Kibizu concentrated liquid suppresses the accumulation of lipid droplets in 3T3-L1 cells

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Abstract Adipocyte size is closely related to the occurrence of diabetes, metabolic syndrome, and insulin resistance. Thus, researchers are searching for active substances that function to reduce adipocyte size. In the present study, we focused on sugar cane vinegar, Kibizu, and evaluated the function of Kibizu to reduce adipocyte size by using an in vitro model system, because people in Amami Oshima famous for longevity regularly consume Kibizu. Results showed that Kibizu treatment significantly reduced the size and number of lipid droplets in 3T3-L1 cells, relative to treatment with Kurozu, another traditional vinegar. Results of an extraction experiment suggest that the active components in Kibizu are lipophilic and hydrophobic. In addition, an in vivo experiment on rats treated with Kibizu showed that the active components were contained in large vein blood. Results of an additional in vivo experiment suggest that metabolites generated by Kibizu-treated rats are

primarily contained or modified specifically in the large vein blood.

Keywords 3T3-L1 cells · Adipocyte size · Kibizu · Kurozu · Lipid droplets · Vinegar

Introduction

Adipocytes are recognized as endocrine cells that are controlled by food intake and energy expenditure, and the endocrine function of adipocytes depends on adipocyte size. In particular, large adipocytes secrete fatty acids and tumor necrosis factor α that might cause insulin resistance (Kubota et al. 1999). Thus, reduction of adipocyte size is a target to prevent obesity and metabolic syndrome. We and other researchers are attempting to identify functional foods that inhibit obesity by using in vitro and in vivo assay systems.

Among traditional foods, vinegar has been used as a condiment and alternative medicine (Horiuchi et al. 1999). Currently, various types of vinegar are produced by using different sources and technologies (Gullo and Giudici 2008). The main component of vinegar, acetic acid, has various beneficial effects against hyperglycemia (Sakakibara et al. 2006), dyslipidemia (Fushimi et al. 2006), and hypertension (Kondo et al. 2001). Thus, many researchers have focused on vinegars and their functionalities.

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The population of Amami Oshima in south Japan is now famous for longevity, which is thought to result from the regular consumption of traditional foods. One of these candidate traditional foods is sugar cane vinegar, Kibizu. Thus, in the present study, we evaluated the function of Kibizu to suppress lipid accumulation in adipocytes.

Materials and methods

Materials

Kurozu and Kibizu are fermented vinegars made from brown rice and sugar cane, respectively. Concentrated liquids of Kurozu and Kibizu were provided by Senriki (Osaka, Japan). Kurozu and Kibizu concentrated liquid contains 6.20 and 6.87 % acetic acid, respectively. We adjusted the pH of both vinegars to 7.2 prior to administering them to the cells. Both vinegars were extracted with water, n-butanol, or diethyl ether in a conventional method. After evaporation, concentrated fractions were used in the assays.

Cell culture and treatment

3T3-L1 preadipocytes (JCRB9014) were maintained in Dulbecco's Modified Eagle Medium (DMEM; Nissui, Tokyo, Japan) containing 10 % fetal bovine serum (FBS; Life Technologies, Carlsbad, CA, USA) at 37 °C in an atmosphere containing 5 % CO₂. The preadipocytes were differentiated in DMEM containing 10 % FBS, 0.5 mM 3-isobutyl-1-methylxanthine (Wako, Osaka, Japan), 0.25 µM dexamethasone, and 10 µg/mL insulin for 72 h (Phase I). After 3 days, culture medium was changed to insulin medium (10 µM insulin, 10 % FBS), and cells were cultured for an additional 24 h (Phase II). After this culture, cells were cultured in DMEM supplemented with 10 % FBS. Samples were added to DMEM during Phase I and II. The same volume of samples was added to DMEM, because pH values of these samples were almost the same.

Quantification of lipid droplets

Differentiated 3T3-L1 cells were fixed with 4 % paraformaldehyde at room temperature for 1 h and stained with BODIPY493/503 (Life Technologies, Gaithersburg, MD, USA) at room temperature for

20 min. Nuclei were counterstained with Hoechst 33342 (Dojin, Kumamoto, Japan) at room temperature for 15 min to count cell number. Fluorescence images for BODIPY493/503 and Hoechst 33342 were obtained by using IN Cell Analyzer 1000 (GE Healthcare, Little Chalfont Buckinghamshire, UK). Area and numbers of lipid droplets and cells were analyzed by Developer software (GE Healthcare) and visualized by using Spotfire DecisionSite Client 8.2 software.

Preparation of rat serum

Ten-week-old male Brown Norway (BN) rats were obtained from Charles River Laboratory Japan (Tokyo, Japan). These rats were divided into two groups (Sham and Kibizu) so that the average body weights and blood glucose levels were comparable among these groups. Kibizu diluted in deionized water were given to the rats at a dose of 100 mg/kg body weight. After 30 min, portal blood, hepatic vein blood, and large vein blood were collected. The blood was incubated at room temperature for 30 min and then centrifuged at 1,000×g for 10 min to collect the serum. Experiment was done by using 1 rat per 1 group, and similar results were obtained in two independent experiments. Serum from rats which were not administered with Kibizu but underwent operation was designated Sham.

This experiment was carried out according to the Guidance for Animal Experiments of the Faculty of Agriculture and the Graduate Course, Kyushu University, Fukuoka, Japan (A24-032-2) and according to Law No. 105 and Notification No. 6 of the Government of Japan.

Statistical analysis

All results were expressed as mean ± standard error of mean. Statistical differences were determined by a two-sided Student's *t* test, and *P* < 0.05 was considered statistically significant (*P < 0.05; **P < 0.01; ***P < 0.001).

Results and discussion

Kibizu concentrated liquid suppressed accumulation of lipid droplets in 3T3-L1 cells

Dietary vinegar reportedly reduces serum total triacylglycerol and cholesterol in rats and human (Fushimi

et al. 2006, 2002; Yamashita et al. 2007), suggesting that vinegar plays a role in decreasing adipocyte size. We previously showed that the number of small adipocytes increased and that of large adipocytes decreased in adipocyte tissues in Kurozu-treated rats (Tong et al. 2010). Here, we evaluated the effects of a novel vinegar made from sugar cane, Kibizu, on lipid accumulation in adipocytes. Results clearly showed that the lipid area and the number of lipid droplets per cell significantly decreased in 3T3-L1 cells treated with vinegars (Fig. 1), which were quantified by using IN Cell Analyzer 1000 and BODIPY493/503. Among these vinegars, Kibizu concentrated liquid most strongly suppressed lipid droplet accumulation in 3T3-L1 cells.

n-Butanol/diethyl ether-extracted vinegar fractions strongly suppressed the accumulation of lipid droplets in 3T3-L1 cells

To characterize the active components, we fractionated the vinegars with water, n-butanol, and diethyl ether, and then we added 50 $\mu\text{g/mL}$ fractions to 3T3-L1 cells. After the induction of differentiation, lipid droplets were quantified as described above. As shown

in Fig. 2, fractions extracted with n-butanol or diethyl ether, but not those extracted with water, strongly suppressed accumulation of lipid droplets in 3T3-L1 cells. In particular, n-butanol- or diethyl ether-extracted fractions of Kibizu strongly suppressed the lipid accumulation in 3T3-L1 cells, relative to those treated with Kurozu, suggesting that hydrophobic ingredients contained primarily or abundantly in Kibizu suppress the lipid accumulation in 3T3-L1 cells. Kibizu contains a large quantity of polyphenols, relative to Kurozu and other vinegars; thus, the functional hydrophobic components should be identified in future studies.

Large vein blood serum from Kibizu-treated rats suppressed lipid accumulation in 3T3-L1 cells

Next, we evaluated the characteristics of active components isolated from rats administered Kibizu concentrated liquid. We collected portal blood, hepatic vein blood, and large vein blood from treated rats and prepared serum samples from these bloods. We added serum at a final concentration of 10 % to 3T3-L1 cells during Phases I and II of differentiation. After the induction of differentiation, we quantified the lipid

Fig. 1 Kibizu concentrated liquid suppressed accumulation of lipid droplets in 3T3-L1 cells. 3T3-L1 cells were seeded onto a 96-well plate; transferred to Dulbecco's Modified Eagle Medium (DMEM) containing 10 % FBS, 0.5 mM 3-isobutyl-1-methylxanthine, 0.25 μM dexamethasone, and 10 $\mu\text{g/mL}$ insulin; and cultured in the presence of 0.5 % vinegar, Kurozu concentrated liquid, or Kibizu concentrated liquid for 72 h. Next, cells were transferred to insulin medium (10 μM insulin,

10 % FBS), and cells were cultured in the presence of 0.5 % vinegar, Kurozu concentrated liquid, or Kibizu concentrated liquid for an additional 24 h. After this culture, cells were cultured in DMEM supplemented with 10 % FBS. The numbers of cells (a) and lipid droplets and the lipid area (b) were quantified by using IN Cell Analyzer 1000 as described in "Materials and methods". Corresponding photographs were also included

Fig. 2 The n-butanol/diethyl ether-extracted fractions strongly suppressed the accumulation of lipid droplets in 3T3-L1 cells. Kurozu and Kibizu concentrated liquids were extracted with water, n-butanol (n-BtOH), or diethyl ether (Et₂O). Differentiation was induced in 3T3-L1 cells in the presence of 50 μg/mL extracted fractions. The numbers of cells (a) and lipid droplets (b) and the lipid area (c) were quantified as described in “Materials and methods”

accumulation in 3T3-L1 cells as described above. Results showed that serum samples tended to suppress the accumulation of lipid droplets in 3T3-L1 cells (Fig. 3). In particular, large vein blood serum significantly suppressed lipid accumulation. These results suggest that Kibizu contains active components that suppress lipid accumulation in 3T3-L1 cells and, furthermore, that Kibizu metabolites generated after exposure of rats to Kibizu are contained primarily or

Fig. 3 Large vein blood serum from Kibizu-treated rats suppressed lipid accumulation in 3T3-L1 cells. We collected portal blood, hepatic vein blood, and large vein blood from Kibizu-treated rat. Differentiation was induced in 3T3-L1 cells in the presence of 10 % serum from these bloods. The numbers of cells (a) and lipid droplets (b) and the lipid area (c) were quantified as described in “Materials and methods”

modified specifically in the large vein blood. Thus, the active components contained in Kibizu concentrated liquid should be identified in future studies.

Conclusions

In our previous study, we showed that average adipocyte size in subcutaneous and perirenal adipose

tissues significantly decreased and the number of small adipocytes in various tissues significantly increased in rats treated with Kurozu concentrated liquid (Tong et al. 2010). However, in this study, we observed that Kibizu more strongly suppressed lipid accumulation in 3T3-L1 cells than Kurozu, suggesting that active components differ between these vinegars. The different results likely arise from different sources, fermentation processes, and/or quantity of active components in Kibizu as compared to Kurozu. Furthermore, Mimura et al. reported that the lipophilic fraction of Kibizu extracted with 40 % methanol showed potent radical-scavenging and growth inhibition in leukemia cells (Mimura et al. 2004). In agreement with those previous results, we also observed strong suppression of lipid accumulation in n-butanol- or diethyl ether-extracted Kibizu fractions, suggesting that similar lipophilic or hydrophobic active components are responsible for these activities.

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