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Antidiabetic Activities of Chalcones Isolated from a Japanese Herb, *Angelica keiskei*

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Diabetes mellitus is a chronic disease that is characterized by hyperglycemia caused by insufficient insulin action. We have explored the edible ingredients from folk medicines in Japan that contain substances complementing insulin action, such as the induction of adipocyte differentiation and the enhancement of glucose uptake. We eventually found that the ethanol extract from a Japanese herb "Ashitaba", *Angelica keiskei*, contained two major chalcones of 4-hydroxyderricin (4-HD) and xanthoangelol that showed strong insulin-like activities via a pathway independent of the peroxisome proliferator-activated receptor-γ activation. The 4-HD especially showed the preventive effects on the progression of diabetes in genetically diabetic KK-A^{y} mice.

KEYWORDS: Diabetes; insulin-like; adipocyte differentiation; glucose-uptake; chalcone; *Angelica keiskei*; 4-hydroxyderricin; xanthoangelol; PPAR-γ

INTRODUCTION

Diabetes mellitus is a chronic disease that is characterized by hyperglycemia caused by insufficiency of insulin action (*1*). Recently, the number of diabetic patients has increased, especially in Asia, due to the prevalence of the western type life style (*2*). To ameliorate diabetes or its complications, many medicines have been investigated, such as insulin secretion agents, aldose reductase inhibitors, α-glycosidase inhibitors, and biguanides (*3*). Recently, peroxisome proliferator-activated receptor (PPAR)-γ ligands have been developed as a target of type II diabetes mellitus (non-insulin-dependent diabetes mellitus; NIDDM). These medicines improve the condition of insulin resistance by inducing differentiation from preadipocytes to small adipocytes that can enhance glucose uptake with insulin stimulation (*4*). More recently, a new type of agent that can directly activate the insulin receptor has been also discovered (*5*).

There are many types of medicines to ameliorate hyperglycemia as described above. However, nondiabetes subjects with impaired fasting glucose are prone to live without medical treatments. Such nontreatment for long periods causes serious complications of diabetes that impair the quality of life of the patients (*3*). Thus, daily edible ingredients that can complement insulin action are desired to prevent and ameliorate diabetes.

We have found that a Japanese herb "Ashitaba", *Angelica keiskei* Koidzumi, had some actions complementing insulin action. *A. keiskei* is a perennial plant belonging to Umbelliferae and has been taken as a health-promoting vegetable around the Hachijo Islands area. In the ethanol extract from *A. keiskei* root (AE), several bioactivities (e.g., preventive effect on hypertension, antitumor activity, and antibacterial activity) have been reported (*6*−*8*). *A. keiskei* contains many bioactive substances such as chalcone, flavanone, and coumarin (*9*). In particular, chalcone is found abundantly in leaf, stem, and root (*10*, *11*). Although about 20 kinds of chalcones are found in AE, two chalcones named 4-hydroxyderricin (4-HD) and xanthoangelol (XA), which are characteristic of *A. keiskei*, are found more abundantly than the other chalcones. In this report, we examined whether the AE and the chalcones, 4-HD and XA, can complement insulin action.

MATERIALS AND METHODS

Reagents. 2-Deoxy-[1,2-^{3}H]glucose was obtained from Parkin Elmer Life Sciences (Boston, MA). Dulbecco's modified Eagle's medium (DMEM) and fetal bovine serum (FBS) were purchased from Cambrex (Walkersville, MD). Calf serum (CS) was purchased from Dainippon Sumitomo Pharma (Osaka, Japan). Bovine insulin was purchased from Takara Bio Inc. (Shiga, Japan). Pioglitazone was purchased from Calbiochem (Darmstadt, Germany) for cotransfection assay, and Actos (including pioglitazone) was obtained from Takeda Chemical Industries Ltd. (Osaka, Japan) for animal experiment. All other reagents were from Sigma (St. Louis, MO) or Nacalai tesque (Kyoto, Japan).

Preparations of AE. AE was prepared by extracting dried, powdered roots of *A. keiskei* (4 g) in 100% ethanol for 30 min (twice) under gentle agitation, followed by concentrating the combined extract in vacuum evaporation and dissolving it in 1 mL of dimethylsulfoxide. This solution contained 12.5 mM XA and 11.1 mM 4-HD.

Isolation and Identification of Chalcones. To isolate active compounds, AE was applied to reverse-phased chromatography (RPC) using cosmosil140 C18-OPN (Nacalai tesque) and eluted stepwise by

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25, 50, 75, and 100% ethanol. Then, the fraction (75% ethanol effluent) that exhibited insulin-like activities was refractionated by RPC on a high-performance liquid chromatography (HPLC) system (Shimadzu, Kyoto, Japan) using a TSKgel ODS80Ts column (Tosoh, Tokyo, Japan) with gradient elution from 50 to 90% acetonitrile. The structures of the active substances were identified as 4-HD or XA by NMR and MS analysis as compared to previously reported values (*12*). The purity of both chalcones used in this study was above 95%.

3T3-L1 Cell Culture and Differentiation Assay. 3T3-L1 preadipocytes (Dainippon Sumitomo Pharma) were maintained at lower than 80% confluence in DMEM containing 10% CS, 200 μM ascorbic acid, and antibiotics. Two days after reaching confluence, differentiation was induced in DMEM containing 10% FBS, 200 μM ascorbic acid, and antibiotics (DMEM/FBS), supplemented with 0.25 μM dexamethasone, 10 μg/mL insulin, and (±) 0.5 mM isobutylmethylxanthine. After 2 days, the medium was changed to DMEM/FBS supplemented with 5 μg/mL insulin. Medium changes were done every 2−4 days. To determine the role of AE or purified chalcones (4-HD or XA) in adipocyte differentiation, AE, 4-HD, or XA was added to the medium to substitute for insulin. After 10−14 more days, cells were harvested for triglyceride analysis or were assayed for glucose uptake activity (*13*).

Determination of Cellular Triglyceride Content. Differentiated 3T3-L1 adipocytes were washed with phosphate-buffered saline (PBS), and cellular triglycerides were extracted by hexane-isopropyl alcohol solution. The triglyceride concentration was quantified by a colorimetric assay kit (Triglyceride E-Test Wako Kit, Wako Pure Chemicals, Osaka, Japan).

Measurement of 2-Deoxyglucose Uptake. 3T3-L1 adipocytes were rinsed with DMEM containing 0.1% bovine serum albumin (DMEM/BSA) and incubated in DMEM/BSA supplemented with or without various samples (AE, 4-HD, XA, or pioglitazone) for 14−15 h. Then, the cells were washed with HEPES-buffered saline solution (HBSS) and incubated in the same buffer supplemented with or without the same samples for 45 min. As a positive control, insulin was added to the well without the addition of any samples for 30 min. Glucose uptake was initiated by the addition of 0.05 μCi/mL 2-deoxy-[1,2-^{3}H]glucose and 100 μM unlabeled 2-deoxyglucose as final concentrations. After 10 min, the reaction was stopped by adding 40 μM cytochalasin B. The cells were washed three times with ice-cold PBS and solubilized with 1% NP-40 in PBS, and the radioactivity was determined by a scintillation counter.

Cotransfection Assay of PPAR-γ. To construct the GAL4-hPPAR-γ-LBD chimera plasmid, human PPAR-γ ligand binding domain was subcloned into the pFA-CMV plasmid (Stratagene, La Jolla, CA). The reporter plasmid was constructed by insertion of five copies of upstream activating sequence (UAS) for the GAL4 DNA binding domain to the upstream region of firefly luciferase gene in pGL3-promoter vector (Promega, Madison, WI). CV-1 cells were obtained from RIKEN Cell bank (Tsukuba, Japan). For cotransfection assay, CV-1 cells were grown in a 24 well plate in DMEM containing 10% FBS overnight. Chimera plasmid, reporter plasmid, and internal control plasmid (renilla luciferase, pRL-SV40, Promega) were cotransfected with lipofectamine-2000 (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. After 24 h, the medium was changed to DMEM containing pioglitazone, 4-HD, or XA and incubated for an additional 24 h. Then, the luciferase assay was conducted by using a Dual-Luciferase Reporter Gene Assay system (Promega) according to the manufacturer's instructions.

Animal Experiments. Animal experiments were conducted in accordance with Ethical Guidelines for our institution. Male 4 week old KK-A^y/Ta mice were purchased from Clea Japan (Tokyo, Japan). Mice were habituated to the novel environment before all experiments. In the experiments, 5 week old mice were divided into three groups of 7−10 mice each by body weight and blood glucose level and fed a powdered CE-2 diet (Clea Japan) or a diet containing test samples (0.15% for chalcones or 0.05% for pioglitazone). Mice were given these diets and water ad libitum. Body weight, food intake, and water intake were measured, and blood samples were collected from the tail vein to quantitate blood glucose levels.

Statistical Analyses. Statistical analyses were performed with StatLight#4 software (Yukms, Tokyo, Japan). Differences in mean values were statistically evaluated by Williams's test, Student's *t* test, or Dunnet's test.

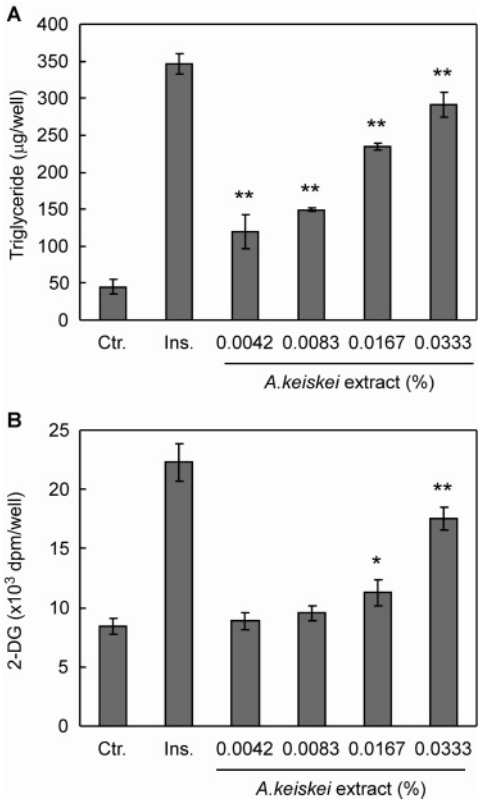

Figure 1. *A. keiskei* extracts show insulin-like activities. Two insulin-like activities, (**A**) preadipocyte differentiation and (**B**) glucose uptake enhancement of AE, were evaluated by the methods indicated in the Materials and Methods. (**A**) The cellular triglyceride content was measured as the index of differentiation. (**B**) Radiolabeled 2-deoxyglucose uptake was measured as the index of glucose uptake. Data are expressed as the mean ± SEM of four wells. *p < 0.05 and **p < 0.01 as compared with control by Williams's test.

RESULTS

Insulin-like Activities of Ethanol Extract from *A. keiskei*. In the differentiation assay, AE instead of insulin was added to 3T3-L1 preadipocytes for induction of differentiation. As shown in **Figure 1A**, incubation of 3T3-L1 cells with AE resulted in conversion of preadipocytes to adipocytes in a dose-dependent manner as indicated by the triglyceride content. On the other hand, the differentiation into adipocytes was not observed in the absence of AE.

In the glucose uptake assay, AE was incubated with the differentiated 3T3-L1 adipocytes overnight, while insulin was incubated with the cells for 30 min. AE significantly enhanced glucose uptake in a dose-dependent manner, as indicated by cellular radioactivity (**Figure 1B**). In the above experiment, it was confirmed that AE, even at high concentrations, did not show cytotoxicity evaluated by the WST-1 assay.

Insulin-like Activities of Two Major Chalcones from *A. keiskei*. To isolate active substances from AE, fractionated samples (see the Materials and Methods) of AE were tested for their activities to induce differentiation into adipocytes or to enhance glucose uptake. The two active fractions were identified as chalcone derivatives, 4-HD (**Figure 2A**) and XA (**Figure 2B**), respectively. In the differentiation assay, 3T3-L1 cells were differentiated to adipocytes by adding 4-HD or XA instead of insulin. The differentiation-inducing activity of 4-HD was almost

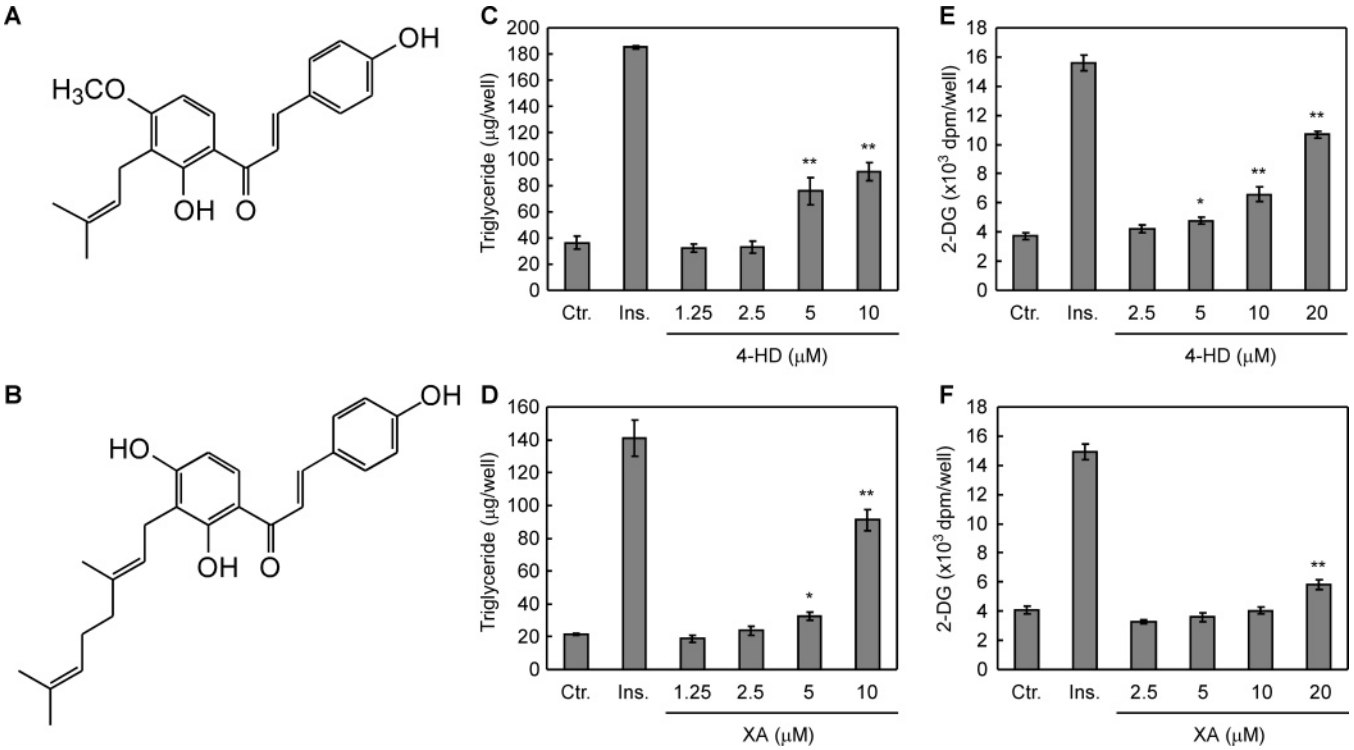

Figure 2. Two major chalcones in *A. keiskei* extract have insulin-like activities. Both 4-HD and XA were isolated from AE. The structures of 4-HD (**A**) and XA (**B**) were indicated. These chalcones were tested for the abilities of induction of differentiation (**C**, 4-HD; **D**, XA) or enhancement of glucose uptake (**E**, 4-HD; **F**, XA) instead of insulin. Data are expressed as the mean ± SEM of four wells. *$p < 0.05$ and **$p < 0.01$ as compared with control by Williams's test.

equal to that of XA (**Figure 2C**,**D**). The glucose uptake-enhancing activity of 4-HD, however, was exhibited several times higher than that of XA (**Figure 2E**,**F**).

Comparison to PPAR-γ Ligand. PPAR-γ is a well-known key molecule for promoting the differentiation of preadipocytes to adipocytes. Activation by PPAR-γ agonists such as pioglitazone or rosiglitazone converts them to adipocytes, and PPAR-γ agonist also increases glucose uptake with a long incubation in 3T3-L1 adipocytes (*14*). Thus, we tested whether pioglitazone also showed these insulin-like activities in our assay system. As shown in **Figure 3A**, the addition of pioglitazone resulted in promotion of the conversion of preadipocytes to adipocytes at low doses (2 μM) but could not increase the glucose uptake even at a high dose (20 μM) (**Figure 3B**). Thus, it was suggested that in our assay system, activation of PPAR-γ was not effective in glucose uptake but in differentiation into adipocytes. Then, we evaluated the ability of XA or 4-HD to activate PPAR-γ to confirm whether the conversion of preadipocytes to adipocytes by chalcones was due to PPAR-γ activation, using well-established GAL4-PPAR-γ ligand-binding domain chimera system (*15*). In this assay, pioglitazone significantly induced luciferase gene expression, whereas neither 4-HD nor XA could activate PPAR-γ (**Figure 3C**). Taken together, these results suggest that the chalcones could induce preadipocyte differentiation into adipocytes without activating PPAR-γ.

Preventive Effect of 4-HD or XA on Progression of Diabetes in KK-A^y Mice. KK-A^y mice develop diabetes and show hyperglycemia with aging, which is attributed to the development of insulin resistance. Because the chalcones were able to complement insulin action, we assumed that 4-HD or XA might prevent the progressive hyperglycemia of the genetically diabetic mice. Male 5 week old KK-A^y mice were fed a powdered diet with or without 0.15% of each chalcone. After 2 and 4 weeks, the blood glucose levels in each animal

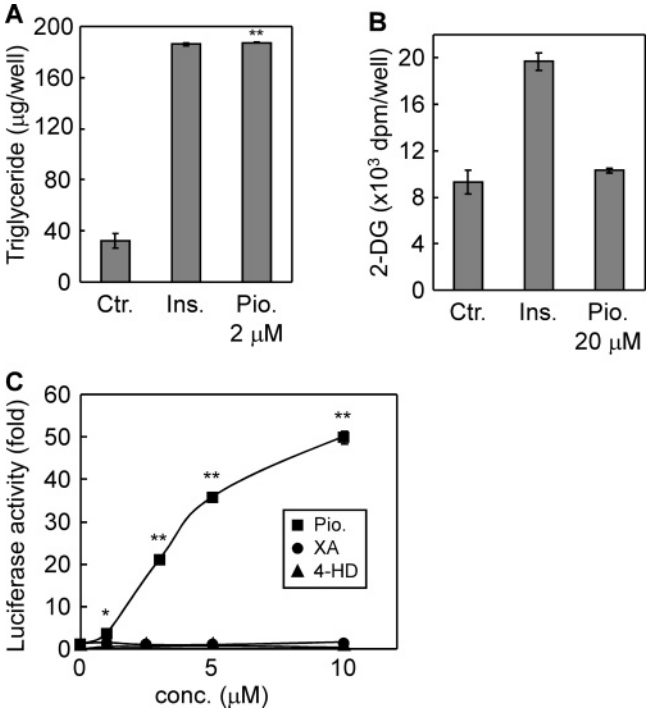

Figure 3. Chalcones cannot activate PPAR-γ. Pioglitazone was tested for the abilities of induction of differentiation (**A**) or the enhancement of glucose uptake (**B**). Data are expressed as the mean ± SEM of four wells. **$p < 0.01$ as compared with control by Student's t test. (**C**) 4-HD and XA were tested for the ability of activating PPAR-γ. Pioglitazone was used as a positive control. The luciferase activity in each sample was presented as the fold activity against vehicle control. Data are expressed as the mean ± SEM of five wells. *$p < 0.05$ and **$p < 0.01$ as compared with control by Williams's test.

Table 1. Chalcones Have Preventive Effects on the Progression of Diabetes [Blood Glucose Level (mg/dL)][a]

sample	day 0	2 weeks	4 weeks
diet only	226.9 ± 9.5	513.3 ± 25.2	474.4 ± 15.6
+4-HD	223.0 ± 11.1	365.9 ± 32.2**	368.1 ± 41.0*
+XA	226.4 ± 8.9	418.4 ± 41.4*	436.8 ± 33.9

[a] Male 5 week old KK-A^y mice were fed a basal diet or basal diet containing 0.15% chalcones ad libitum. On days 0 and 2 and 4 weeks after feeding samples, blood samples were collected to measure the blood glucose levels in each animal. Data are expressed as the mean ± SEM of 9–10 animals. *$p < 0.05$ and **$p < 0.01$ as compared with the group fed basal diet only at each point by Dunnet's test.

were measured. Two weeks feeding of basal diets containing 0.15% 4-HD or XA resulted in suppression of the elevation of the blood glucose levels, with about 50% reduction by 4-HD and 33% reduction by XA (**Table 1**). In this experiment, the polydipsia associated with insulin resistance was moderated in the 4-HD-fed group while no significant body weight difference was seen between each group (data not shown).

Then, we investigated the effect of 4-HD intake for a long period (7 weeks) on hyperglycemia, polydipsia, and body weight in the same animal model. In this experiment, pioglitazone was also tested for the ability to prevent progression of diabetes. In the 4-HD-fed groups, the elevation of blood glucose levels was suppressed during the observation period, as compared with the control groups (**Figure 4A**). Moreover, polydipsia was mitigated by 4-HD administration, probably due to the attenuation of insulin resistance (**Figure 4B**). Both hyperglycemia and polydipsia were almost completely suppressed by feeding of basal diets containing 0.05% pioglitazone (**Figure 4A**,**B**). However, an increase of body weight was observed at this dose of pioglitazone, whereas this side effect was not seen in 4-HD groups (**Figure 4C**). These results indicate that 4-HD has a preventive effect on the progression of diabetes. Also, it was observed that there were no obvious toxicities or any other adverse effects on 4-HD or XA groups in the experiments.

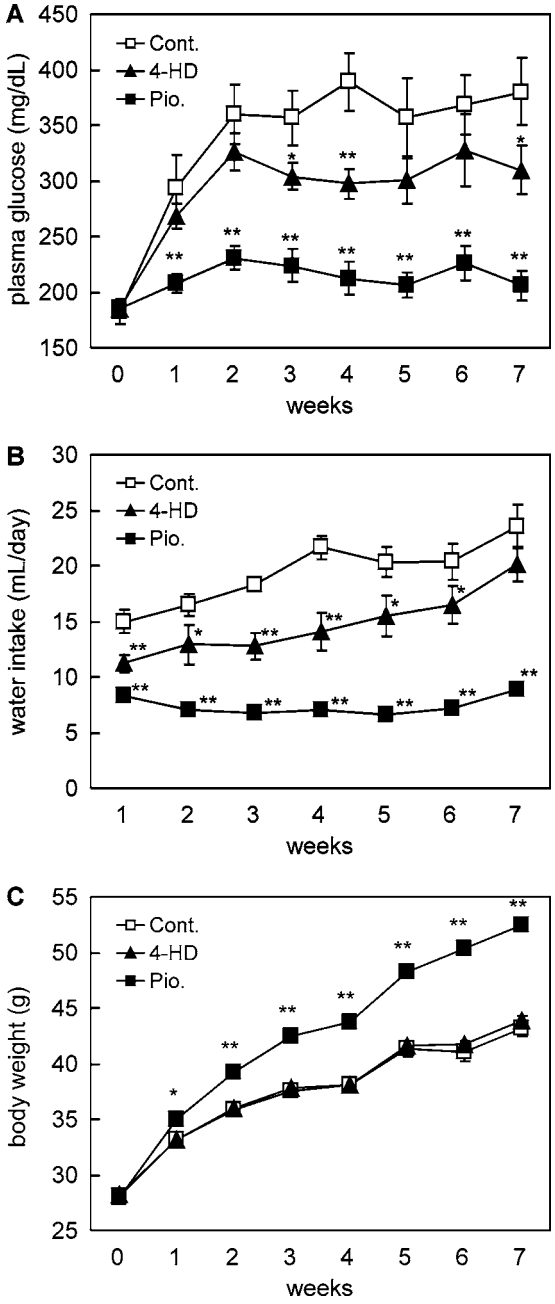

Figure 4. 4-HD has a preventive effect on the progression of diabetes for long time periods without adverse effects. Male 5 week old KK-A^y mice were fed powdered diet containing 0.15% 4-HD or 0.05% pioglitazone ad libitum. In every week, blood samples were collected to quantitate the blood glucose levels, and body weight and water intake were measured in each animal. Data are expressed as the mean ± SEM of 7–8 animals. *$p < 0.05$ and **$p < 0.01$ as compared with control-fed group at each point by Dunnet's test.

DISCUSSION

The chalcones, 4-HD and XA, are major ingredients in the ethanol extract from *A. keiskei*. It has been known that these chalcones exhibit several bioactivities (*6*−*8*). In this report, we showed that AE (the ethanol extract from *A. keiskei* root) and its active components, 4-HD and XA, have insulin-like activities, the induction of preadipocyte differentiation to adipocytes and the enhancement of glucose uptake. Both 4-HD and XA have an almost equal relative activity in the preadipocyte differentiation assay, whereas the glucose uptake-enhancing activity of 4-HD was several times higher than that of XA. These data suggest that the target molecules of the chalcones are different between two assay systems to evaluate insulin-like activities. Unfortunately, these target molecules are presently unknown. However, because our preliminary experiment indicates that chalcone is incorporated into the cell, it may act on intracellular molecules (data not shown).

It is known that some of the other plant extracts and their active substances also show insulin-like activities. For example, the water extract from cinnamon increases glucose uptake in 3T3-L1 adipocyte cells (*16*). Genistein from soy extract induces adipogenesis through the activation of PPAR-γ (*17*). Banaba extract has glucose uptake activity but no adipocyte conversion activity (*18*). However, well-known polyphenols such as catechin, chlorogenic acid, or naringenin did not exhibit insulin-like activities. The present study showed that AE and chalcones stimulate differentiation into adipocyte and increase glucose uptake in the absence of insulin. This is a unique action as compared with those reported plants and substances.

The preadipocyte differentiation into adipocyte is a tightly controlled process, in which PPAR-γ is the predominant molecular target. Pioglitazone, one of the PPAR-γ agonists, is prescribed to treat and improve insulin resistance in NIDDM patients. The mechanism of action of this medicine is reported that augmentation of small adipocytes through the activation

of PPAR-γ results in alleviation of insulin resistance (*4*). Indeed, pioglitazone could induce adipogenesis as well as AE and chalcones in our differentiation assay. Pioglitazone, however, could not enhance glucose uptake. Furthermore, we confirmed that neither 4-HD nor XA could induce luciferase gene expression in the GAL4-PPAR-γ LBD chimera system. Collectively, these results suggest that the addition of chalcones instead of insulin leads to induction of differentiation into adipocytes and an increase of glucose uptake without activating PPAR-γ signaling.

Because the activities of 4-HD and XA were thought to be unique with respect to anti-diabetes, two chalcones were tested for their ability to prevent progression of diabetes in genetically diabetic KK-A^y mice. As shown in **Table 1**, both chalcones suppressed the elevation of blood glucose levels as compared to the control groups. In this case, the stronger activity of 4-HD as compared with XA was ascribed to the enhancement of glucose uptake because the activity of 4-HD was several times higher than that of XA in glucose uptake assay. The action mechanism of the chalcones was thought to be different from that of PPAR-γ agonist. Thus, we investigated the preventive effect of 4-HD and pioglitazone on progression of hyperglycemia in KK-A^y mice. The administration of pioglitazone led to nearly complete suppression of the development of diabetes, while the increase of body weight was observed at this dose. On the other hand, although administration of 4-HD resulted in modest suppression of the elevation of blood glucose levels, no adverse side effects were observed. Taken these findings together, daily consumption of *A. keiskei* (chalcones) may be beneficial for the people who have the glycemia without having drug treatment for diabetes, because *A. keiskei* (chalcones) is a food ingredient that has been eaten in Japan for a long time without any adverse effects. Furthermore, our results suggest that chalcone derivatives may become the leading compounds in a new type medicine for diabetes mellitus, since the action mechanism to complement insulin action is quite unique.

ABBREVIATIONS USED

AE, ethanol extract from *Angelica keiskei* root; 4-HD, 4-hydroxyderricin; XA, xanthoangelol; PPAR, peroxisome proliferator-activated receptor; NIDDM, non-insulin-dependent diabetes mellitus.

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