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# Hypoglycemic effect of aspalathin, a rooibos tea component from *Aspalathus linearis*, in type 2 diabetic model db/db mice

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## Abstract

Effects of aspalathin, a green rooibos tea component, on glucose metabolism were studied *in vitro* and *in vivo*. We first examined the effect of aspalathin on glucose uptake by cultured L6 myotubes and on insulin secretion from cultured RIN-5F pancreatic β-cells *in vitro*, and then investigated the effect of dietary aspalathin on fasting blood glucose level and conducted an intraperitoneal glucose tolerance test (IPGTT) using type 2 diabetes model mice *in vivo*. Aspalathin dose-dependently and significantly increased glucose uptake by L6 myotubes at concentrations 1–100 μM. It also significantly increased insulin secretion from cultured RIN-5F cells at 100 μM. Dietary aspalathin (0.1–0.2%) suppressed the increase in fasting blood glucose levels of db/db mice for 5 weeks. In IPGTT, aspalathin improved impaired glucose tolerance at 30, 60, 90, and 120 min in db/db mice. These results suggest that aspalathin has beneficial effects on glucose homeostasis in type 2 diabetes through stimulating glucose uptake in muscle tissues and insulin secretion from pancreatic β-cells.


***Keywords:*** Hypoglycemic effect; Aspalathin; *Aspalathus linearis*; Type 2 diabetes; db/db mice; Myotubes; Pancreatic β-cell

## Introduction

Diabetes mellitus is a metabolic disorder altering the metabolism of three major nutrients, namely, carbohydrate, lipid, and protein. The total number of people with diabetes is increasing worldwide, and predicted to rise from 171 million in 2000 to 366 million in 2030 (Wild et al., 2004). To reduce the hyperglycemia, several trials have been conducted; for instance, inhibition of α-glucosidase to interfere with glucose absorption from the intestine, stimulation of pancreatic islet cells to secret insulin, reduction of hepatic glucose production, and insulin itself to suppress glucose production and augment glucose utilization (Moller, 2001; Banskota et al., 2006). Type 2 (non-insulin-dependent) diabetes is associated with metabolic syndrome and a marked increase in the risk of coronary heart disease (Haffner et al., 1998). Insulin resistance is a cardinal feature of type 2 diabetes (Moller, 2001). Muscle is the largest tissue in our body and plays an important role in blood glucose regulation. Thus, factors insulin-independently enhancing the uptake of glucose by muscle cells and tissues may overcome insulin resistance in the diabetic state and regulate the blood glucose level. Houstis et al. (2006) recently suggested that reactive oxygen species (ROS) have a causal role in multiple forms of insulin resistance, suggesting that compounds possessing

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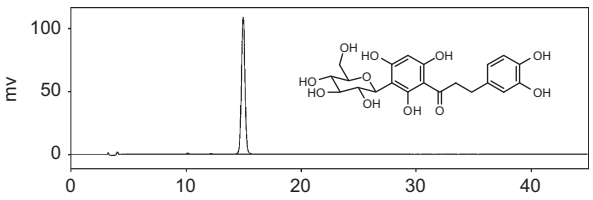


**Fig. 1.** The structure and a typical HPLC chromatogram of aspalathin.

antioxidant activities have a possibility to overcome insulin resistance in type 2 diabetes and hence to regulate blood glucose concentration.

Rooibos (*Aspalathus linearis*) is a leguminous shrub native to the mountainous areas in South Africa (Morton, 1983). Its leaves and fine stems are used for unfermented and fermented rooibos tea. Fermented rooibos tea has been reported to show various biological activities such as calming digestive disorders, reducing nervous tension, and alleviating allergy (Morton, 1983; McKay and Blumberg, 2007). Diverse flavonoids have been identified and quantified in fermented rooibos tea (Bramati et al., 2002) and unfermented rooibos tea (Bramati et al., 2003). The main flavonoid in unfermented rooibos aqueous extract was aspalathin (49.92 mg/g), a dihydrochalcone-glucoside (Fig. 1), which occupied 84.5% of the total flavonoid contents (59.08 mg/g), while aspalathin content in fermented rooibos aqueous extract (1.243 mg/g) was 22.4% of the total flavonoid contents (5.521 mg/g) (Bramati et al., 2002, 2003). The total antioxidant activity of unfermented rooibos was twice as high as that of fermented rooibos (Bramati et al., 2003).

In the present study, we first examined the effect of aspalathin on glucose uptake by cultured muscle cells and insulin secretion from cultured pancreatic β-cells *in vitro*, and then investigated the effect of dietary aspalathin on fasting blood glucose level and conducted a glucose tolerance test using type 2 model mice *in vivo*.

## Materials and methods

### Materials

L6 myoblasts derived from a rat (Yaffe, 1968) were generously provided by Dr. T. Amano, Mitsubishi-Kasei Institute of Life Sciences, Tokyo, Japan. RIN-5F cells derived from rat pancreatic β-cells (Chick et al., 1977) were purchased from American Type Culture Collection (ATCC® number: CRL-2058), Dulbecco's modified Eagle medium (DMEM) and RPMI 1640 medium were from Nissui Pharmaceutical Co., Tokyo, fetal bovine serum (FBS) was from JRH Biosciences, Lenexa, KS, USA, and streptomycin and penicillin G were from Nacalai Tesque, Inc., Kyoto, Japan. A glucose detecting kit (Glucose CII Test Wako) and D(+)-glucose were from Wako Pure Chemical Industries, Ltd., Osaka, Japan. Rat insulin detecting kit (High Range Rat Insulin ELISA) was from Mercodia AB, Uppsala, Sweden. Triton X-100 was purchased from Sigma Chemical Co., MO, USA. LDH Cytotoxicity Detection Kit was purchased from Takara Bio Inc., Shiga, Japan. Aspalathin, with purity greater than 98.5%, was prepared from unfermented rooibos as described below (Fig. 1). All other chemicals were of the best grade commercially available, unless otherwise noted. Plastic multiwell plates and tubes were obtained from Nunc A/S (Roskilde, Denmark) or Iwaki brand (Asahi Glass Co., Ltd., Tokyo, Japan).

### Purification of aspalathin

Aspalathin was purified from Green Rooibos Extract containing more than 20% of aspalathin produced by Tama Biochemical Co., Ltd., Tokyo, Japan; 86.4% of aspalathin was obtained with column chromatography of silica gel (Wako Pure Chemical Industries) and DIAION HP20SS resin (Mitsubishi Chemical Corp., Tokyo, Japan). The aspalathin was dissolved in a mixture of 70% (v/v) ethyl acetate and 30% (v/v) acetone and crystallized for 48 h at room temperature. Aspalathin was identified by HRMS, NMR, and IR (Fig. 1). The spectroscopic and other physical data are in full agreement with those of the literature (Rabe et al., 1994). The purity was confirmed by HPLC. HPLC separation was performed using a JASCO LC-2000 plus series (JASCO International Co., Ltd., Tokyo, Japan). The column was a 5 mm Sunfire $C_{18}$ (250 mm × 4.6 mm i.d.) from Waters Corp., MA, USA. The eluent was acetonitrile/distilled water/acetic acid (160/840/1, by volume). Separation was performed at 35 °C isocratically at a flow-rate of 1.0 ml/min. Acquisition was set at 287 nm. Retention time of aspalathin was 14.9 min (Fig. 1).

### Determination of glucose uptake by cultured myotubes

Stock cultures of L6 myoblasts were maintained in DMEM supplemented with 10% (v/v) FBS, streptomycin (100 μg/ml), and penicillin G (100 U/ml) (10% FBS/DMEM) under an atmosphere of 5% $CO_2$/95% humidified air at 37 °C as described previously (Yagasaki et al., 2003). Effect of aspalathin was examined by the procedure described previously (Doi et al., 2003) with slight modifications. Briefly, the prefused cells ($5 \times 10^4$ cells/well) were subcultured into Nunc 24-place multiwell plates and grown for 11 days to form myotubes in 0.4 ml of 10% FBS/DMEM. The medium was

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renewed every 3 days. Later, the 11-day-old myotubes were kept for 2 h in filter-sterilized Krebs–Henseleit buffer (pH 7.4, 141 mg/l $MgSO_4$, 160 mg/l $KH_2PO_4$, 350 mg/l KCl, 6900 mg/l NaCl, 373 mg/l $CaCl_2 \cdot 2H_2O$ and 2100 mg/l $NaHCO_3$) containing 0.1% bovine serum albumin, 10 mM Hepes and 2 mM sodium pyruvate (KHH buffer). Then the myotubes were cultured in KHH buffer containing 11 mM glucose without or with aspalathin (0–100 μM) for another 4 h. Differences in glucose concentrations between before and after culture were determined with a microplate reader (Appliskan 523000, Thermo Fisher Scientific Inc., MA, USA) and a glucose detecting kit at 508 nm, and the amounts of glucose consumed were calculated.

## Determination of insulin secretion from cultured pancreatic cells

RIN-5F cells derived from rat pancreatic β-cells were maintained in RPMI 1640 supplemented with 10% (v/v) FBS, streptomycin (100 μg/ml), and penicillin G (100 U/ml) (10% FBS/RPMI 1640) under an atmosphere of 5% $CO_2$/95% humidified air at 37 °C. The medium was renewed every 3 days. Effect of aspalathin on insulin secretion was examined by the procedure described previously (Nomura et al., 2003) with slight modifications. Briefly, the cells ($2.5 \times 10^5$ cells/well) were subcultured into Nunc 24-place multiwell plates. After being cultured for 72 h in 1 ml of 10% FBS/RPMI 1640, the medium in each well was removed and the cells were washed once with $Ca^{2+}$-, $Mg^{2+}$-free phosphate-buffered saline. Thereafter, RIN-5F cells received 1 ml of fresh medium (1% FBS/RPMI 1640) without or with aspalathin for another 3 h. Aliquots of culture media of all wells were centrifuged, and the insulin concentration of each supernatant was determined at 450 nm with an ELISA kit and a microplate reader (Model 680, Bio-Rad Laboratories, CA, USA) using 10 μl of supernatant.

## Cytotoxicity test

To carry out the cytotoxicity test, RIN-5F cells were cultured for 3 h in 1% FBS/RPMI 1640 without or with 100 μM aspalathin as described above. As the positive control, RIN-5F cells were cultured for 3 h in 1% FBS/RPMI 1640 containing 2% (v/v) Triton X-100. At the end of the culture, aliquots of culture media of all wells were centrifuged, and LDH activity of each supernatant was determined at 490 nm with an LDH Cytotoxicity Detection Kit and a microplate reader (Model 680, Bio-Rad Laboratories). The mean value of media containing Triton X-100 was regarded as maximum cytotoxicity (100%).

## Effect of aspalathin on blood glucose levels in db/db mice

All animal experiments were conducted in accordance with guidelines established by the Animal Care and Use Committee of Tokyo University of Agriculture and Technology and were approved by this committee. To determine the effect of aspalathin on fasting blood glucose levels and conduct a glucose tolerance test, db/db mice were used as a model of type 2 diabetes. Male db/db and misty control (normal) mice (5 weeks of age) were obtained from Charles River Japan, Kanagawa, Japan. Animals were individually housed in stainless-steel cages with wire bottoms in an air-conditioned room with a temperature of $22 \pm 2$ °C, a relative humidity of $60 \pm 5$%, and an 8:00–20:00 light cycle. All mice were maintained on a stock CE-2 pellet diet (CLEA Japan, Tokyo, Japan) for 3 days and thereafter a basal 20% casein diet (20C) for 4 days. The composition of the 20C diet was as follows (dry weight basis): 20% casein (Oriental Yeast Co., Tokyo, Japan), 7% corn oil (Hayashi Chemicals Co., Tokyo, Japan), 13.2% α-cornstarch (Nihon Nosan Kogyo Co., Yokohama, Japan), 49.75% β-cornstarch (Nihon Nosan Kogyo Co.), 3.5% mineral mixture (AIN-93G composition, Nihon Nosan Kogyo Co.), 1% vitamin mixture (AIN-93 composition, Nihon Nosan Kogyo Co.), 0.25% choline bitartrate (Wako Pure Chemical Industries), 0.3% L-cystine (Wako Pure Chemical Industries), and 5% cellulose powder (Oriental Yeast Co.). After preliminary feeding for 1 week, mice were deprived of their diet at 9:00 but allowed free access to water until blood collection from tail vein 4 h later. Blood (10 μl) was burst in water (40 μl), 20% (w/v) trichloroacetic acid aqueous solution (50 μl) was added and test tubes containing the mixture were kept in ice-cold water. The mixture was then centrifuged at 13,000*g* and 4 °C for 5 min. The resultant supernatant (80 μl) was subjected to glucose determination with a commercial kit and a spectrophotometer (Model U-1100, Hitachi Science Systems, Ltd., Ibaraki, Japan) at 505 nm, and db/db mice (6 weeks of age at the moment) were divided into two groups of similar fasting blood glucose levels and body weights (0 week). Diabetic mice of each of the two groups were given either the 20C as the control group or the 20C supplemented with 0.1% or 0.2% aspalathin as the test group for 5 weeks. Aspalathin was supplemented to the 20C at the expense of β-cornstarch. Likewise, misty control mice were given the 20C as the normal group for 5 weeks. In a separate experiment, db/db mice (6 weeks of age) treated as mentioned above were divided into three groups of similar fasting blood glucose levels and body weights. Diabetic mice of each of the three groups were given either the 20C, 20C supplemented with 0.2% aspalathin, or 20C supplemented with 0.005% pioglitazone hydrochloride (Wako Pure Chemical Industries) as a positive control drug (Ishida et al., 2004) for

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5 weeks. Water and each diet were available at all times except for the experiments to determine fasting blood glucose levels, which were carried out every week as mentioned above.

Intraperitoneal glucose tolerance test (IPGTT) was performed after the final determination of fasting blood glucose level. Briefly, two groups of db/db mice (11 weeks of age) were deprived of their diet at 20:00 but allowed free access to water. After fasted for 15 h, aspalathin (test group) was given orally at a dose of 20 mg/ml/100 g body weight. Water alone (1 ml/100 g body weight) was orally given to the control group. Two hours later, blood was collected from the tail vein of db/db mice (0 min). Immediately after blood collection, diabetic mice received an intraperitoneal injection of glucose (0.2 g/ml/100 g body weight). Blood samples were successively collected at appropriate time intervals (30, 60, 90, and 120 min), and blood glucose levels were determined as mentioned above.

## Statistical analyses

Data are expressed as means ± standard errors of means (SEM). Multigroup comparisons were carried out by one-way analysis of variance followed by the Tukey–Kramer multiple comparisons test and differences between two group means were compared by Student's *t*-test (Instat ver. 2.00, GraphPad Software Inc., San Diego, CA, USA). Values of $p<0.05$ were considered statistically significant.

## Results

We first examined the effect of aspalathin on glucose uptake by L6 myotubes in culture (Fig. 2A). Aspalathin dose-dependently increased glucose uptake at concentrations of 1–100 μM irrespective of insulin absence. Maximum stimulatory effect of aspalathin was attainable at around 10 μM.

We next examined the effect of aspalathin on insulin secretion from RIN-5F cells in cuture (Fig. 2B). Aspalathin also stimulated insulin secretion from pancreatic β-cells, but a significant effect was seen at 100 μM. Because of this stimulatory effect, a possibility that aspalathin effect might be due to its cytotoxicity against RIN-5F cells could not be excluded. As shown in the inset of Fig. 2B, cytotoxicity of 100 μM of aspalathin did not increase as compared with that of the control (0 μM of aspalathin), indicating that the stimulatory effect of aspalathin on insulin secretion was not due to its cytotoxicity. The weak activity of LDH seen in the control medium was considered to derive, at least in part, from 1% FBS in the medium.

To study the *in vivo* effect of aspalathin, we employed db/db mice, a model for type 2 diabetes. Fig. 3A shows the effect of aspalathin on the fasting blood glucose level. The blood glucose level gradually and almost linearly increased in diabetic control mice for 5 weeks, while it was unchanged and constant in normal mice. Aspalathin (0.1% in diet) tended to suppress until the second week and significantly suppressed at the third week after feeding. After the third week, aspalathin content in diet increased to 0.2%, so that its suppressive effect was strengthened and significant at the fourth and fifth weeks after feeding. As shown in Table 1, food intake of the aspalathin group for 5 weeks was not significantly different from that of the control group, suggesting that the suppressive effect of aspalathin on fasting blood glucose level was not due to reduced food intake but due to its pharmacological action.

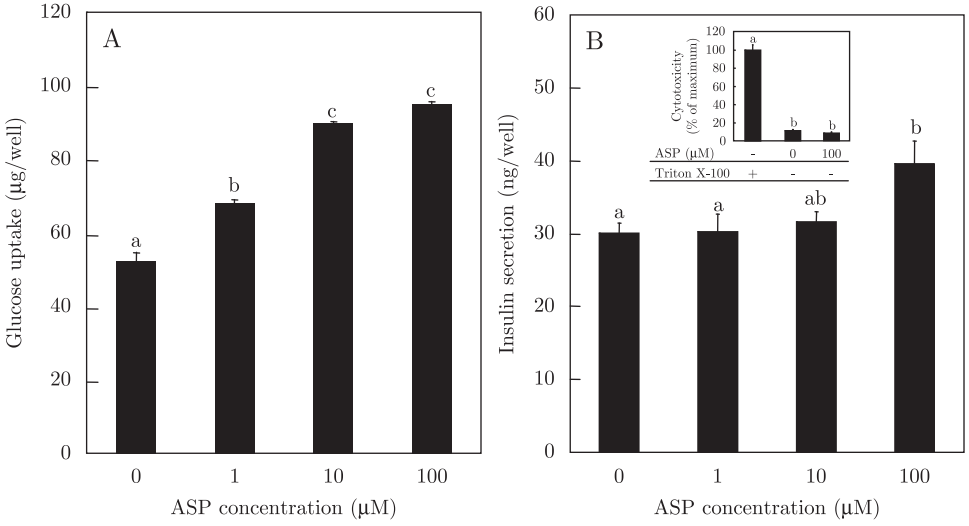


**Fig. 2.** Stimulatory effect of aspalathin on glucose uptake by cultured L6 myotubes (A) and insulin secretion from cultured RIN-5F cells (B). Inset of B indicates cytotoxicity of aspalathin on RIN-5F cells. Each value represents the mean ± SEM of six wells. Values not sharing a common letter are significantly different at $p<0.05$ by the Tukey–Kramer multiple comparisons test.

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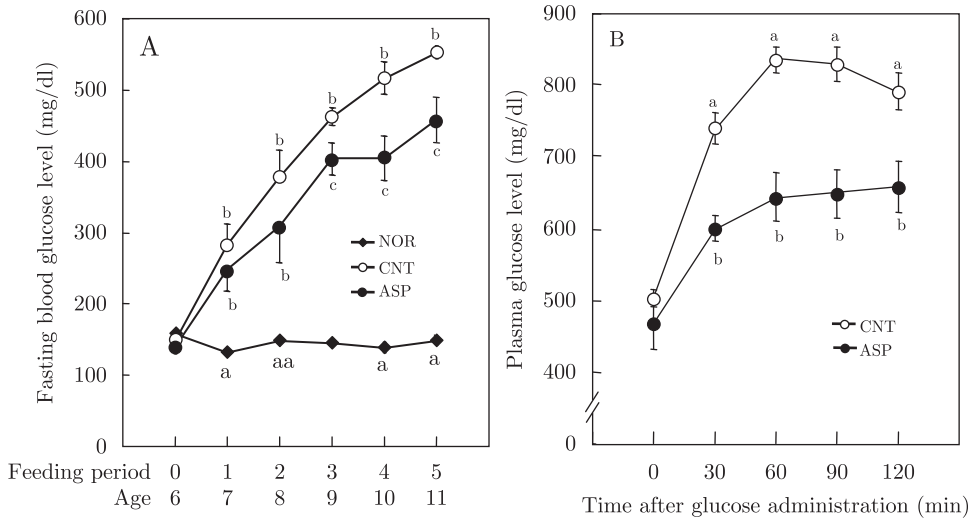


**Fig. 3.** Effect of aspalathin on fasting blood glucose level (A) and impaired glucose tolerance (B) in db/db mice. (A) Mice were fasted for 4 h before blood collection from the tail vein. Each value represents the mean ± SEM of six [normal (NOR) and control (CNT) groups] or four [aspalathin (ASP) group] mice. Values not sharing a common letter are significantly different at $p < 0.05$ by the Tukey–Kramer multiple comparisons test. (B) IPGTT was performed after the final determination of fasting blood glucose level. Diabetic db/db mice of 11 weeks of age were fasted for 15 h and given orally aspalathin at a dose of 20 mg/ml/100 g body weight or water (1 ml/100 g body weight). Two hours later, blood was collected from the tail vein of db/db mice (0 min). Immediately after blood collection, diabetic mice received an intraperitoneal injection of glucose at a dose of 0.2 g/ml/100 g body weight. Blood samples were successively collected at 30, 60, 90, and 120 min after glucose injection, and blood glucose levels were determined as described in "Materials and methods" section. Each value represents the mean ± SEM of six (CNT) or four (ASP) mice. Values not sharing a common letter are significantly different at $p < 0.05$ by Student's *t*-test.

**Table 1.** Initial body weight, food intake, body weight gain in db/db mice.

| Measurement | NOR | CNT | ASP |
|---|---|---|---|
| Initial body weight (g/mouse) | $18.0 \pm 0.3^{a}$ | $27.4 \pm 0.2^{b}$ | $28.2 \pm 0.5^{b}$ |
| Food intake (g/5 weeks) | $182.0 \pm 11.1^{a}$ | $253.8 \pm 9.3^{b}$ | $230.7 \pm 11.1^{b}$ |
| Body weight gain (g/5 weeks) | $3.5 \pm 0.3^{a}$ | $2.8 \pm 0.9^{a}$ | $6.9 \pm 1.6^{b}$ |

Each value represents the mean ± SEM of six (NOR, CNT) and four (ASP) mice. Values not sharing a common letter are significantly different at $P < 0.05$ by the Tukey–Kramer multiple comparisons test.

To compare the hypoglycemic effect of aspalathin with that of an existing drug, effects of aspalathin and pioglitazone were examined at the same time. Fasting blood glucose levels of diabetic control, aspalathin, and pioglitazone groups at the fifth week after treatment were 527 ± 50, 452 ± 36, and 472 ± 34 (mean ± SEM, $n = 6$), respectively. The hypoglycemic effect of aspalathin was almost comparable to that of pioglitazone at doses used here.

Finally, we tried to examine the effect of aspalathin on impaired glucose tolerance in db/db mice (Fig. 3B). Blood glucose level in the control group linearly increased until 60 min and thereafter kept the high level up to 120 min after the intraperitoneal injection of glucose. In contrast, the rise in blood glucose level was significantly suppressed at all time points except for the initial point (0 min) in the aspalathin group, suggesting that this polyphenol could improve the impaired glucose tolerance in db/db mice.

## Discussion

In the present study, aspalathin was demonstrated to significantly stimulate glucose uptake at 1, 10, and 100 μM by myotubes under the condition of insulin absence. L-Leucine in muscle tissue (Nishitani et al., 2002) and L-isoleucine in $C_2C_{12}$ myotubes (Doi et al., 2003) have been reported to promote glucose uptake independently of insulin. These effects of two branched chain amino acids on glucose uptake were mediated by phosphatidylinositol 3-kinase (PI3K), but was independent of the mammalian target of rapamycin (mTOR). In case of L-isoleucine, insulin-independent glucose uptake

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in skeletal muscle cells has been shown to contribute to the plasma glucose-lowering effect in normal rats (Doi et al., 2003). Thus, insulin-independent stimulation by aspalathin of glucose uptake in myotubes would contribute to the plasma glucose-lowering effect *in vivo* by overcoming insulin resistance. Aspalathin was also demonstrated to stimulate insulin secretion from RIN-5F pancreatic β-cells, but a significant effect was seen at a high concentration of 100 μM. Although the stimulatory effect of aspalathin on insulin secretion was weaker than that on glucose uptake, these two properties of aspalathin prompted us to conduct *in vivo* experiment.

*In vivo* effect of aspalathin was conducted by feeding a basal diet containing this polyphenol to db/db mice for 5 weeks. Aspalathin treatment commenced to suppress the rise in fasting blood glucose level from the first week of feeding and significant suppression was observed at the third week of feeding and thereafter. Since aspalathin had no influence on α-amylase activity (data not shown), we attempted the intraperitoneal glucose tolerance test instead of the oral glucose tolerance test (OGTT). Aspalathin clearly improved impaired glucose tolerance in db/db mice. These *in vivo* results suggest aspalathin possesses hypoglycemic and anti-diabetic effects in db/db mice, a type 2 diabetic animal model. In addition, the hypoglycemic effect of aspalathin is suggested to be comparable to that of pioglitazone, an existing drug.

Oxidative stress has been reported to impair insulin action in L6 myotubes, in particular reducing glucose uptake in response to the hormone (Blair et al., 1999). Increased ROS levels have been suggested to be an important trigger for insulin resistance and the suppression of oxidative stress by antioxidants has been considered to result in the improvement of insulin sensitivity and glucose homeostasis (Houstis et al., 2006). In addition, because gene expression of antioxidant enzymes such as glutathione peroxidase and catalase was very low in pancreatic cells (Lenzen et al., 1996; Tiedge et al., 1997), β-cells are vulnerable to oxidative stress, thus leading to the reduction of insulin secretion. Under conditions of IPGTT, treatment of db/db mice with *N*-acetyl-L-cystein, an antioxidant, retained glucose-stimulated insulin secretion and moderately decreased blood glucose levels (Kaneto et al., 1999). Aspalathin is capable of scavenging intracellular ROS (unpublished observation). Thus, its antioxidative function may be involved in the stimulation of glucose uptake and insulin secretion, and hence glucose homeostasis. Precise mechanisms by which aspalathin promotes glucose uptake by myotubes and insulin secretion from pancreatic β-cells remain to be elucidated.

In conclusion, aspalathin was demonstrated for the first time to increase both glucose uptake by muscle cells and insulin secretion from pancreatic β-cells. It significantly suppressed the rise in fasting blood glucose levels, and improved the impaired glucose tolerance in db/db mice.

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