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ABSTRACT

Procyanidins, the main ingredient of apple polyphenols, are known to possess anti-oxidative and anti-inflammatory effects associated closely with the pathophysiology of insulin resistance and type 2 diabetes. We investigated the effects of orally administered apple procyanidins (APCs) on glucose metabolism using diabetic ob/ob mice. We found no difference in body weight or body composition between APCs-treated and untreated mice. 4-week oral administration of APCs containing water (0.5% w/v) ameliorated glucose tolerance, insulin resistance, and hepatic gluconeogenesis in ob/ob mice. APCs also suppressed the increase of pancreatic β-cell. Insulin-stimulated Akt phosphorylation was significantly enhanced, pro-inflammatory cytokine expression levels were significantly decreased, and c-Jun N-terminal kinase (JNK) phosphorylation was down-regulated in the liver of those APCs-treated mice. In conclusion, APCs ameliorate insulin resistance by improving hepatic insulin signaling through suppression of hepatic inflammation in ob/ob mice, which may be a mechanism of possible beneficial health effects of APCs in disturbed glucose metabolism.

Keywords: apple procyanidins, insulin resistance, inflammation, ob/ob mouse
INTRODUCTION

Insulin resistance induced by obesity readily develops into type 2 diabetes and leads to elevated risk of cardiovascular disease. The pathogenesis of type 2 diabetes is characterized by two major features, insulin resistance and impaired insulin secretion. If insulin demand due to insulin resistance is over the capacity of pancreatic β-cells, blood glucose homeostasis cannot be maintained, leading to chronic hyperglycemia. Reducing insulin resistance is therefore clinically important for the prevention and management of type 2 diabetes.

The mechanism of insulin resistance is still unclear. Insulin resistance is reported to be associated with a state of chronic and low-grade inflammation in insulin target tissues including adipose tissue, liver and skeletal muscle. Tumor necrosis factor-α (TNF-α), a pro-inflammatory cytokine produced from accumulated fat, activates various signaling cascades, including c-Jun N-terminal kinase (JNK). JNK leads to serine phosphorylation of insulin receptor substrate (IRS)-1 and 2, and consequently induces insulin resistance.\(^1\) Oxidative stress also activates the JNK pathway and induces insulin resistance.\(^2\)

Epidemiological studies suggest that consumption of fruits and vegetables reduces
the risk of developing type 2 diabetes.\textsuperscript{3,4} The benefits of fruits and vegetables have been attributed to their dietary fiber and various phytochemicals, such as polyphenol.

Apple is one of the most commonly consumed fruits in the world. Apple polyphenols are known to have various physiological effects including antioxidant activity,\textsuperscript{5} anti-inflammation activity,\textsuperscript{6} and anti-tumor activity.\textsuperscript{7}

Apple procyanidins (APCs) are the main ingredient of apple polyphenols\textsuperscript{8} and consist of flavanol units such as (+)-catechin and (−)-epicatechin, which are linked together through 4→8 and 4→6 interflavonoid bonds, and have many isomeric forms depending on the extent of polymerization and the nature of their constituent units (Figure S1). Recent research has indicated that APCs have various beneficial effects on health, including anti-aging effects in Caenorhabditis elegans,\textsuperscript{9} an inhibitory effect on triglyceride absorption through inhibition of pancreatic lipase activity in mouse and human,\textsuperscript{10} and anti-inflammatory and immunomodulatory effects on intestinal epithelial cells.\textsuperscript{11}

However, there are few reports regarding ingestion of APCs and risk of type 2 diabetes. In this study, we investigated the effects of APCs on glucose metabolism using model mice for obesity and type 2 diabetes. Our findings may lead to a strategy for development of therapeutic agents for impaired glucose tolerance and type 2
diabetes.

MATERIALS AND METHODS

Preparation of apple polyphenol extracts

The procyanidin fraction was prepared from apple (*Malus pumila* cv. Fuji) by preparative column chromatography with the method of previous study. Briefly, the apple polyphenol fraction was prepared from apple juice using the preparative column with aromatic synthetic adsorbents, Sepabeads SP-850 (Mitsubishi Kasei Co., Ltd., Japan). Apple polyphenol extracts were lyophilized, and the powder obtained was dissolved in distilled water and adjusted to pH 6.5 with 5N NaOH. The sample was applied to a Diaion HP-20ss (Mitsubishi Kasei Co., Ltd., Japan) column, and after rinsing the column with distilled water, the procyanidin fraction was eluted with 25% ethanol. Finally, the eluate was concentrated by rotary evaporation at 45°C and lyophilized as the APCs fraction. APCs were analyzed using by reversed-phase HPLC equipped with an LC-10AD VP pump (Shimadzu, Kyoto, Japan), an SIL-10AD VP autosampler (Shimadzu), and a Inertsil ODS-3 (GL Sciences Inc., Tokyo, Japan) reversed-phase column (150 x 4.6 mm i.d.) at 40°C. Mixtures of 10 mM KH$_2$PO$_4$ solution (adjusted to pH 1.8 with H$_3$PO$_4$) and methanol was used as the mobile phase.
[mobile phase A, 10 mM KH$_2$PO$_4$:MeOH (8:2) and mobile phase B, 10 mM KH$_2$PO$_4$:MeOH (5:5)] were used as the mobile phases with a flow rate of 1.0 ml/min.

Detection was performed using a SPD-10A VP UV-vis detector (Shimadzu) at 280 nm.

For the first 10 min, the initial eluent used was 0% mobile phase B, followed by a linear gradient from 100% mobile phase B for 40 min; subsequently the concentration was held at 100% mobile phase B for 15 min and then returned to the initial conditions.

This fraction did not include phloretin glucoside (phlorizin) or chlorogenic acid (Figure 1). The former has a blood glucose lowering effect by inhibiting sodium glucose cotransporter (SGLT1 and SGLT2),$^{13}$ and the latter has several beneficial biological properties including blood pressure lowering and anti-oxidative effects.$^{14,15}$

**Mice**

5-week-old male B6.Cg-Lepob/J mice (C57BL/6J background) were purchased from Charles River Japan Inc. (Kanagawa, Japan). The phenotype is obese and insulin resistance but hyperglycemia is not so severe. They were divided into two groups: an APCs-treated and an untreated group. The mice were housed in individual under controlled environment at 23°C and 12-hour light/dark cycle with free access to water and a commercial nonpurified diet (MF, Oriental Yeast Co., Tokyo, Japan). All animal
experiments were approved by the Kyoto University Animal Care Committee.

Beginning at 8 weeks of age, mice were administered APCs dissolved in drinking water (0.5%, w/v) ad libitum for 4 weeks. Body weight and food intake were measured once every week and water intake was measured every day. During continuation of the APCs administration to 16 weeks of age, insulin tolerance test (ITT), oral glucose tolerance test (OGTT), and pyruvate tolerance test (PTT) were performed.

Measurement of energy expenditure

Energy expenditure of the mice at the age of 16 weeks was measured for 48 hours using indirect calorimetry. Oxygen consumption and CO₂ production were determined every 5 min in an open chamber with the mass spectrometry-based O₂ and CO₂ analyzer ARCO-2000 (ARCO system, Chiba, Japan). Oxygen consumption was normalized by lean body mass.

Measurement of blood glucose, oral glucose tolerance test and insulin tolerance test

After 4 weeks administration of water with or without 0.5% APCs, APCs-treated and untreated ob/ob mice were fasted overnight for 16 h, and received an oral dose of
1 g/kg glucose. Blood glucose levels and serum insulin concentrations were measured at 0, 15, 30, 60, and 120 min after oral injection by the glucose oxidase method (Sanwa Kagaku Kenkyusho, Nagoya, Japan) and an ELISA kit (Shibayagi Co. Ltd, Gunma, Japan), respectively. After 5 weeks administration of APCs, APCs-treated and untreated ob/ob mice were fasted for 16 h, and regular insulin (2 units/kg) was injected intraperitoneally. Blood glucose levels were measured at 0, 15, 30, 45, 60, 90, and 120 min after injection.

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**Pyruvate tolerance test**

Pyruvate was dissolved with 0.9% (wt/vol) sterile saline. APCs-treated and untreated ob/ob mice were fasted overnight for 16 h, and pyruvate (1 g/kg) was injected intraperitoneally. Blood glucose levels were measured at 0, 15, 30, 60, 90, and 120 min after injection.16

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**Calculation of homeostasis model assessment of insulin resistance**

The homeostasis model assessment of insulin resistance (HOMA-IR) was calculated using fasting blood glucose and insulin concentrations based on OGTT data. HOMA-IR is used to estimate insulin resistance in human and animals.17,18 HOMA-IR
was calculated using the following formula: \[^{19}\]

\[
\text{HOMA-IR} = \text{insulin (mU/L)} \times \left[ \frac{\text{glucose (mg/dL)}}{405} \right]
\]

**Measurement of body fat composition**

Body fat mass was measured by CT scan (LaTheta LCT-100, Aloka, Tokyo, Japan). The mice were anesthetized, and the images were analyzed using LaTheta software, version 1.00 and values of subcutaneous and visceral fat mass were quantified in grams (g).

**Measurement of serum adiponectin**

Mice at the age of 16 weeks were sacrificed and blood samples were taken. Serum adiponectin concentration was measured by ELISA kit (Otsuka Pharmaceutical Co. ltd, Tokyo, Japan) according to the instruction manuals.

**Histomorphology and immunohistochemistry**

The sections of paraffin embedding pancreas in mice at the age of 16 weeks were incubated with anti-glucagon mouse monoclonal antibody (cloneK79bB10, 1:2000
dilution; Abcam plc, Cambridge, UK) and polyclonal rabbit anti-insulin (H-86) antibody (1:100 dilution; Santa Cruz Biotechnology, Inc., Texas, U.S.A.). The sections were then incubated with goat anti-mouse IgG and goat anti-rabbit fluorescein-conjugated secondary antibody (1:200 dilution, Alexa Fluor 488; Alexa Fluor 546; Invitrogen/Life Technologies Japan, Tokyo, Japan). Two slides randomly selected from each pancreas were analyzed. After immunostaining, quantification of β-cell area was performed by immunofluorescent microscope using BZ-II Analyzer software (Keyence Corp., Osaka, Japan). Results are expressed as percentage of total surveyed area containing cells positive for insulin. The insulin-positive cells were counted as the number of islets per area of pancreas.20, 21

Sections of liver tissues of mice at the age of 16 weeks were stained with Oil Red O and Hematoxylin and Eosin (H&E). For immunohistochemistry, the liver sections were incubated with anti-F4/80 rat-monoclonal antibody (1:100 dilution; Abcam plc, Cambridge, UK). The sections were then treated with anti-rat fluorescein-conjugated secondary antibody (Alexa Fluor 546; Invitrogen/Life Technologies Japan, Tokyo, Japan) and anti-rat horseradish peroxidase (HRP)-conjugated antibody (polyclonal rabbit anti-rat immunoglobulins/HRP; DakoCytomation, Glostrup, Denmark).22
Measurement of total cholesterol and lipid contents in liver

Hepatic lipids were extracted as described previously. Total cholesterol and triglyceride were measured at Skylight Biotech, Inc. (Akita, Japan) using cholesterol and triglyceride assay kits (choletest-CHO and choletest-TG, Sekisui Medical Co., Ltd., Tokyo, Japan). Hepatic lipid content was defined as weight per gram of liver tissue.

Immunoblotting

Liver isolated from APCs-treated and untreated ob/ob mice were lysed in ice-cold lysis buffer (10 mmol/l Tris [pH 7.2], 100 mmol/l NaCl, 1 mmol/l EDTA, 1% Nonidet P-40, and 0.5% sodium deoxycholate) containing protease inhibitor cocktail (Complete; Roche, Mannheim, Germany), phosphatase inhibitor cocktail (Calbiochem, Darmstadt, Germany), and 5 mmol/l sodium pyrophosphate. Immunoblotting was performed. Primary antibodies used were rabbit anti-phospho-Akt (Ser473) and anti-Akt from Cell Signaling (Danvers, MA); mouse anti-phospho-JNK and anti-JNK were from Sigma (St. Louis, MO). Secondary antibodies used were horseradish peroxidase-conjugated anti-rabbit and mouse antibody (GE Healthcare). Band intensities were quantified with Multi Gauge software (Fujifilm, Tokyo, Japan).
Isolation of total RNA and quantitative RT-PCR

Total RNA was isolated from liver of APCs-treated mouse using Trizol (Invitrogen).

SYBR Green PCR Master Mix (Applied Biosystems, Foster, CA, USA) was prepared for the quantitative RT-PCR run using TNF-α primer with the following sequence:

5’-AAATGGGCTTTCCGAATTCA-3’ and 5’-CAGGGGAAGAATCTGGAAAGGT-3’,

IL-6 primer with the following sequence: 5’-GGAGGCTTAATTACACATGTT-3’ and 5’-TGATTTCAGATGAATTGGAT-3’, IL-1β primer with the following sequence: 5’-ATCTTTGGGTCCGTAACCTGAPDH-3’ and 5’-GCAACTGTTCTGAACCTCAACT-3’. GAPDH mRNA was used as an internal control. The sequences of GAPDH primer are as follows:

5’-AAATGGTGAAGGTCGG-3’ and 5’-TCGTTGATGGCAACAA-3’. The thermal cycling conditions were denaturation at 95 °C for 10 min followed by 50 cycles at 95 °C for 15 s and 60 °C for 1 min. mRNA levels were measured by real-time quantitative RT-PCR using ABI PRISM 7000 Sequence Detection System (Applied Biosystems/Life Technologies Japan, Tokyo, Japan).
Statistical analysis

The data are expressed as means ± SE. Statistical significance was determined by unpaired Student’s t-test. \( P < 0.05 \) was considered significant.

RESULTS

Body weight, food intake, and energy expenditure

We first evaluated the effect of APCs on body weight of ob/ob mice. There was no difference between APCs-treated and untreated mice during the test period (Figure 2A).

Dietary food intake (Figure 2B) and water intake (APCs-treated mice: 6.24 ± 0.32 ml of 0.5% APCs water per day, untreated mice: 7.11 ± 0.24 ml of water per day) were also unchanged. Similarly, energy expenditure was not significantly different between APCs-treated and untreated mice (Figure 2C).

Glucose tolerance test and insulin tolerance test

In OGTT, blood glucose levels were significantly lower at 15 min and 30 min in APCs-treated ob/ob than those in untreated mice (Fig. 3A). The serum insulin levels did not differ at these time points (Figure 3B). The value of HOMA-IR was
significantly lower in APCs-treated (27.3 ± 7.9) than that in untreated mice (76.0 ± 13.3). In ITT, blood glucose levels were significantly lower in APCs-treated ob/ob (Figure 3C). These data suggest that APCs ameliorate insulin resistance in ob/ob mice.

Insulin resistance contributes to an adaptive change in pancreatic β-cell mass. We therefore observed pancreatic islets morphologically by immunohistochemistry using anti-insulin and anti-glucagon antibodies (Figure 3D). β-cell area was decreased by 21% in APCs-treated compared with that in untreated mice (Figure 3E). On the other hand, there was no difference in the number of islets (Figure 3F). These data suggest that hypertrophy of pancreatic islets was suppressed by treatment of APCs.

Effects of APCs on body composition, adipocyte size, and serum adiponectin level

We then examined the effects of APCs on adipose tissue, a target organ for insulin. There was no difference in lean mass and fat composition between APCs-treated and untreated mice (Figure 4A). In addition, the size of adipocytes and serum adiponectin levels did not change (Figure 4B and C). It is therefore unlikely that APCs have an effect on insulin resistance in adipose tissue.

Effects of APCs on hepatic insulin signals and lipid content
Hepatic gluconeogenesis is enhanced in the state of insulin resistance.\textsuperscript{25} In PTT, APCs-treated ob/ob mice displayed lower blood glucose levels at 15 min and 30 min after pyruvate injection, indicating that APCs treatment suppresses hepatic gluconeogenesis (Figure 5A). It was reported that Akt phosphorylation of ob/ob mice was down-regulated, indicating impairment of the insulin signaling.\textsuperscript{26} Insulin-stimulated Akt phosphorylation was elevated in APCs-treated compared with untreated mice (Figure 5B). Accumulation of fat in liver induces insulin resistance.\textsuperscript{27} We therefore estimated lipid content in liver. Interestingly, total cholesterol and TG contents in liver did not differ between APCs-treated and untreated mice (Figure 5C and D). Similarly, change of lipid content was not observed by using other methods such as H&E staining and Oil Red O staining (Figure 5E). These data indicate that APCs suppress hepatic gluconeogenesis by improving the insulin signal without altering fat accumulation in liver.

**Effects of APCs on inflammation in liver**

We then considered whether APCs influence inflammation in liver to ameliorate insulin resistance, and evaluated macrophage infiltration into liver by immunostaining using anti-F4/80 anti-body, a marker for mature mouse macrophage.\textsuperscript{28} The number of...
macrophage in liver was decreased in APCs-treated ob/ob mice (Figure 6A). We examined the mRNA expression levels of pro-inflammatory cytokine in liver, and found that mRNAs of TNF-α and IL-6 were down-regulated by APCs treatment (Figure 6B). TNF-α activates TNF receptors on hepatocytes to induce JNK activation. We therefore examined JNK activation by immunoblotting using anti-phospho-JNK and anti-JNK antibody. Phosphorylation of JNK was decreased by APCs treatment in liver (Figure 6C). These data suggest that APCs treatment attenuates inflammation in liver.

**DISCUSSION**

In this study, we show that oral administration of APCs ameliorates glucose intolerance in obese diabetic ob/ob mice. Continuous but not single (Figure S2) oral administration of APCs ameliorated glucose intolerance, and suppressed the expression of inflammation-related genes and phosphorylation of JNK in liver, suggesting that APCs improve insulin sensitivity in liver through suppression of chronic inflammation. In our preliminary experiments, it was confirmed that the dosage of 0.5% APCs in drinking water is the appropriate concentration that has no effects on water intake, food intake or body weight.

Obesity is associated with chronic, low-grade, and systemic inflammation that may
contribute to the development of insulin resistance and type 2 diabetes. Generally, adipose tissue inflammation is considered to initiate adipocyte hypertrophy and hyperplasia and to influence release of adipocytokines and pro-inflammatory signaling. On the other hand, obesity also is associated with the regulation of adipocytokine secretion, and causes adverse effects on inflammation and insulin sensitivity. Akiyama reported that apple proanthocyanidins do not affect body weight or food intake in \( W/W^V \) and B10A mice. There are several reports suggesting that procyanidins improve glycemic control. In accord with these data, while the body weight, food intake, and fat mass of APCs-treated ob/ob mice were unchanged compared with those of untreated mice, the insulin sensitivity was significantly improved in our study. The beneficial effects of procyanidins from various plants have been investigated. Procyanidin from cinnamon was reported to lower levels of blood glucose, total cholesterol, low-density cholesterol, and hemoglobin A1c in type 2 diabetes. Persimmon peel proanthocyanidins decrease blood glucose levels and glycosylated protein concentrations and have a protective effect against diabetes-induced oxidative stress in streptozotocin-induced diabetic rats. Recently, tetrameric procyanidins from cacao liquor were shown to increase the levels of plasma glucagon-like peptide-1.
(GLP-1), an incretin hormone that potentiates insulin secretion.\textsuperscript{35} Our data showing that APCs ameliorate impaired hepatic insulin signaling in ob/ob mice may be useful in clarifying the therapeutical actions of substances from vegetables and fruits.

Hepatic insulin resistance is a key feature of obesity-related type 2 diabetes.\textsuperscript{36} Kupffer cells, which are liver-resident macrophage-like cells, are activated by inflammation, apoptosis, and necrosis of hepatocytes. Activation of Kupffer cells causes the release of inflammatory cytokines such as TNF-\(\alpha\) and IL-6 in liver.\textsuperscript{37} It is reported that accumulation of fat in hepatocyte induces inflammation in liver.\textsuperscript{38} However, APCs treatment decreased the number of macrophages and the expression levels of TNF-\(\alpha\) and IL-6 in liver without changing the lipid content in the liver of ob/ob mice. These data suggest that the effect of APCs on liver inflammation is not due to suppression of fat accumulation in hepatocytes.

It was suggested that intestinal bacteria may contribute to the pathogenesis of inflammation in liver. Lipopolysaccharide (LPS), one of the gut-derived endotoxins, was reported to cause liver damage via activation of Kupffer cells and release of TNF-\(\alpha\) and other cytokines.\textsuperscript{39} Obesity alters the ecology of intestinal microbiota.\textsuperscript{40} It is reported that ob/ob mice have higher endotoxin levels in the portal blood than that in wild-type mice.\textsuperscript{41} Recent studies suggested that procyanidins can be degraded by some
kinds of intestinal microbiota\textsuperscript{42} and also that administration of apple flavonoid alters intestinal microbiota.\textsuperscript{43} In addition to our preliminary experiment using ob/ob mouse (data not shown), we have carried out an experiment whether APCs could change the microbiota of high-fat/high-sucrose (HFHS)-fed C57BL/6J male mice.\textsuperscript{44} We found the chronic oral administration of high polymeric APCs prevent obesity associated with gut microbial and metabolic changes. It is therefore possible that APCs suppress inflammation in liver not by decreasing fat accumulation but through effects on intestinal microbiota. Glycolysis, gluconeogenesis, glycogenolysis and glycogen synthesis is involved in the maintenance of blood glucose levels.\textsuperscript{45} Both gluconeogenesis and glycogenolysis are important in glucose production in liver. Pyruvate is one of well-known substances of gluconeogenesis. In this study, we showed APCs are considered to be suppress pyruvate induced gluconeogenesis (Figure 5A). However, the effect of APCs on glycogen metabolism is not clear. Further studies are needed to clarified the details of how APCs ameliorate glucose resistance in diabetic state.

In conclusion, our data indicate that oral administration of APCs ameliorates insulin resistance by improving hepatic insulin signaling through suppression of inflammation in ob/ob mice. Moreover, further investigation of the mechanism of the effects of APCs
on glucose metabolism could shed light on the pathophysiology of insulin resistance and suggest new targets for type 2 diabetes therapy.

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**Notes**

The authors declare no competing financial interest.
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ABBREVIATIONS USED

APCs (apple procyanidins), OGTT (oral glucose tolerance test), ITT (insulin tolerance test), PTT (pyruvate tolerance test), TNF (tumor necrosis factor), IL (interleukin), JNK (c-Jun N-terminal kinase), IRS (insulin receptor substrate), SGLT (sodium glucose cotransporter), HOMA-IR (homeostasis model assessment of insulin resistance), GLP (glucagon-like peptide)

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Figure Legends

Figure 1
Reversed phase HPLC profile of apple polyphenol (upper) and apple procyanidins (lower). These panels show that our apple procyanidins do not include chlorogen acid or phloretin glucoside (Phlorizin) fractions, which are included as apple polyphenols.

Figure 2
Effects of APCs administration on body weight, food intake, and energy expenditure in ob/ob mice. (A) Body weight (n=12) and (B) food intake (n=12) were measured once every week. (C) Energy expenditure (n=4) was measured for 48 h. APCs (-) and APCs (+) indicate APCs-untreated and APCs-treated ob/ob mice, respectively. Results are presented as mean ± SE. *P<0.05
Figure 3

Effects of APCs administration on OGTT, ITT, and pancreatic islet size in ob/ob mice.

(A) Blood glucose levels and (B) serum insulin levels were measured during OGTT (load of glucose 1g/kg body weight). (C) Blood glucose levels measured during ITT (load of insulin 2U/kg body weight) (D) immunocytochemistry of pancreatic islets. (E) β-cell area in the total pancreatic area (n=4) and (F) the number of islets per area of pancreas (n=4). APCs (-) and APCs (+) indicate APCs-untreated and APCs-treated ob/ob mice, respectively. Results are presented as mean ± SE. *P<0.05

Figure 4

Effects of APCs administration on adipose tissue adipocyte size and serum adiponectin level in ob/ob mice. (A) Visceral, subcutaneous, and total fat mass in APCs-treated and untreated mice were measured using CT images of transverse abdominal sections (n=4). (B) Representative images of HE staining of adipose tissue section. (C) Serum adiponectin concentrations (n=8). APCs (-) and APCs (+) indicate APCs-untreated and treated ob/ob mice, respectively. Results are presented as mean ± SE.
Figure 5

Effects of APCs administration on liver tissue in ob/ob mice. (A) Glucose levels measured during PTT (load of pyruvate 1g/kg body weight, n=8) (B) The ratio of phosphorylated Akt to total Akt (t-Akt) in liver tissues (n=3). (C) Total cholesterol (n=4) level and (D) triglycerides level in liver were measured (n=4). (E) HE and Oil red O staining of the liver. APCs (-) and APCs (+) indicate APCs-untreated and treated ob/ob mice, respectively. Results are presented as mean ± SE. *P<0.05

Figure 6

Effects of APCs administration on liver inflammation in ob/ob mice.

(A) Immunohistochemistry with anti-F4/80 antibody in liver of APCs-treated and untreated mice. Arrows indicate representative macrophages in liver. (B) mRNA levels of TNF-α, IL-6, and IL-1β in the liver (n=8). (C) Effects of APCs administration on the ratio of phosphorylated JNK to total JNK (t-JNK) in liver in ob/ob mice (n=3). APCs (-) and APCs (+) indicate APCs-treated and untreated ob/ob mice, respectively. Results are presented as mean ± SE.
FIGURE 1

[Graph showing chromatograms labeled 'Apple Polyphenol' and 'Apple Procyanidin'. Peaks are labeled 'Chlorogenic acid' and 'Phloretin glucoside'.]
**FIGURE 2**

A

**Body weight (g)**

- **APCs (−)**
- **APCs (+)**

0 1 2 3 4 5 6 7 8

weeks after administration

B

**Food intake (g/day)**

- **APCs (−)**
- **APCs (+)**

0 1 2 3 4

weeks after administration

C

**Energy expenditure (cal/min/kg)**

- **APCs (−)**
- **APCs (+)**

0 50 100 150 200 250 300

(time)

**dark**

ACS Paragon Plus Environment
FIGURE 3

A. Blood glucose (mg/dL) over time with APCs (-) and APCs (+).

B. Serum insulin (ng/mL) over time with APCs (-) and APCs (+).

C. Blood glucose (%) over time with APCs (-) and APCs (+).

D. Images showing β-cell area in APCs (-) and APCs (+) with corresponding scales of 100 μm.

E. Bar graph showing β-cell area (% of pancreas) with APCs (-) and APCs (+).

F. Bar graph showing islet number per pancreas area (× 10^3/mm^2) with APCs (-) and APCs (+).
FIGURE 4

A

APCs(-)  APCs(+)

Viseral fat  Subcutaneous fat  Lean mass

Lean and fat mass (g)

B

APCs (-)  APCs (+)

100μm  100μm

C

Serum adiponectin (ng/mL)

APCs (-)  APCs (+)
FIGURE 5

A

Blood glucose (mg/dL)

APCs (-)  
APCs (+)

(min)

0 30 60 90 120

B

insulin

p-Akt
t-Akt

60kD

insulin

-  +  +  +  +

-  +  +  +  +

APCs (-)  APCs (+)

pAkt/t-Akt (ratio)

C

Total cholesterol (mg/g)

APCs (-)  APCs (+)

D

Triglycerides (mg/g)

APCs (-)  APCs (+)

E

APCs (-)  APCs (+)

HE

Oil red O

ACS Paragon Plus Environment
FIGURE 6

A

FITC

APCs (-)

100μm

APCs (+)

100μm

HRP

B

TNF-α

IL-6

IL-1β

Relative mRNA/GAPDH

0

0.2

0.4

0.6

0.8

1

1.2

1.4

*  

APCs (-)  

APCs (+)

C

p-JNK

APCs (-)

APCs (+)

100μm

54kDa

46kDa

t-JNK

pJNK/t-JNK (ratio)

0

0.2

0.4

0.6

0.8

1

1.2

1.4

*  

APCs (-)  

APCs (+)
Ameliorate insulin resistance via suppression of pro-inflammatory cytokines expression in liver

**OGTT**

<table>
<thead>
<tr>
<th>Blood glucose (mg/dL)</th>
<th>APCs (-)</th>
<th>APCs (+)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>200</td>
<td>100</td>
</tr>
<tr>
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<td>400</td>
<td>300</td>
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<tr>
<td>60</td>
<td>500</td>
<td>400</td>
</tr>
<tr>
<td>90</td>
<td>600</td>
<td>500</td>
</tr>
<tr>
<td>120</td>
<td>700</td>
<td>600</td>
</tr>
</tbody>
</table>

**ITT**

<table>
<thead>
<tr>
<th>Blood glucose (%)</th>
<th>APCs (-)</th>
<th>APCs (+)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>20</td>
<td>10</td>
</tr>
<tr>
<td>30</td>
<td>40</td>
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</tr>
<tr>
<td>60</td>
<td>60</td>
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<tr>
<td>90</td>
<td>80</td>
<td>70</td>
</tr>
<tr>
<td>120</td>
<td>100</td>
<td>90</td>
</tr>
</tbody>
</table>

**PTT**

<table>
<thead>
<tr>
<th>Blood glucose (mg/dL)</th>
<th>APCs (-)</th>
<th>APCs (+)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>200</td>
<td>100</td>
</tr>
<tr>
<td>30</td>
<td>400</td>
<td>300</td>
</tr>
<tr>
<td>60</td>
<td>500</td>
<td>400</td>
</tr>
<tr>
<td>90</td>
<td>600</td>
<td>500</td>
</tr>
<tr>
<td>120</td>
<td>700</td>
<td>600</td>
</tr>
</tbody>
</table>

**Immunohistochemistry with anti-F4/80 antibody in liver**

ACS Paragon Plus Environment