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Ellagic acid in pomegranate suppresses resistin secretion by a novel regulatory mechanism involving the degradation of intracellular resistin protein in adipocytes

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ABSTRACT

Resistin, an adipocytokine, is considered the link between obesity and type 2 diabetes. Pomegranate is a rich source of compounds used to treat metabolic diseases including type 2 diabetes. In this study, we found that consumption of pomegranate fruit extract (PFE) predominantly reduced the serum resistin levels in ovariectomized mice, an animal model with elevated resistin levels in serum and upregulated resistin mRNA expression in white adipose tissue. Moreover, the PFE significantly reduced the secretion and intracellular protein levels of resistin in differentiated murine 3T3-L1 adipocytes, but it did not alter resistin mRNA expression. When *de novo* protein synthesis was inhibited by the protein synthesis inhibitor cycloheximide, the intracellular resistin protein levels were drastically reduced by the PFE, suggesting that the PFE promoted the degradation of resistin at the protein level. We also found that ellagic acid (EA), a main component of pomegranate, had the same effects on the secretion and intracellular protein level of resistin. These results suggest that EA in pomegranate suppresses resistin secretion by a novel mechanism involving the degradation of intracellular resistin protein in adipocytes.

1. Introduction

Type 2 diabetes is a major, chronic disease associated with obesity. Adipose tissue is considered to play a central role in the development of type 2 diabetes because it secretes hormones—called adipocytokines—that regulate insulin resistance, a contributing factor for this disease [1,2].

Expression of *Retn*, encoding the adipocytokine resistin, is downregulated by the antidiabetic drug thiazolidinedione in mouse adipose tissue [3], suggesting that resistin is the link between obesity and type 2 diabetes [3,4]. Both high-fat diet-fed and *ob/ob* mice show elevated serum levels of resistin [5]. Humans with obesity and type 2 diabetes also have elevated plasma levels of this adipocytokine [6,7]. Further, *Retn* knockout improves insulin sensitivity and blood glucose levels [4,8] whereas overexpression or exogenous administration of this gene impairs insulin activity and glucose metabolism in rodents [3,4,9,10]. Therefore, type 2 diabetes is potentially preventable by suppressing elevated serum resistin levels.

Pomegranate (*Punica granatum*, Punicaceae) contains compounds such as anthocyanins, hydrolyzable tannins, and estrogenic flavonols and flavones [11,12]. In humans with type 2 diabetes, pomegranate consumption reportedly lowers lipid peroxide and serum cholesterol levels, improving cardiovascular disease [13,14]. Further, pomegranate seed oil lowers the elevated blood glucose levels in a high-fat diet-fed disease model (CD-1 mice), accompanied by improvements in elevated serum leptin and adiponectin levels [15]. Punicic acid (PUA), a main component of pomegranate seeds, reportedly ameliorates the elevated fasting blood glucose levels and glucose intolerance in a diabetic animal model (*db/db* mice) [16]. However, the effects of pomegranate on resistin remain unknown.

Ovariectomy can increase the resistin levels in serum and mRNA expression in white adipose tissue, which is due to the hormonal stimulated obesity [17,18]. In this study, we fed pomegranate fruit extract (PFE) to ovariectomized (OVX) mice as an animal model with high resistin levels to investigate the effects of pomegranate on resistin *in vivo*. Moreover, we investigated the effect of PFE on resistin secretion in differentiated murine 3T3-L1 adipocytes to investigate the mechanism of the action of PFE on resistin.

2. Materials and methods

2.1. Preparation of PFE

Pomegranate fruit juice from California (the Wonderful species, quintuple strength, Brix 65) was kindly provided by Japan Food &

Abbreviations: CHX, cycloheximide; EA, ellagic acid; MMP, matrix metalloproteinase; OVX, ovariectomized; PFE, pomegranate fruit extract; PUA, punicic acid; SNARE, soluble *N*-ethylmaleimide-sensitive factor attachment.

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Liquor Alliance (Kyoto, Japan). The extract was dialyzed through a cellulose tube (pore size of 36/32, Sanko Jun-yaku Co., Tokyo, Japan) in water overnight. The dialysate was evaporated and freeze-dried to obtain PFE in powdered form.

2.2. Animal experiments

Twelve-week-old female ddY mice (Japan SLC, Shizuoka, Japan) were ovariectomized (OVX group) or sham operated (Sham group, controls). They housed individually and were maintained on a standard 12-h light–dark cycle during the study. The OVX mice were divided into two groups according to the type of feed: the OVX group was fed commercial diet (MF; Oriental Yeast Co., Tokyo, Japan) only and the OVX–PFE group received the MF containing PFE (30 mg/kg body weight/day) for 12 weeks. All animal experiments were approved by the Institutional Animal Care and Use Committee and carried out according to the Kinki University Animal Experimentation Regulations.

Body weight, food intake, and water intake were measured twice weekly by using a digital scale, and the data were averaged to yield the weekly values. After 12 weeks, the mice were sacrificed under anesthesia with pentobarbital and their parametrial adipose-tissue weight was measured after collecting. Blood samples were collected at room temperature, and serum was obtained by centrifugation at 3000*g* for 10 min and stored at −80 °C until use. The serum levels of glucose, triglyceride, and total cholesterol were determined by enzymatic assay (Wako Pure Chemical Industries, Osaka, Japan). Further, the serum levels of resistin and adiponectin were measured by using an ELISA Duo Kit (R&D Systems, Minneapolis, MN, USA) and those of leptin were measured by using a Leptin ELISA Kit (Morinaga Institute of Biological Science, Kanagawa, Japan).

2.3. Cell culture and adipocyte differentiation

Mouse 3T3-L1 cells were purchased from American Type Culture Collection (ATCC, Manassas, VA, USA). They were maintained in DMEM (Nissui Pharmaceutical Co., Tokyo, Japan) supplemented with 10% FBS (Gibco®, Life Technologies, Rockville, MD, USA) and 4.5 g/L glucose at 37 °C under an atmosphere of 5% CO_2 and seeded on 6-well plates. Two days after the cells reached confluence, their differentiation into adipocytes was induced by incubation first in DMEM containing 0.5 nM 3-isobutyl-1-methylxanthine, 2 μM dexamethasone, and 10 μg/mL insulin (Takara Bio, Shiga, Japan) for 2 days and then in DMEM containing 10 μg/mL insulin for 2 days. Thereafter, the culture medium was refreshed with DMEM every 2 days until the cells were used at 10–12 days after the induction of differentiation.

2.4. Adipocyte experiments

The PFE was dissolved in dH_2O and sterilized by membrane filtration (0.22-μm pore size). Cycloheximide (CHX; Nacalai Tesque, Kyoto, Japan), PUA (Larodan Fine Chemicals, Malmö, Sweden), and ellagic acid (EA; Nacalai Tesque) were dissolved in DMSO. In addition, all the reagents were dissolved in serum-free DMEM at the concentration used in the experiments. The final concentration of DMSO added to the 3T3-L1 adipocyte culture was 1% (v/v).

2.5. Western blot analysis

Rabbit polyclonal antibody against mouse resistin was generated previously [19]. Antibodies against adiponectin (MAB11192, R&D Systems) and β-actin (MAB1501, Millipore, Billerica, MA, USA) were also used for western blotting.

First, 3T3-L1 adipocytes were washed twice with PBS and added to 200 μL/well RIPA buffer (Nacalai Tesque). The plates were shaken for 15 min on ice, and the lysates were harvested with a cell scraper and rinsed with 100 μL/well RIPA buffer. They were then shaken for 1 h on ice and centrifuged at 9000*g* for 10 min. After removal of fats and residues, the protein concentration of the supernatant was measured by using a Pierce BCA Protein Assay Kit (Thermo Fisher Scientific, Rockford, IL, USA). Thirty micrograms of protein from each lysate was boiled for 5 min in SDS sample buffer [20]. Medium samples that collected at 12 h after treatment of PFE with 3T3-L1 adipocytes were also boiled in SDS sample buffer. Proteins were separated by SDS–PAGE and transferred onto PVDF membranes (Immobilon™-P, Millipore). All immunoreacted proteins were detected by using ECL™ Western Blotting Detection Reagents (GE Healthcare, Buckinghamshire, UK).

2.6. RNA extraction and real-time RT-PCR

Total RNA was extracted by using a High Pure RNA Isolation Kit (Roche, Mannheim, Germany) and reverse-transcribed by using Super Script® III First-Strand Synthesis SuperMix (Invitrogen™, Life Technologies).

Real-time RT-PCR was performed by using the Thermal Cycler Dice™ Real Time System (TP800; Takara Bio) with 2× SYBR® Premix Ex Taq™ (Takara Bio). The primer sequences used for the amplification of resistin cDNA were 5′-GACTTCAACTCCCTGTTTCC-3′ (forward) and 5′-CTCAAGACTGCTGTGCCTTC-3′ (reverse). The PCR cycle consisted of denaturation at 95 °C for 30 s, 40 cycles of annealing at 95 °C for 5 s, and extension at 60 °C for 30 s. Reactions were performed in triplicate for each sample, and gene expressions were normalized to *Gapdh* expression. All assays were carried out in 96-well format plates.

2.7. Statistical analysis

Data represent means ± SE and each of animal experiments and cell experiments were analyzed by Scheffe's method and Student's *t*-test, respectively. Values of $p < 0.05$ were considered statistically significant.

3. Results

3.1. Effect of PFE on elevated serum resistin levels in OVX mice

We first investigated whether the PFE affected the serum resistin levels in OVX mice. The OVX group had significantly higher serum resistin levels than the Sham group (Table 1). However, the OVX-induced increase in serum resistin levels was significantly reduced in the PFE group. Further, the OVX group exhibited significantly increased body weight, parametrial adipose-tissue weight, and serum levels of total cholesterol, adiponectin, and leptin compared with the Sham group. The OVX–PFE group tended to have lower parametrial adipose-tissue weight and serum total-cholesterol levels than the OVX group. However, PFE consumption had no effect on the serum levels of adiponectin, an adipocytokine associated with insulin sensitivity [21,22].

3.2. Effect of PFE on resistin secretion by 3T3-L1 adipocytes

We then investigated whether the PFE could suppress resistin secretion by 3T3-L1 adipocytes. Resistin secretion increased time-dependently from 3 to 9 h in the control group (cultured in serum-free medium). In the groups treated with 50 and 100 μg/mL of the PFE, resistin secretion during this period was significantly suppressed (Fig. 1A). It was concerned that PFE disturbs res-