phosphorylation of Akt was significantly decreased in these mice. In the skeletal muscle, on the other hand, insulin-stimulated phosphorylation of IRS-1 and Akt was similar in degree between the wild-type and *adipo(-/-)* mice. In fact, while the EGP and expression levels of PEPCK and G6Pase were increased, the Rd was not found to be significantly changed in the *adipo(-/-)* mice during the euglycemic-hyperinsulinemic clamp study.

Triglyceride content in the liver was not increased in the *adipo(-/-)* mice, despite the decrease in PPAR α expression involved in lipid combustion, since the expressions of lipogenic genes such as SREBP-1 and SCD-1 were decreased in association with the increased leptin sensitivity. Consistent with this, down-regulation of SREBP-1 and SCD-1 observed in the *adipo(-/-)* mice was no longer observed in the *adipo(-/-)*ob/ob mice, and the hepatic triglyceride content was significantly increased in the *adipo(-/-)*ob/ob mice as compared with that in the ob/ob mice. On the other hand, the triglyceride content in the skeletal muscle was significantly decreased in the *adipo(-/-)* mice, probably due to the up-regulated muscle AMPK activity associated with the increased leptin sensitivity in these mice [17]. In fact, the increase in AMPK activity [17] and decrease triglyceride content in the skeletal muscle were no longer observed in the *adipo(-/-)*ob/ob mice. Therefore, increase in the leptin actions appears to compensate for the adiponectin deficiency in both the liver and the skeletal muscle of the *adipo(-/-)* mice, accounting for the unexpected absence of a increase in the hepatic triglyceride content and rather decreased

muscle triglyceride content in the *adipo(-/-)* mice [13].

Why was the degree of insulin resistance different between the liver and skeletal muscle of the *adipo(-/-)* mice? We recently demonstrated that adiponectin induces the expression of IRS-2 in the liver (Awazawa M, Ueki K and Kadowaki T, manuscript in preparation). IRS-2 is a major IRS in the liver, but not in the skeletal muscle [25], suggesting that the reduction of IRS-2 due to adiponectin deficiency may have little effect on the insulin signaling in the skeletal muscle of *adipo(-/-)* mice.

A similar degree of phosphorylation of AMPK was seen in the liver of the wild-type and *adipo(-/-)* mice, even though adiponectin is known to activate AMPK [9, 10]. SCD-1 expression was down-regulated in the *adipo(-/-)* mice. Increased phosphorylation of AMPK has been reported in the liver of SCD-1-knockout mice [26]. It is suggested that the down-regulation of AMPK resulting from adiponectin deficiency may be balanced by the up-regulation of AMPK occurring as a result of the decreased SCD-1 expression, resulting in the absence of any net change in the phosphorylation level of AMPK in the liver of the *adipo(-/-)* mice. In Nawrocki's study, there appeared to be no differences in the phosphorylation level and activity of AMPK between the wild-type and *adipo(-/-)* mice [16].

In conclusion, *adipo(-/-)* mice showed impaired insulin signaling in the liver to cause hepatic insulin resistance, however, no increase in the triglyceride content was observed in either the liver or the skeletal muscle, presumably on account of the increased leptin sensitivity.

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