Original Article

Ipomoea batatas and *Agarics blazei* ameliorate diabetic disorders with therapeutic antioxidant potential in streptozotocin-induced diabetic rats

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***Ipomoea batatas*, *Agaricus blazei* and *Smallanthus sonchifolius* are known to favorably influence diabetes mellitus. To clarify their antidiabetic efficacy and hypoglycemic mechanisms, we treated streptozotocin-induced diabetic rats with daily oral feeding of powdered *Ipomoea batatas* (5 g kg^{-1} d^{-1}), *Agaricus blazei* (1 g kg^{-1} d^{-1}) or *Smallanthus sonchifolius* (4 g kg^{-1} d^{-1}) for 2 months. Treatments with *Ipomoea batatas* or *Agaricus blazei*, but not *Smallanthus sonchifolius*, significantly suppressed the increases of fasting plasma glucose and hemoglobin A1c levels, and restored body weight loss during diabetes. Serum insulin levels after oral glucose administration tests increased along the treatments of *Ipomoea batatas* or *Agaricus blazei*. Moreover, *Ipomoea batatas* and *Agaricus blazei* reduced superoxide production from leukocytes and vascular homogenates, serum 8-oxo-2'-deoxyguanosine, and vascular nitrotyrosine formation of diabetic rats to comparable levels of normal control animals. Stress- and inflammation-related p38 mitogen-activated protein kinase activity and tumor necrosis factor-α production of diabetic rats were significantly depressed by *Ipomoea batatas* administration. Histological examination also exhibited improvement of pancreatic β-cells mass after treatments with *Ipomoea batatas* or *Agaricus blazei*. These results suggest that hypoglycemic effects of *Ipomoea batatas* or *Agaricus blazei* result from their suppression of oxidative stress and proinflammatory cytokine production followed by improvement of pancreatic β-cells mass.**

***Key Words*:** **hypoglycemic effect, oxidative stress, pancreatic islet β-cells, *Ipomoea batatas* (Caiapo), *Agaricus blazei* (Agaricus)**

Nowadays, diabetes mellitus in the industrialized countries has spread to younger generation and the number of patients is prospected to increase to 300 million or more worldwide by the year 2025. Diabetes mellitus is a serious complex chronic condition that is a major source of ill health worldwide. Even optimal control of blood glucose can not prevent clinical diabetic complications, so that diabetes therapy has revolved around dietary measures including the use of traditional antihyperglycemic medicines. Moreover, insulin or oral hypoglycemic drugs have serious side effects on overdose medication. Therefore, there is a real need today for further pharmacological study on the effectiveness and side effects caused by the alternative medicines.

In fact, about 800 species of plants and mushrooms have been reported to improve the metabolism of carbohydrates and to be effective against diabetes mellitus.[1–3] However, only a few compounds of antihyperglycemic plants and mushrooms have shown the efficacy on the management of diabetes in randomized trials.[3,4] Their hypoglycemic mechanisms in chronic treatment are not clear and scientific evidences are very poor. In the present study, we selected the three of plants and mushroom based on the previous reports, *Ipomoea batatas* L. (Convolvulaceae), i.e., Caiapo, *Agaricus blazei* Murill (Agaricaceae), i.e., Agaricus, and *Smallanthus sonchifolius* Poepp. & Endl. (Compositae), i.e., Yacon, which may improve on the balance of glucose and insulin through a safe and effective program.

Ipomoea batatas is a white-skinned sweet potato originating in Amazonis Brazil, and it has been used in Shikoku region of Japan, as a folk medicine for the treatments of diabetes and other metabolic diseases.[5,6] In concerning diet therapy of *Ipomoea batatas*, the treatment for 3 months has been reported to lower the plasma glucose and cholesterol levels in patients with type 2 diabetes.[7,8] Oral administration of *Ipomoea batatas* to diabetic animal models for longer than 6 weeks was shown to prevent and improve the symptoms of diabetes and hypoglycemia in streptozotocin (STZ)-induced diabetic and obese Zucker rats.[9,10] Further, *Ipomoea batatas* has abundant phenolic compounds, such as caffeic acid and its derivatives,[11] whose efficacious functions[12] are expected to prove a number of health benefits.

Agaricus blazei is a medical mushroom that grows in North America and Brazil, and it is widely taken, and described in the world.[13] There are many reports on the immunological beneficial properties such as anti-tumor,[14,15] anti-viral,[16] and disinfectant[17] activities. Moreover, it is recently reported that β-glucan extracts from *Agaricus blazei* could reduce blood glucose, triglyceride, and cholesterol levels[18] as well as insulin-like action.[19]

Smallanthus sonchifolius roots are a rich source of fructo-oligosacharides and have a long use tradition as food in the Andean region. Expansion to other counties including New Zealand, Japan and Brazil has been stimulated further by presumed medical properties of both roots and leaves.[20] The water-soluble extract of its leaves has been recognized to have hypoglycemic effect,[21,22] antioxidant property,[23] and enhancement of liver metabolism.[24] Natural and neutraceutical products including the above three materials are highly expected on diabetes control.

In the present study, *Ipomoea batatas*, *Agaricus blazei*, or *Smallanthus sonchifolius* were fed with diet for 2 months to STZ-induced type 2 diabetes rats, and their efficacy against diabetes and diabetes-associated pathophysiologic changes including pancreatic β-cells, insulin secretion, oxidative stress, and inflammatory cytokine production were investigated.

Materials and Methods

Preparation of experimental materials. *Ipomoea batatas* (Caiapo) was grown in Kagawa Prefecture (Japan). Whole extract

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was obtained including the peel, because *Ipomoea batatas* contains more phenolic compounds in the outer part than in the center,[25] and then lyophilized to prepare a uniform powder.[26] The powder was purchased from Fuji Sangyo Co., Ltd. (Kagawa, Japan). All ingredients were measured by Japan Food Research Laboratories (Tokyo, Japan) using the standard protocols recommended by the Resources Council, the Science and Technology Agency of Japan. The main composition was as follows: protein, 5.2%; carbohydrate, 78.3%; fiber, 11.0%. *Ipomoea batatas* had also abundant phenolic compounds such as chlorogenic acid, caffeic acid and its derivatives,[27] and high levels of vitamins including ascorbic acid and tochopherol. It contained many minerals including zinc, copper, manganese and selenium.

Agarics blazei (Agarics) fruit body was cultivated outdoors in Brazil. Fruit bodies were air dried by ventilator with a blowing temperature low than 60°C to maintain their enzyme activities, powdered, and imported by Toei Pharmaceutical Co., Ltd. (Tokyo, Japan). The main composition was as follows: protein, 38.5%; carbohydrate, 27.7%; fiber, 20.6%; β-glucan, 12.4%. It had also enzymes such as polyphenol oxidase, peroxidase, glucanase, and laccase. It contained many minerals including large amounts of zinc and copper, and certain amounts of manganese and selenium.[28–30]

Smallanthus sonchifolius (Yacon) plants were grown in Brazil. The tuberous roots and stems of *Smallanthus sonchifolius* were peeled, sliced and dried at 40°C in a forced air circulation oven. Partially dried slices were subjected to 60°C for 2 h to stop rapid degradation of fructooligosaccharides,[31] milled, and imported by Toei Pharmaceutical Co., Ltd. The main composition was as follows: protein, 9.6%; carbohydrate, 66.0%; fiber, 11.6%. The major proportion of carbohydrates was in the form of inulin-type oligofructans and β-fructooligosaccharides.[20] The tubers had chlorogenic acid and caffeic acid derivatives.[32,33] It also contained ascorbic acid, and many minerals including zinc, copper, manganese and selenium.

All diets for animals were prepared by mixing powdered test materials and powdered standard diet (SP; Funabashi Farm, Funabashi, Japan). The doses of *Ipomoea batatas*, *Agarics blazei*, or *Smallanthus sonchifolius* were kept at 5 g/kg of body weight/day, 1 g/kg of body weight/day, or 4 g/kg of body weight/day, respectively. This is a dose likely to be in the upper limit of what can be tolerated in long-term experiments in human as indicated by the manufacturers.

Animals. Male Wistar rats weighing 130 g to 150 g (6 weeks of age, Japan SLC, Inc., Shizuoka, Japan) were housed in an air-conditioned room at 22 ± 2°C with 60 ± 5% humidity, under 12 h light-dark cycle in the center for animal experiments of Kinki University School of Medicine. Rats were given laboratory diets as mentioned above and water *ad labium*. Rats were treated according to the ethical guideline of Kinki University School of Medicine Animal Committee.

Experimental procedure and sample collection. After initial determination of fasting blood glucose levels at 7 weeks of age, rats were intravenously injected the streptozotocin fluid (STZ, Sigma Chemical Co., St. Louis, MO) at the dose of 45 mg/kg (freshly dissolved in 3 mM citrate buffer pH 4.5). Blood glucose levels were monitored on the fifth day after STZ injection and thereafter at a fasting period of 18 h prior to the monitoring once a week. Animals showing above 250 mg/dl of blood glucose levels on the fifth day after STZ injection were selected as diabetic rats for this study and randomly divided into 4 groups: (i) normal diet ($n = 5$), (ii) *Ipomoea batatas* diet ($n = 5$), (iii) *Agarics blazei* diet ($n = 5$), (iv) *Smallanthus sonchifolius* diet ($n = 5$). The treatments with *Ipomoea batatas*, *Agarics blazei* or *Smallanthus sonchifolius* were performed for 8 weeks during 8 to 15 weeks of age. For normal reference, non-diabetes control ($n = 5$) was also examined.

Body weight of normal and diabetic rats was weekly recorded in fasting state. Food and water intake, and urine output for 24 h were measured every other week (1, 3, 5, and 7th week after treatments) individually in metabolic cages. Blood samples were collected from the tail vein. Urine and serum samples were stored at −80°C until the assay. Animals were sacrificed under pentobarbital anesthesia in the eighth week after treatments, and the pancreas, kidney and aorta were collected for the pathophysiological examination.

Plasma glucose and HbA1c. Plasma glucose values were obtained by the electric tip using glucose oxidase method[34] (MediSense Xtra; Abbott Laboratories Inc., Tokyo, Japan). Levels of glycosylated hemoglobin A1c (HbA1c) and total hemoglobin[35] after 7 weeks of treatments were determined according to manufacture's instruction (Liquitech HbA1cII; Roche Diagnostic Systems, Branchburg, MO).

Serum insulin. Serum insulin levels 20 min after oral administration of 20% glucose solution were determined by radioimmunoassay[36] using Rat Insulin radioimmunoassay (RIA) kit (Linco Research Inc., St. Charles, MO) 1, 3, 5 and 7 weeks after treatments. Fasting insulin levels were also measured in rats fasted overnight in the eighth week after treatments using Ultrasensitive Rat Insulin enzyme-linked immunosorbent assay (ELISA) kit (Mercodia, Uppsala, Sweden).

Superoxide production by peripheral leukocytes. Superoxide production by polymorphonuclear leukocytes (PMNs) and monocytes (MNCs) was measured using a gated flow cytometry according to the methods described by Perticarari *et al.*[37] with some modifications. Seven weeks after treatments fresh blood was collected and erythrocytes were removed by Tris-bufferd ammonium chloride lysis. White blood cells were preincubated for 15 min in a shaking water bath in 37°C with 500 ng/ml hydroethidium (HE) (Invitrogen Life Technologies, Carlsbad, CA) in Hank's Balanced Salt Solution containing 1% bovine serum albumin (Sigma Chemical Co.), and then the reaction was immediately stopped on ice. HE, a nonfluorescent compound which can diffuse through cell membrane, is rapidly oxidized to ethidium bromide by oxidative products, giving red fluorescence emission. Superoxide levels produced by PMNs and MNCs were evaluated by mean fluorescence intensity. Superoxide levels in stimulated leukocytes in the presence of 1 μg/ml phorbol mirystate acetate (PMA; Sigma-Aldrich Co.) were also measured.

Lucigenin-enhanced chemiluminescence detection of vascular superoxide. Vascular superoxide production was measured using lucigenin (bis-N-methyl acridinium; Sigma-Aldrich Co.)-enhanced chemiluminescence as described by Guzik *et al.*[38] Chemiluminescence in the vascular homogenates 8 weeks after treatments was measured in Krebs-HEPES (N-2-hydroxyethylpiperazine-N-2-ethanesulphonic acid) buffer containing lucigenin (100 μM) using luminometer analyzer (PICOLITE 6100; Packard Instrument Co. Inc., Downers Grove, IL). Specificity for superoxide was determined by coincubation with tiron (20 mM) (4,5-dihydroxy-1,3-benzene disulfonic acid; Sigma-Aldrich Co.). Superoxide production was expressed as chemiluminescence counts/10 min/30 mg vessel wet weight.

Serum 8-oxo-2'-deoxyguanosine (8-OHdG). Serum samples of 8 weeks after treatments of the experimental diets were filtrated to remove high molecular weight products above 10,000 MW (Ultrafree-MC; Millipore Co., Bedford, MA). Thereafter, 8-OHdG levels of the samples were determined[39] by high sensitive commercial ELISA kit (Japan Institute for The Control of Aging, Nikken SEIL Co., Shizuoka, Japan).

Urinary Tumor necrosis factor-α (TNF-α). Urine samples of 5 and 7 weeks after treatments of the experimental diets were centrifuged at 5,000 × g for 10 min. TNF-α level in the supernatants was measured[40] by BD Opt EIA Rat TNF ELISA kit II (BD biosciences, San Jose, CA).

Western blot analysis. Protein from aortas were extracted in a lysis buffer containing 0.1% sodium dodecylsulfate (SDS),

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2% Triton X-100, 1% dithiothreitol and 9 M urea (Wako Pure Chemical Industries, LTD., Osaka, Japan). For extraction from tissues, aortas were excised and snap-frozen in liquid nitrogen before homogenization in lysis buffer. Equal amounts of protein (30 μg) were separated by SDS-polyacrylamide gel. Anti-nitrotyrosine (Upstate biotechnology, Lake Placid, NY),[41] anti-p38 mitogen-activated protein (MAP) kinase, and anti-phospho p38 MAP kinase (Cell Signaling Technology, Danvers, MA)[42] antibodies were used for western blot analysis. Bands were visualized by enhanced chemiluminescence (Amersham Biosciences, Buckinghamshire, UK) and were quantified by using luminescent image analyzer (LAS-1000, Fuji Photo Film Co., LTD, Tokyo, Japan) and Image Gauge software version 3.12 (Fuji Photo Film). All antibodies were used as indicated by the manufacturers.

Histochemical analysis of pancreatic-β cells. Whole pancreatic tissues were fixed in 15% formalin neutral buffer solution and embedded in paraffin. Sections were stained by hematoxylin and eosin, and aldehyde fuchsin[43] for pancreatic β-cells population. To quantify the contents of pancreatic β-cells, the 12 randomly selected sections with the maximal lesion in each animal were chosen. A computer-assisted morphometric analysis was performed with a semiautomatic image computer system (Lumina Vision version 1.5; Mitani Co., Maruoka, Japan) and software for image analysis (Mac SCOPE version 2.5; Mitani Co.). The numbers of islets per field were manually counted.

Statistical analysis. Values were expressed as mean ± standard error of the mean (SEM). Data were analyzed by one-way analysis of variance (ANOVA), followed by post hoc Scheffé test. Differences were considered statistically significant at $p<0.05$.

Results

Body weight. Body weight significantly lowered in all the diabetic groups compared with normal control (NC) group ($p<0.05$, all diabetic groups vs NC) (Fig. 1). Two weeks after treatments with the experimental diets, the body weight of *Ipomoea batatas*-treated diabetic (Caiapo) and *Agarics blazei*-treated diabetic (Agarics) groups was significantly increased compared with that of untreated diabetic control (DC) group. The weight of Caiapo group was 221.5 ± 11.2 g and that of Agaricus group was 208.8 ± 16.6 g ($p<0.05$, Caiapo or Agarics group vs DC 142.5 ± 6.1 g) 7 weeks after treatments. On the other hand, *Smallanthus sonchifolius*-treated diabetic (Yacon) rats showed no significant restoration of the body weight.

NC showed greater daily food intake than those of all the diabetic groups for 3 weeks after the start of treatments, and thereafter there were no significant differences observed among any groups (data not shown). There were also no significant differences in food intakes among DC, Caiapo, Agaricus and Yacon groups during the experimental period.

Plasma glucose and HbA1c levels. Fasting blood glucose levels of NC ranged from 82.5 ± 9.4 mg/dl to 111.2 ± 2.6 mg/dl during the experiment. On the other hand, blood glucose levels of DC were increased after injection of STZ, and 5 weeks later those reached to the peak level (474.2 ± 34.1 mg/dl). In comparison with DC, Caiapo and Agaricus groups showed significant suppression on blood glucose levels after 3 weeks of treatments ($p<0.05$, Caiapo or Agarics group vs DC). In contrast, Yacon showed no significant hypoglycemic effect during the experimental period (Fig. 2).

There was a significant increase in the level of glycosylated HbA1c in DC after 7 weeks of STZ injection compared with that of NC ($p<0.01$). Of note, the value of Caiapo group was suppressed to 4.1 ± 0.2%, which was approximately the same level as NC ($p<0.01$ vs DC, 7.6 ± 0.8%). Suppressive tendency was also observed in Agaricus group (5.8 ± 0.7%). In contrast, HbA1c level of Yacon group did not differ from that of DC (Fig. 3).

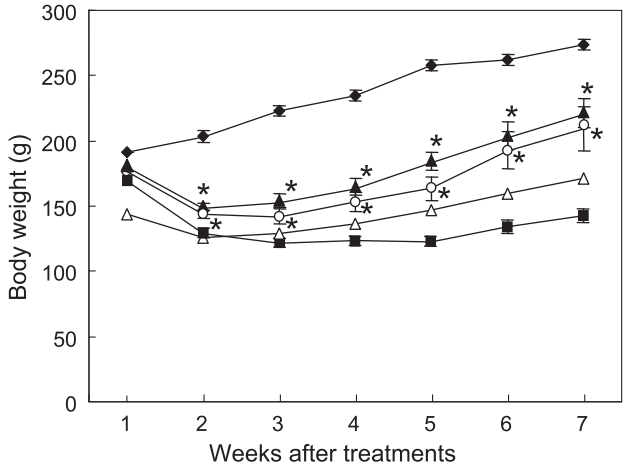

Fig. 1. Effects of treatments with *Ipomoea batatas* (Caiapo), *Agaricus blazei* (Agarics) or *Smallanthus sonchifolius* (Yacon) on body weight in STZ-induced diabetic rats. Streptozotocin-induced diabetic rats were treated with daily oral feeding of powdered *Ipomoea batatas* (5 g/kg body weight/day), *Agaricus blazei* (1 g/kg body weight/day) or *Smallanthus sonchifolius* (4 g/kg body weight/day) for 2 months. Treatments with Caiapo or Agaricus significantly restored weight loss during diabetes, but Yacon had no effect on body weight loss. Normal control (filled diamond), untreated diabetic control (filled square), *Ipomoea batatas* (Caiapo)-treated diabetic (filled triangle), *Agaricus blazei* (Agarics)-treated diabetic (open circle), and *Smallanthus sonchifolius* (Yacon)-treated diabetic (open triangle) rats. Values are mean ± SEM ($n = 5$). *$p<0.05$ vs untreated diabetic control.

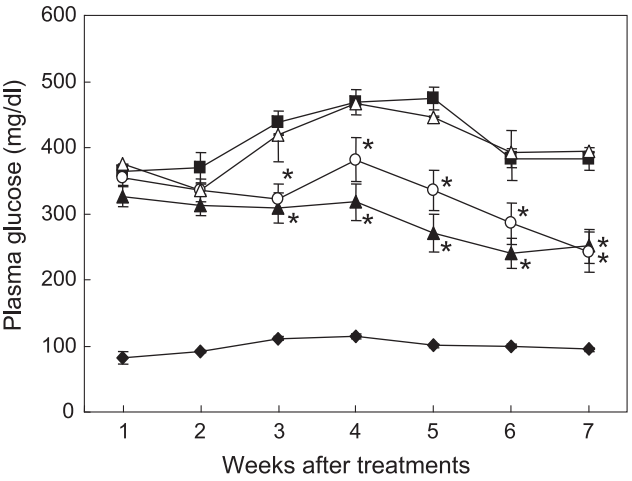

Fig. 2. Effects of treatments with *Ipomoea batatas* (Caiapo), *Agaricus blazei* (Agarics) or *Smallanthus sonchifolius* (Yacon) on fasting blood glucose levels in STZ-induced diabetic rats. Blood glucose levels were monitored on the fifth day after STZ injection and thereafter at a fasting period of 18 h prior to the monitoring once a week. Hyperglycemia was significantly suppressed by the treatments with Caiapo or Agarics, but not with Yacon. Normal control (filled diamond), untreated diabetic control (filled square), *Ipomoea batatas* (Caiapo)-treated diabetic (filled triangle), *Agaricus blazei* (Agarics)-treated diabetic (open circle), and *Smallanthus sonchifolius* (Yacon)-treated diabetic (open triangle) rats. Values are mean ± SEM ($n = 5$). *$p<0.05$ vs untreated diabetic control.

Furthermore, urinary ketone levels were increased in all of the diabetic groups compared with NC after 5 weeks of STZ injection. The levels were suppressed in Caiapo and Agaricus groups compared with DC. On the other hand, Yacon had no effect on urinary ketone (data not shown).

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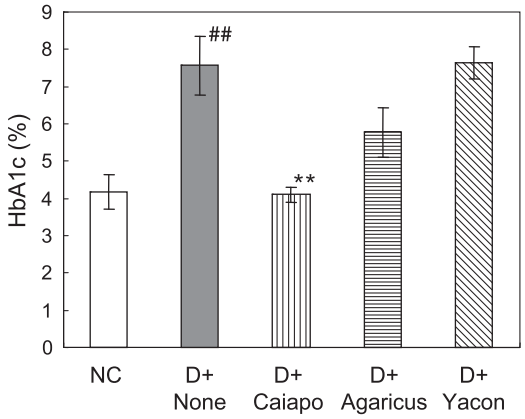

Fig. 3. Levels of HbA1c in STZ-induced diabetic rats treated with *Ipomoea batatas* (Caiapo), *Agaricus blazei* (Agarics) or *Smallanthus sonchifolius* (Yacon) in the seventh week after treatments. HbA1c values were reduced with treatment with Caiapo. Values are mean ± SEM (n = 5). ##p<0.01 vs normal control, **p<0.01 vs untreated diabetic control. NC; normal control, D + None; untreated diabetic control, D + Caiapo; *Ipomoea batatas*-treated diabetic group, D + Agarics; *Agaricus blazei*-treated diabetic group, D + Yacon; *Smallanthus sonchifolius*-treated diabetic group.

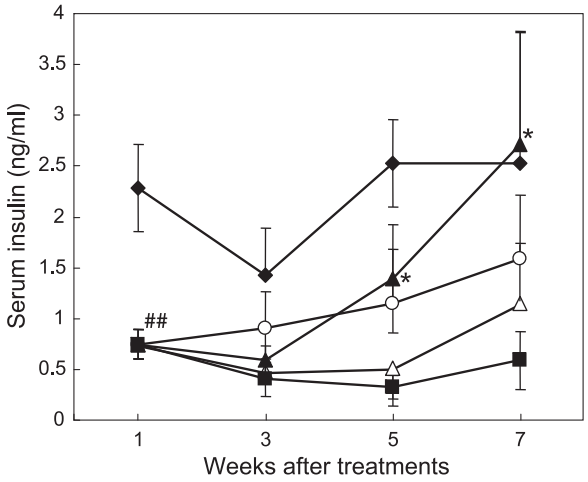

Fig. 4. Serum insulin levels after oral glucose administration in *Ipomoea batatas*-treated diabetic (Caiapo), *Agaricus blazei*-treated diabetic (Agarics) or *Smallanthus sonchifolius*-treated diabetic (Yacon) rats. The levels were determined 20 min after oral administration of 20% glucose solution by radioimmunoassay. Treatment with Caiapo for 2 months restored to serum insulin level comparable to that of normal control. Normal control (filled diamond), untreated diabetic control (filled square), Caiapo-treated diabetic (filled triangle), Agaricus-treated diabetic (open circle), and Yacon-treated diabetic (open triangle) rats. Values are mean ± SEM (n = 5). ##p<0.01 vs normal control, *p<0.05 vs untreated diabetic control.

Serum insulin levels after glucose loading. Changes of serum insulin levels 20 min after 400 mg/rat glucose loading were shown in Fig. 4. In the first week of experiment, mean serum insulin levels in all of the diabetic groups were remarkably decreased to 0.74 ng/ml (p<0.01 vs NC, 2.3 ± 0.4 ng/ml). After 5 weeks of treatments with the experimental diets, the increases in insulin levels were observed in Caiapo and Agaricus groups after glucose loading. The levels of Caiapo groups was significantly higher compared with DC (p<0.05). In the seventh week after treatment, the mean value of Caiapo group was 2.7 ng/ml, and thus recovered to the level comparable to that of NC, although the error range was great (p<0.05 vs DC). By contrast, the levels of serum insulin in Yacon group did not differ from those of DC during the experiment. On the other hand, fasting insulin levels remained unchanged in all the diabetic groups.

Oxidative stress. One major mechanism underlying STZ toxicity is cytokine-mediated β-cell destruction in which oxidative stress plays a key role.[44] First, we examined the changes of superoxide production from leukocytes in normal or diabetic rats with or without the treatments of Caiapo, Agarics, or Yacon for 8 weeks. Mean fluorescence intensity, which is equivalent to the ability of superoxide production by a single monocyte or neutrophil, under phorbol mirystate acetate stimulation (1 μg/ml) showed in DC group 1.6 times as high as that of NC groups 7 weeks after experiment (arbitrary units, mean fluorescence intensity; DC 773.1 vs NC 474.8). Of note, mean intensity of Caiapo group was suppressed by 495.7, approximately the same level as the NC. Agarics or Yacon treatment did not significantly influence on superoxide production from blood cells (arbitrary units, mean fluorescence intensity; Agarics 634.9, Yacon 801.6). There were also no differences in superoxide production without stimulation between the groups.

Next, the levels of 8-OHdG, an additional oxidative stress maker, were measured in the serum from normal or diabetic rats. Level of serum 8-OHdG was 240.8 ± 58.3 pg/ml in untreated DC. Only Yacon-treated rats showed significant increase of 8-OHdG and the value was 104.0 ± 40 pg/ml 8 weeks after treatments. In contrast, 8-OHdGs of Caiapo and Agaricus groups were suppressed below the detectable limit (0.1 ng/ml).

Finally, in order to investigate the effects on oxidative stress in the tissue under high blood glucose, lucigenin-enhanced chemiluminescence was measured in aortas of diabetic rats of the eighth week after treatments with Caiapo, Agarics, or Yacon. Fluorescence level in aortas of DC was approximately 2.5 times as high as that of NC, showing a significant increase in superoxide production (p<0.05, DC 13,376.8 ± 1,994.9 counts vs NC 5,186.3 ± 715.8 counts). Caiapo group significantly reduced the levels of superoxide production of tissue (5,985.3 ± 1,162.5 counts, p<0.05 vs DC) (Fig. 5a). In addition, formation of nitrotyrosine, another oxidative stress marker, was assessed in aortas by western blotting. Nitrotyrosine formation was also accompanied with the development of diabetes. Treatment of Caiapo for 8 weeks significantly attenuated the formation (p<0.05, Fig. 5b).

Urinary TNF-α. Urinary TNF-α level 5 and 7 weeks after treatments was illustrated in Fig. 6. In the fifth week of treatment, urinary TNF-α level of the diabetic rats was increased by 6 to 9 times as high as that of NC (p<0.01, NC 23.6 ± 6.3 pg/ml vs DC 180.4 ± 11.2 pg/ml). The levels of Caiapo (141.1 ± 10.6 pg/ml) and Agarics (150.9 ± 6.2 pg/ml) groups were significantly less than that of DC (p<0.05 vs DC). In contrast, Yacon group remained unchanged. In the seventh week, TNF-α levels showed similar tendencies with those of the fifth week, although significant differences between the groups were not observed. We also measured the levels of serum TNF-α in the experimental groups. The values were low (range of the mean from 9 pg/ml to 13 pg/ml) and were not significantly different between the groups.

Mean urinary albumin was increased to 4.02 mg/day urine albumin excretion (UAE) in DC group 5 weeks after the experiment. Caiapo, Agaricus or Yacon reduced UAE to the level comparable to that of NC group (1.5 mg/day mean UAE) (data not shown).

After 1 week of the experiment, water intakes in all of the diabetic rat groups were increased by 3-fold as much as that of NC, and their urine excretions were also increased by 5-fold, with no difference between DC and the other treated diabetic groups. In addition, the ratio of kidney/body weight 8 weeks after treatments became approximately twice in the diabetic rat groups

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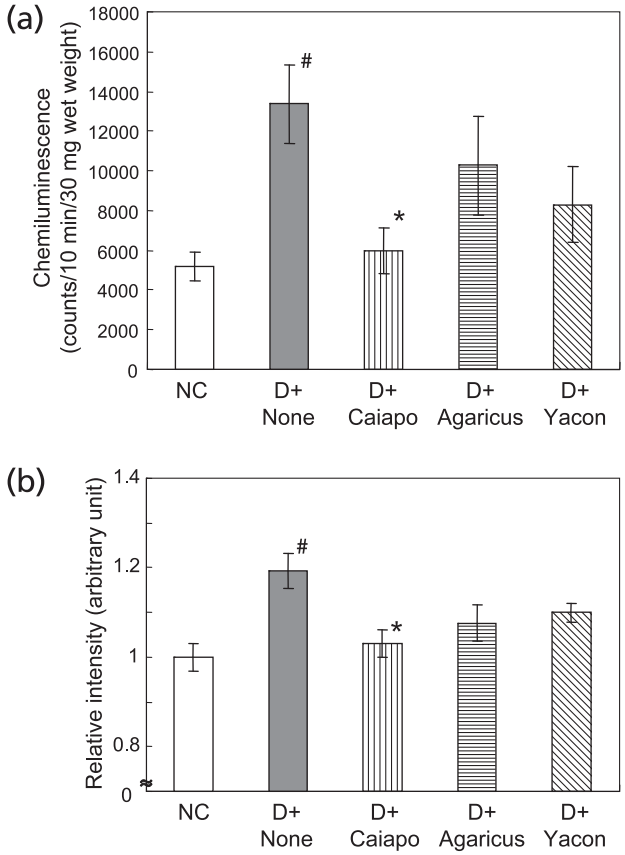

Fig. 5. (a) Lucigenin-enhanced chemiluminescence intensity in aortas after the treatments with *Ipomoea batatas* (Caiapo), *Agaricus blazei* (Agarics) or *Smallanthus sonchifolius* (Yacon). Chemiluminescence in the vascular homogenates 8 weeks after the treatments was measured by lucigenin-enhanced chemiluminescence using luminometer analyzer. Values were shown as counts/10 min/30 mg wet tissue weight. Superoxide production was increased in diabetic rats. Treatment with Caiapo significantly suppressed the oxidative stress in aorta. Values are mean ± SEM (n = 4–5). $^{\#}p<0.05$ vs normal control, $^{*}p<0.05$ vs untreated diabetic control. NC; normal control, D + None; untreated diabetic control, D + Caiapo; *Ipomoea batatas*-treated diabetic group, D + Agarics; *Agaricus blazei*-treated diabetic group, D + Yacon; *Smallanthus sonchifolius*-treated diabetic group.
(b) Nitrotyrosine formation in aortas after the treatments with *Ipomoea batatas* (Caiapo), *Agaricus blazei* (Agarics) or *Smallanthus sonchifolius* (Yacon). The values were measured by western blotting in the vascular homogenates 8 weeks after the treatments. Data are expressed as the relative ratio to normal control, which were assigned as value of 1. Nitrotyrosine formation was significantly increased in diabetic rats. Treatment with Caiapo significantly suppressed the oxidative stress in aorta. Values are mean ± SEM (n = 5). $^{\#}p<0.05$ vs normal control, $^{*}p<0.05$ vs untreated diabetic control. NC; normal control, D + None; untreated diabetic control, D + Caiapo; *Ipomoea batatas*-treated diabetic group, D + Agarics; *Agaricus blazei*-treated diabetic group, D + Yacon; *Smallanthus sonchifolius*-treated diabetic group.

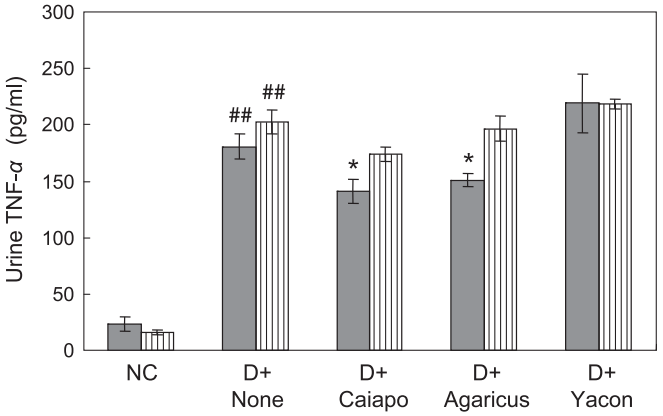

Fig. 6. Urinary TNF-α excretion after 5 weeks (filled bar) and 7 weeks (striped bar) of treatments with *Ipomoea batatas* (Caiapo), *Agaricus blazei* (Agarics) or *Smallanthus sonchifolius* (Yacon). TNF-α levels were measured in the urine of 5 and 7 weeks after the treatments using enzyme-linked immunosorbent assay. Urinary TNF-α excretion was markedly increased in untreated diabetic rats. Caiapo and Agaricus significantly reduced urinary TNF-α excretion after 5 weeks with treatments. Values are mean ± SEM (n = 5). $^{\#\#}p<0.01$ vs normal control, $^{*}p<0.05$ vs untreated diabetic control. NC; normal control, D + None; untreated diabetic control, D + Caiapo; *Ipomoea batatas*-treated diabetic group, D + Agarics; *Agaricus blazei*-treated diabetic group, D + Yacon; *Smallanthus sonchifolius*-treated diabetic group.

compared with that of the NC, with no difference between DC and the other treated diabetic groups (data not shown).

Activation of p38 mitogen-activaed protein kinase. Data of the superoxide production and TNF-α level in STZ-induced diabetes rats suggest that Caiapo or Agaricus is capable of depressing the increases in oxidative stress and inflammatory responses. We examined p38 MAP kinase activation by western blotting. P38 MAP kinase is potently and preferentially activated by a variety of stress including oxidative stress and inflammatory cytokines and receptor systems of the TNF family.[45]

Increases in p38 MAP kinase expression and its phosphorylation level were noted in aortas of diabetic rats compared with normal rats ($p<0.05$, DC vs NC). Caiapo significantly decreased these levels ($p<0.05$ vs DC), but Agaricus and Yacon were not effective (Fig. 7a–c).

Pancreatic β-cells. Representative light micrographs of pancreatic β-cells in NC, Agarics-treated, and DC rats were shown in Fig. 8. Area and numbers of islet of Langerhans were recorded as an index of pancreatic β-cells mass (Table 1). STZ treatment caused a remarkable degranulation of pancreatic β-cells. In the DC, the β-cells area for an islet and number of islets in pancreas were decreased by 1/6 and 1/4, respectively, as compared with those of NC ($p<0.01$, DC vs NC). Eight weeks after treatments, the β-cells mass in Caiapo and Agarics groups were increased by twice volume and more as compared with that of DC ($p<0.05$, Caiapo or Agarics group vs DC). Yacon treatment showed no effects on pancreatic β-cells recovery.

Discussion

Ipomoea batatas L. (Caiapo), *Agaricus blazei* Murill (Agaricus), and *Smallanthus sonchifolius* Poepp. & Endl. (Yacon) are known to favorably influence diabetes mellitus. However, their antidiabetic efficacy and hypoglycemic mechanisms are not fully determined. In the present study, we have shown that the treatments of *Ipomoea batatas* and *Agaricus blazei* suppress the increase in oxidative stress and TNF-α production in STZ-induced diabetes, and are useful in pancreatic β-cells recovery.

There has been much recent attention given to the relationship between diabetes and oxidative stress. Excessive superoxide production is likely to injury pancreatic β-cells and to weaken the insulin production. In this study, superoxide production by peripheral blood monocytes and neutrophils was increased in diabetic rats, accompanied by an increase in the levels of serum 8-OHdG, which displays cellular DNA damage as described in the text. We also demonstrated that the levels of plasma glucose and HbA1c were correlated with increased oxidative stress in diabetic rats. These findings are coincident with the reports that superoxide production and 8-OHdG levels from peripheral blood

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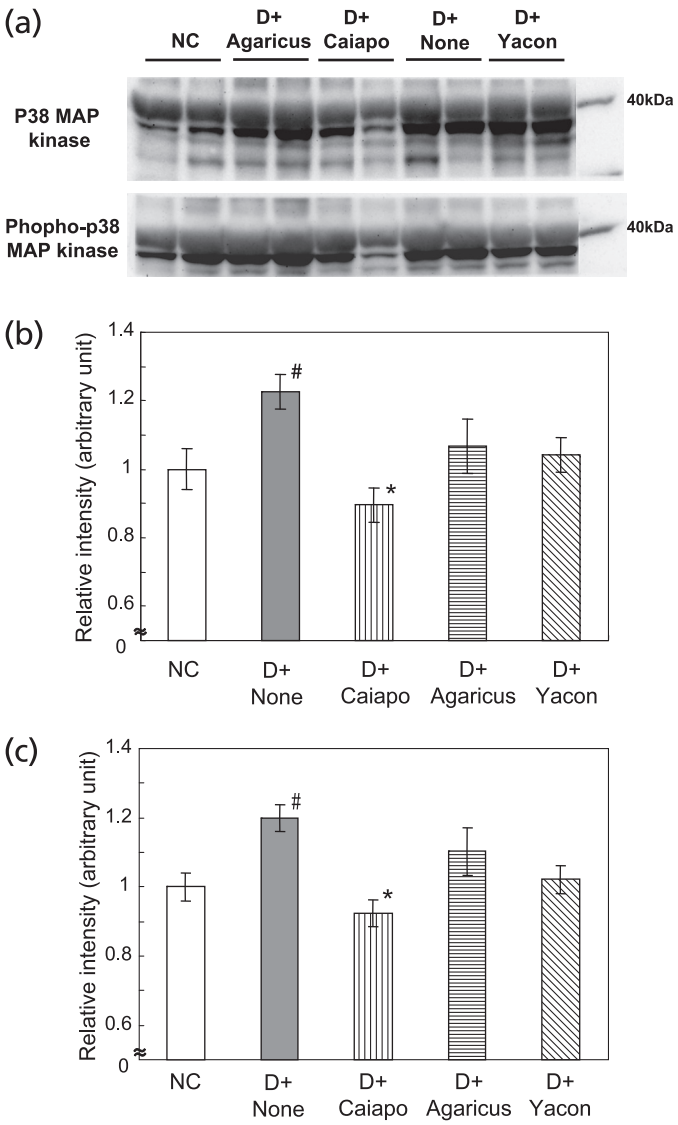

Fig. 7. Activation of p38 mitogen-activated protein kinase (MAP kinase) in aortas after the treatments with *Ipomoea batatas* (Caiapo), *Agaricus blazei* (Agarics) or *Smallanthus sonchifolius* (Yacon). The values were measured by western blotting in the vascular homogenates 8 weeks after the treatments. (a) shows a typical western blot analysis of vascular homogenates, using p38 MAP kinase antibody (upper) and phopho-p38 MAP kinase antibody (Thr180/Thr182) (lower). P38 expression (b) and its phosphorylation (c) were significantly increased in diabetic rats. Treatment with Caiapo significantly suppressed p38 MAP kinase activation in aorta. Data are expressed as the relative ratio to normal control, which were assigned as value of 1. Values are mean ± SEM ($n = 5$). $^{\#}p<0.05$ vs normal control, $^{*}p<0.05$ vs untreated diabetic control. NC; normal control, D + None; untreated diabetic control, D + Caiapo; *Ipomoea batatas*-treated diabetic group, D + Agarics; *Agaricus blazei*-treated diabetic group, D + Yacon; *Smallanthus sonchifolius*-treated diabetic group.

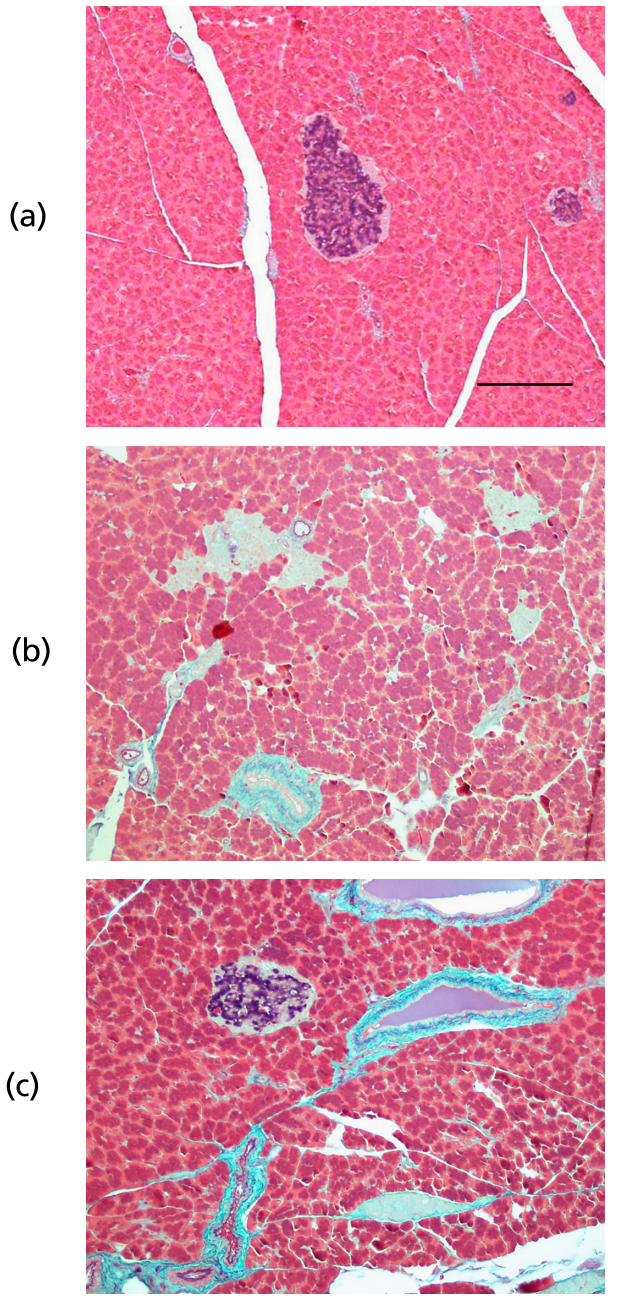

Fig. 8. Typical pancreatic islets in (a) normal control, (b) untreated diabetic control (DC), and (c) *Agaricus blazei* (Agarics)-treated diabetic rats. Pancreatic β-cells showed blue or purple by aldehyde fuchsin staining. Following STZ injection, remarkable degranulation of pancreatic β-cells was observed. The remaining β-cells of DC or *Smallanthus sonchifolius* (Yacon)-treated diabetic rats were interspersed between the pancreatic cells and exhibited little to no cytoplasmic staining. Treatments with *Agaricus blazei* (Agarics) or *Ipomoea batatas* (Caiapo) tended to increase β-cells mass in STZ-induced diabetic rats. Scale bar; 200 μm.

monocytes were significantly higher in diabetic patients than in healthy people,[46] and that there was a correlation between the rise of HbA1c and oxidative stress levels from peripheral blood monocytes in diabetic patients.[47] Here, it is of note that long-term treatments with *Ipomoea batatas* or *Agaricus blazei* significantly suppressed the increase in HbA1c and oxidative stress in diabetes animals (Figs. 1 and 3).

Furthermore, Sheetz and King[48] and Aslan *et al.*[49] have reported that free radicals induced oxidative stress-related gene expressions and peroxidization of plasma membrane, and that contributed to the development of various diabetic complications. Consistently, the present study showed that oxidative stress levels in the aortas of diabetic rats were markedly increased, as shown in the results in superoxide production (Fig. 5a) and nitrotyrosine

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Table 1. Histochemical analysis of pancreatic islets in diabetic rats

	Treatment				
	NC	D + None	D + Caiapo	D + Agarics	D + Yacon
Islet number	3.77 ± 0.09	1.06 ± 0.12##	1.46 ± 0.12*	1.52 ± 0.11*	0.96 ± 0.08
β-cells area (mm², ×1/100)	3.58 ± 0.23	0.56 ± 0.17##	1.03 ± 0.30	1.25 ± 0.35	0.68 ± 0.21

Values are mean ± SEM (n = 5). ##p<0.01 vs NC, *p<0.05 vs DC.
Twelve fields were examined in each animal. Islet number is count of pancreatic islets per field. Positive staining area by aldehyde fuchsin for an islet is calculated as β-cells area.
NC; normal control, D + None; untreated diabetic control, D + Caiapo; Ipomoea batatas-treated diabetic group, D + Agarics; Agaricus blazei-treated diabetic group, D + Yacon; Smallanthus sonchifolius -treated diabetic group.
Twelve fields were examined in each animal. Islet number is count of pancreatic islets per field. Positive staining area by aldehyde fuchsin for an islet is calculated as β-cells area.

formation (Fig. 5b). Nitrotyrosine is a footprint of increased peroxynitrite presence, which is the reactive product of superoxide and nitric oxide, and is a highly reactive radical species. Lakey *et al.*[50] have reported that peroxynitrite is a mediator of cytokine-induced destruction of human pancreatic islet β-cells. Of profound importance, treatments with *Ipomoea batatas* or *Agaricus blazei* significantly reduced the production of reactive oxygen species in vascular cells as well as peripheral blood cells.

Recent study shows the evidence for evaluation of nitrotyrosine and TNF-α as potential biomarkers of the presence, severity and progress of diabetes and its complication.[51] TNF-α, a proinflammatory cytokine, is mainly secreted from monocytes and macrophages, and also produced in various other tissues. It has reported that TNF-α affects not only peripheral insulin resistance,[52] but also directly pancreatic β-cells, followed by their apoptosis and reduction of insulin secretion.[53] It has also demonstrated that high glucose participate in the production of inflammatory cytokines including TNF-α through the activation of nuclear transcription factor.[54]

Here we shows that TNF-α production remarkably increased in STZ-induced diabetic rats and that treatments of *Ipomoea batatas* or *Agaricus blazei* had significant suppressive effect on urinary TNF-α level (Fig. 6). Additionally, *Ipomoea batatas* depressed p38 MAP kinase expression and activation (Fig. 7). P38 MAP kinase is potently and preferentially activated by a variety of stress including oxidative stress and inflammatory cytokines and receptor systems of the TNF family.[45] Excretion of urinary albumin was also significantly increased in diabetic rats and suppressed after 5 weeks of treatments with *Ipomoea batatas*, *Agaricus blazei*, and *Smallanthus sonchifolius*. It supports our result that increase in urinary TNF-α level is associated with the onset of diabetic nephropathy.[40]

Ipomoea batatas-treated and *Agaricus blazei*-treated rats exhibited the recovery tendency on serum insulin levels along the treatments (Fig. 4). In the histochemical examination of pancreatic islets (Table 1, Fig. 8), a recovery tendency of islet β-cells mass was also observed in *Ipomoea batatas* or *Agaricus blazei* groups. These results suggest that there is a possibility that *Ipomoea batatas* or *Agaricus blazei* has a protective effect on islet cell destruction and increases the secretion of insulin. On the other hand, Kusano and Abe[10] have reported that *Ipomoea batatas* extract directly secretes insulin and improves insulin resistance. Gray and Flatt[19] have found the insulin-like activity and the insulin-releasing effect of *Agaricus blazei*, although the nature or the mechanisms of action was not determined.

Taken together, hypoglycemic effects of *Ipomoea batatas* or *Agaricus blazei* are likely to be their antioxidant and anti-inflammatory action. The leaves and roots of *Ipomoea batatas* used in this study have abundant phenolic compounds, such as chlorogenic acid, caffeic acid, and its derivatives,[11] which exhibit high radical-scavenging activity, antimutagenic, and antidiabetic effects.[55,56] *Ipomoea batatas* is rich in ascorbic acid, a well-known antioxidant molecule. The plant also contains large amounts of dietary fibers, which may interfere with glucose absorption and further reduce blood glucose levels.

On the other hand, the fruit body of *Agaricus blazei* has highly branched 1,3-glucan as the major carbohydrate component.[57] β-glucan is a well-known biological response modifier which is widely distribution in nature and used as an alternative medicine. Although the previous reports concerning the oxidative/anti-oxidative activity of β-glucan are controversial, β-glucan treatment was found to be effective against oxidative injury through the inhibition of TNF-α response.[58–60] In addition, *Agaricus blazei* contains polyphenol oxidase, an antioxidant enzyme.[28,29] Cupper, zinc, and selenium contained richly in *Ipomoea batatas* and *Agaricus blazei* may be beneficial for the protection against radical generation, because the minerals have radical-scavenging activities and are contained in antioxidant enzymes. It is important that neither significant adverse effects nor biochemical changes of blood were observed in our other experiment using *Ipomoea batatas*, *Agaricus blazei*, or *Smallanthus sonchifolius*.

In opposition to previous reports,[21,22] *Smallanthus sonchifolius* did not improve in glucose balance, oxidative stress or inflammatory response. We used primarily dried tuber and stem powder of *Smallanthus sonchifolius* in this study, and there may be a possibility of discrepancy between our results and the results of other reports which utilized the decoction from the leaves.

Collectively, we provide that cellular oxidative is a critical step in STZ-mediated islet β-cells injury, and that *Ipomoea batatas* and *Agaricus blazei* protect the organs from oxidative damage by those immunomodulatory (inhibition of TNF-α formation and its signaling pathway) and antioxidant properties. Excessive NO production is recently thought to be involved in the pathogenesis of metabolic disorders such as atherosclerosis and obesity-linked type 2 diabetes.[61,62] Proinflammatory cytokines including TNF-α increase nitric oxide (NO) production through the expression of inducible NO synthase in adipocytes and in skeletal muscle of obese human subjects and several diabetic animal models.[62,63] In the present study using STZ-induced diabetic rats, rise of plasma or urine NOx levels was not detected. However, we do not exclude the possible mechanism, because nitrotyrosine formation was markedly increased in vascular cells of untreated diabetic rats (Fig. 5b).

In conclusion, treatments with *Ipomoea batatas* or *Agaricus blazei* suppressed the increases of blood glucose and HbA1c levels in streptozotocin-induced type 2 diabetes model rats, and restored insulin release and their body weight loss. The results suggest that the antidiabetic effects result from the suppression of oxidative stress and proinflammatory cytokine, TNF-α, and improvement in β-cells mass. Further precise mechanism of suppression on oxidative stress and/or TNF-α production by the components of *Ipomoea batatas* or *Agaricus blazei* remains to be elucidated.

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Abbreviations

ANOVA	analysis of variance
DC	untreated diabetic control
ELISA	enzyme-linked immunosorbent assay
HbA1c	hemoglobin A1c
HE	hydroethidium
HEPES	N-2-hydroxyethylpiperazine-N-2-ethanesulphonic acid
MAP kinase	mitogen-activated protein kinase
MNCs	monocytes
NADPH	nicotinamide adenine dinucleotide phosphate
NC	normal control
NO	nitric oxide
8-OHdG	8-oxo-2'-deoxyguanosine
PMA	phorbol mirystate acetate
PMNs	polymorphonuclear leukocytes
RIA	radioimmunoassay
SDS	sodium dodecylsulfate
SEM	standard error of the mean
STZ	streptozotocin
TNF-α	tumor necrosis factor-α
UAE	urine albumin excretion

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