RESEARCH ARTICLE

The anti-diabetic effect of anthocyanins in streptozotocin-induced diabetic rats through glucose transporter 4 regulation and prevention of insulin resistance and pancreatic apoptosis

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Hyperglycemia, abnormal lipid and antioxidant profiles are the most usual complications in diabetes mellitus. Thus, in this study, we investigated the anti-diabetic and anti-oxidative effects of anthocyanins (ANT) from black soybean seed coats in streptozotocin (STZ)-induced diabetic rats. The administration of ANT markedly decreased glucose levels and improved heart hemodynamic function (left ventricular end diastolic pressure, $\pm dp/dt$ parameters). ANT not only enhanced STZ-mediated insulin level decreases, but also decreased the triglyceride levels induced by STZ injection in serum. Diabetic rats exhibited a lower expression of glucose transporter 4 proteins in the membrane fractions of heart and skeletal muscle tissues, which was enhanced by ANT. In addition, ANT activated insulin receptor phosphorylation, suggesting an increased utilization of glucose by tissues. Moreover, ANT protected pancreatic tissue from STZ-induced apoptosis through regulation of caspase-3, Bax, and Bcl-2 proteins. Furthermore, ANT significantly suppressed malondialdehyde levels and restored superoxide dismutase and catalase activities in diabetic rats. Interestingly, the observed effects of ANT were superior to those of glibenclamide. Taken together, ANT from black soybean seed coat have anti-diabetic effects that are due, in part, to the regulation of glucose transporter 4 and prevention of insulin resistance and pancreatic apoptosis, suggesting a possible use as a drug to regulate diabetes.

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1 Introduction

The simple metabolic characterizations of type 1 diabetes as an insulin deficiency syndrome and type 2 diabetes as a primarily insulin-resistant state have been superseded by a more refined understanding of the pathophysiology of nonautoimmune forms of diabetes. Hyperglycemia, which defines the diagnosis, is now viewed as a consequence of a

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Abbreviations: ANT, anthocyanins; **CAT,** catalase; **DM,** diabetes mellitus; **GLUT4,** glucose transporter 4; **HR,** heart rate; **IR,** insulin receptor; **LV,** left ventricle; **LVEDP,** left ventricular end diastolic pressure; **MDA,** malondialdehyde; **ROS,** reactive oxygen species; **SOD,** superoxide dismutase; **STZ,** streptozotocin; **TBS,** Tris-buffed saline; **T2DM,** type 2 diabetes mellitus; **TUNEL,** terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling

complex interplay between insulin sensitivity and secretion, with a failure of pancreatic beta cells to compensate sufficiently for the increased insulin requirement induced by insulin resistance [1]. Thus, in metabolic studies of β cell function, insulin secretion should always be interpreted in the context of concomitant insulin sensitivity [2]. Recent studies have reported that insufficient insulin secretion represents the primary defect in the pathogenesis of type 2 diabetes mellitus (T2DM) [3, 4]. Additionally, it has been well established that insulin signaling, including activation of insulin receptor (IR) tyrosine kinase activity, is impaired in most patients with T2DM [5]. Impaired insulin signaling leads to hyperglycemia and other metabolic abnormalities [6]. Therefore, pharmacological agents known as IR activators that restore IR auto-phosphorylation in insulin-resistant cells could be useful for treating T2DM [7, 8].

One of the major roles of insulin is to maintain whole-body glucose homeostasis by stimulating the transport of glucose into peripheral tissues *via* glucose transporter 4 (GLUT4), which is mainly expressed in skeletal and cardiac muscle and adipose tissue [9, 10]. In response to acute insulin stimulation, intracellular vesicles that store GLUT4 translocate to the plasma membrane. This translocation results in the redistribution of GLUT4 to the plasma membrane, where it facilitates glucose uptake [11, 12]. In a diabetic state, reduced expression of GLUT4 causes impairment of insulin signaling in skeletal muscle and stimulates glucose production in the liver. These changes lead to high glucose concentrations in blood.

Diet, exercise, oral anti-hyperglycemic medications, and insulin are the main treatments for T2DM. Oral agents are used by the people, who fail to meet glycemic goals with diet or exercise. Insulin is usually reserved for those who fail or cannot tolerate oral therapy. Glibenclamide is a second-generation sulfonylurea a K^+ channel blocker, resulting in an increased insulin excretion. It increases plasma levels of insulin and causes hypoglycemia; however, approximately 2–5% of patients report side effects with sulfonylureas [13]. People started on sulfonylurea should be counseled that hypoglycemia and weight gain are the primary adverse effects of prolonged therapy [14, 15]. Sulfonylureas have been associated with a decrease in microvascular events compared with diet treatment, but there have been no significant differences in mortality or macrovascular events. The lack of benefit with regard to cardiovascular complications may be related to the fact that these drugs cause hyperinsulinemia, which is associated with the metabolic syndrome [14]. In addition, due to a sulfonyl component in the chemical structure of sulfonylureas, hypersensitivity can occur in people with sulfa-allergies. This treatment should thus be prescribed to people with a sulfa-allergy with caution. Therefore, it becomes more interesting to determine whether any alternative agents, especially those from natural products, produce a hypoglycemic effect in diabetes. Furthermore, as oxidative stress is produced under diabetic conditions and is likely involved in the progression of pancreatic β cell dysfunction [16], an agent that possesses antioxidant activity would be better for diabetic patients.

Streptozotocin [2-deoxy-2-(3-methyl-3-nitrosoureido)-D-glucopyranose] (STZ) is a naturally produced antibiotic from *Streptomyces achromogenes*. STZ-induced hyperglycemia is a widely used experimental model for screening the activity of hypoglycemic agents. In this model, hyperglycemia arises because of the irreversible destruction of the β-islet cells of the pancreas by STZ and consequently reduces insulin secretion. DNA strand breaks and poly ADP-ribose polymerase-1 over-activation are prime modes of STZ-induced islet cell death [17]. It is also known that the usage of antioxidants, such as metallothionine and poly ADP-ribose polymerase inhibitors, prevents β-cell destruction *in vitro* and *in vivo* [17, 18].

Anthocyanins (ANT) are polyphenols that are responsible for many fruit and floral colors, and consist of a basic skeleton of either 2-phenylbenzopyrylium or flavylium glycoside. ANT are widely distributed in the human diet through crops, berries, fruits, vegetables, and red wine. ANT are especially abundant in the epidermis palisade layer of black soybean seed coats [19–21]. Three main ANT, that is, cyanidin-3-glucoside, delphinidin-3-glucoside, and petunidin-3-glucoside, were characterized in black soybean seed coats [19, 21]. Previous reports have demonstrated the health-promoting benefits of ANT to include antioxidant effects, a reduced risk of coronary heart disease, regulation of adhesion molecules, and protection from ischemia and reperfusion heart injury [22–24]; In addition, it has been reported that the ANT or each component affects hyperglycemia and insulin secretion in diabetic animal model [25, 26]. However, although one of the diabetic complications is heart dysfunction, which can lead to heart failure [27, 28], few studies have reported the effect of ANT on heart function in diabetic condition. Moreover, there is no report for the potential of ANT from black soybean seed coats for T2DM; in particular, their mechanism for inducing hypoglycemia.

Thus, this study investigated the possible anti-hyperglycemic and anti-oxidative activities of ANT from black soybean seed coats in STZ-induced diabetic rats. In addition, the effect of ANT on heart function in diabetic rats was evaluated and these effects were compared with glibenclamide, which was used as a reference hypoglycemic drug for type 2 diabetes treatment.

2 Materials and methods

2.1 Chemicals

*A*nti-Bax, anti-Bcl-2, and anti-GLUT4 antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA); an antibody that recognized the cleaved form of caspase-3 was obtained from Cell Signaling Technology

(Beverly, MA, USA). An enhanced chemiluminescence Western blotting detection reagent was obtained from Amersham (Buckinghamshire, UK). All other chemicals, including STZ, were supplied by Sigma-Aldrich (St. Louis, MO, USA).

2.2 ANT extraction and purification

ANT, extracted from black soybean (*Glycine max* (*L.*) *Merr*) and purified as described by Kim *et al.* [23], were provided by Dr. Sung Chul Shin (Department of Chemistry, Research Institute of Life Science, Gyeongsang National University). The purity ($\geq$99%) and compositions of ANT were analyzed using HPLC. ANT consisted of cyanidin-3-glucoside (72%), delphinidin-3-glucoside (20%), and petunidin-3-glucoside (6%).

2.3 Animal experiments

Adult male Sprague-Dawley rats (weighing 220–250 g) were used for the experiment. The rats were fed standard laboratory chow and water before the experiment. The rats were divided into five equal groups ($n = 5$) and housed in cages. All experiments were performed in compliance with institutional guidelines set by the Institutional Animal Care and Use Committee at the Gyeongsang National University. After overnight fasting (rats were deprived of food for 16 h but allowed free access to water), four groups of rats (each $n = 5$) were injected intraperitoneally with a freshly prepared solution of STZ (50 mg/kg) in a 0.1 M citrate buffer with a pH of 4.5. Control rats were injected with only a citrate buffer. After a 5-day waiting period, rats with hyperglycemia (a blood glucose range above 300 mg/dL) were considered as diabetic rats and used for further experiments. One group of STZ-injected rats (the same-day treatment group) was pretreated with ANT (50 mg/kg, the dose enough to exert its anti-inflammatory and anti-oxidative properties as referred by Kim *et al.* [23]) 1 h before the STZ injection. The post-treatment rats (two groups of STZ-injected rats) began to receive treatment on the fifth day after STZ injection; this was considered the first day of post-treatment. Treatment by gavages was continued for 30 days. Control rats (group 1) were given vehicle (distilled water) only (no diabetic); STZ-treated rats (group 2) were given distilled water every day; STZ+ANT same-day treatment rats (group 3) were given ANT (50 mg/kg) from the day on which STZ was injected until the 30th day; STZ+ANT post-treatment rats (group 4) and STZ+glibenclamide (1 mg/kg) post-treatment rats (group 5) were given ANT or glibenclamide from the fifth day after STZ injection until the 30th day. Body weight and blood glucose levels were measured at 5, 10, 15, 20, 25, and 30 days of treatment. Blood samples were collected from the tail vein and measured with a glucometer (Accu-CHEK Sensor kit, Roche, GmbH, Germany). Hemodynamics were measured, blood samples were taken by cardiac puncture, and the heart, skeletal muscles, and pancreas were removed.

2.4 Hemodynamic measurements

A Millar catheter (Millar Instruments, Houston, TX, USA), was cannulated to the right common carotid artery to measure heart function (left ventricular end diastolic pressure; LVEDP, heart rate; HR, and $\pm dp/dt$) in each group. After completion, the right common carotid artery was exposed and cannulated with a 2 F Millar catheter into the ascending aorta to measure systolic and diastolic blood pressures, mean arterial pressure, and HR. The pressure transducer was then advanced into the left ventricle (LV) to measure left ventricular systolic pressure, LVEDP, LV-developed pressure, and the first derivatives (positive and negative) of LV pressure over time.

2.5 Collection of serum, heart, and skeletal muscle

After 30 days of feeding, rats were sacrificed and blood samples (800 μL from each rat) were collected by cardiac puncture in normal tubes. The collected blood was kept at room temperature for 5 min for coagulation. After that, the serum was obtained from the coagulated blood by centrifugation at $1600 \times g$ for 15 min at 4°C. The separation of serum was finished within 30 min. The serum was immediately stored at −80°C until use. The hearts and skeletal muscles were removed and then immediately frozen using liquid nitrogen and stored at −80°C until use.

2.6 Western blot analysis

For analysis, Bax, Bcl-2 and caspase 3 protein expressions, heart and skeletal muscle tissues of rats were homogenized and lysed in a PRO-PREP protein extract solution. The samples were centrifuged at 13 000 rpm × 20 min at 4°C and the supernatant was used as a total protein. For analysis of GLUT4 expression, the tissues were homogenized and the plasma membrane fractioned with a Mem-PER Eukaryotic Membrane Protein Extraction kit, according to the manufacturer's protocol (PIERCE, Rockford, IL, USA). Protein concentrations were determined by the Bradford method. Aliquots of 30 μg of protein were subjected to 10% SDS-polyacrylamide gel electrophoresis for 1 h 30 min at 110 V. The separated proteins were transferred to a PVDF membrane for 2 h at 20 mA with a SD Semi-dry Transfer Cell (Bio-Rad, Hercules, CA, USA). The membranes were blocked with 5% nonfat milk in Tris-buffed saline (TBS) containing 0.05% Tween 20 (TBS-T) for 2 h at room temperature. The membranes were then incubated with anti-GLUT4, anti-Bax, anti-Bcl-2, and anti-cleaved caspase 3 antibodies at a 1:500 concentration (4 μg/mL) in 5% skim milk in TBS-T overnight at 4°C, and the bound antibody was

detected by horseradish peroxidase-conjugated anti-rabbit IgG. The membranes were washed and then developed using a Western blotting Luminol Reagent system (Amersham, Piscataway, NJ, USA).

2.7 Terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling assay

Pancreatic tissues were removed and then fixed overnight in 10% buffered formalin, followed by paraffin infiltration and embedding. Tissue blocks were cut into 5 μm sections and later underwent a deparaffinization procedure. The slides were then washed with 0.1% Tween 20 in PBS (PBS-T) and permeabilized for 90 min at 37°C in PBS containing 0.5% Triton X-100. Apoptotic cells were determined using an In Situ Cell Death Detection Kit (Roche Applied Science, Penzberg, Germany). After staining, slides were washed for 5 min in PBS-T three times and were observed using a confocal laser-scanning microscope. Terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL)-positive cells were imaged in at least four randomly selected fields.

2.8 Measurement of serum insulin, triglycerides, malondialdehyde, superoxide dismutase (Cu/Zn-, Mn-, and Fe- SOD), catalase, and IR auto-phosphorylation levels

The serum insulin levels were measured using a commercial ELISA kit according to the manufacturer's directions (Rat insulin ELISA kit, Bachem, San Carlos, CA, USA). The serum triglyceride levels were measured by a commercial kit according to the manufacturer's protocol (Triglyceride Quantification kit, BioVision, Mountain View, CA, USA). Auto-phosphorylation of IRβ was detected by an ELISA kit from Cell Signaling Technology (Beverly). A malonyl dialdehyde assay was performed using a commercially available kit (OxiSelect TBARS assay kit, Cell Biolabs, San Diego, CA, USA). Superoxide dismutase (SOD) and catalase (CAT) activities were measured by commercial kits (Cayman, Ann Arbor, MI, USA).

2.9 Statistical evaluation

Data are presented as the mean ± SD of results obtained from the number (n) of animals used. Differences between data sets were assessed by one-way analyses of variance (ANOVA) followed by Newman–Keuls tests. $p < 0.05$ was accepted as statistically significant.

3 Results

3.1 Effect of ANT on the change of body weight and blood glucose levels in STZ-induced diabetic rats

The upper panel of Table 1 lists the body weight changes in the control (no diabetic) and experimental animals in each group. STZ-induced diabetic rats exhibited a significant loss ($p < 0.01$) in body weight 10 days after STZ injection compared with the controls (220 ± 12 and 275 ± 15 g, respectively), as well as very prominent decreases in body weight (175 ± 12 g, about 52% of the control body weight of 335 ± 20 g) at 30 days after STZ injection. ANT treatment (50 mg/kg) significantly ($p < 0.01$) recovered the loss of body weight caused by STZ. At 30 days after STZ injection, post-treatment or same-day treatment with ANT increased body weight from 175 to 275 or 280 g, respectively, indicating a 30 or 31% increase compared with STZ-induced diabetic rats. Glibenclamide, which is currently used as a medicine for type 2 diabetes, showed a comparable amount of recovery (282 ± 12 g) to the control group.

Figure 1 shows the blood glucose level changes in the control and experimental animals of each group. STZ-induced diabetic rats showed increases of blood glucose levels beginning at 5 days after STZ injection, as well as 5.6-fold increases at 30 days relative to the control animals (105 ± 7 and 585 ± 16 mg/dL, respectively). STZ-induced increases of blood glucose levels were effectively reduced by post- or same-day treatment with ANT beginning 10 days after STZ injection. Finally, at 30 days, ANT decreased the

Table 1. Body weight in control, STZ-induced diabetic rats, and anthocyanins before and after STZ treatment or GLB after STZ treatment

	Day 5	Day 10	Day 15	Day 20	Day 25	Day 30
Body weight (g)						
Control ($n = 5$)	255 ± 15	275 ± 15	295 ± 20	305 ± 16	325 ± 18	335 ± 20
STZ ($n = 5$)	254 ± 13	220 ± 12**	198 ± 12**	185 ± 12**	180 ± 10**	175 ± 12**
STZ+ANT (same-day, $n = 5$)	255 ± 10	254 ± 12†	256 ± 17††	255 ± 10††	268 ± 10††	275 ± 14††
STZ+ANT (post, $n = 5$)	255 ± 10	255 ± 14†	258 ± 15††	265 ± 12††	272 ± 10††	280 ± 12††
STZ+GLB (post, $n = 5$)	257 ± 14	256 ± 12†	260 ± 14††	268 ± 14††	275 ± 15††	282 ± 12††

Data are presented as means ± SD of three independent experiments. One-way analysis of variance (ANOVA) was used to compare multiple group means and was followed by a Newman–Keuls test (significance compared with control, $^{**}p < 0.01$; significance compared with STZ-treated rats, $^{\dagger}p < 0.05$ or $^{\dagger\dagger}p < 0.01$).

blood glucose level from 585 to 255 mg/dL (post-treatment) or 260 mg/dL (same-day treatment); these decreases were at least as efficient (and more so) as that of glibenclamide (265 mg/dL).

3.2 Improvement of hemodynamic parameters by ANT in STZ-induced diabetic conditions

Functional cardiac performance was assessed by measuring the left ventricular response to changes in left atrial filling pressure *per* second in terms of LVED, $+dp/dt$, and $-dp/dt$. The value of LVEDP in STZ-induced diabetic rats was significantly reduced ($p<0.01$) as compared with that in the control rats. Table 2 summarizes the hemodynamic measurements, which clearly show that left ventricular systolic pressure decreased after the onset of diabetes. ANT or glibenclamide treatment significantly improved this parameter, and ANT demonstrated greater effects to improve this parameter than did glibenclamide in both groups (same-day and post-treatment). The result shows that hyperglycemia decreased the $+dp/dt$ to 3202.2 ± 218.7 mmHg/s, whereas the glibenclamide and ANT treatment increased the $+dp/dt$. The $-dp/dt$ in the STZ-treated group was dramatically elevated compared with the control rats, whereas the glibenclamide and ANT treatment decreased this parameter in all experimental groups (same-day and post-treatment); however, HR did not change in any groups of the experimental animals. Therefore, we conclude from this data that ANT markedly improve heart functions after the onset of diabetes at least as efficiently (and possibly more so) as glibenclamide.

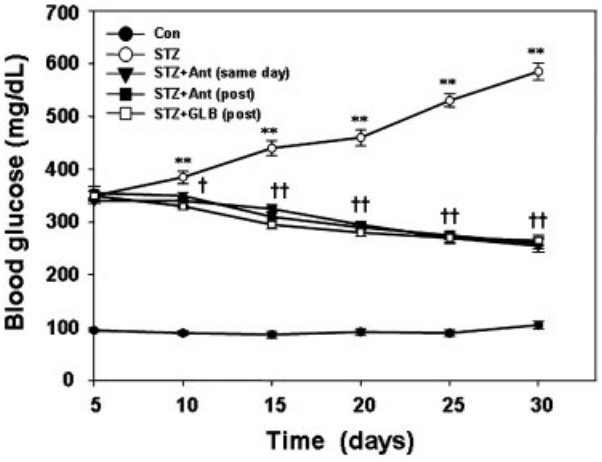

Figure 1. ANT down-regulate blood glucose concentrations that are increased in STZ-induced diabetic rats. The STZ-induced diabetic model and ANT (same-day or post-treatment) or glibenclamide treatment were performed as described in Section 2. Every 5th day after the induction of diabetes (measured blood glucose levels $\geq$300 mg/dL) by STZ injection, blood glucose levels were measured by a glucometer in each group ($n=5$). Bars represent mean$\pm$SD of three independent experiments (significance compared with control, $^{**}p<0.01$; significance compared with STZ-treated rats, $^{\dagger}p<0.05$ or $^{\dagger\dagger}p<0.01$).

3.3 The effect of ANT on insulin and triglyceride levels in STZ-induced diabetic rat

The onset of diabetes in rats resulted in a significant reduction in serum insulin levels 30 days after STZ treatment, whereas ANT or glibenclamide treatment significantly increased the concentration of insulin (Fig. 2A). Serum triglycerides were also increased in diabetic rats (Fig. 2B), which were markedly decreased by ANT or glibenclamide treatment compared with the STZ-treated group. Importantly, ANT seem to have a more marked effect on insulin production and triglyceride levels than does glibenclamide in the post-treated groups of diabetic rats. Moreover, when ANT were given on the same day as the STZ injection, their effect was much stronger than that of observed in the post-treated group. These data suggest that the STZ-induced diabetic rats used in this study manifested hyperglycemia, hyperlipidemia, and hypoinsulinemia, and that ANT regulated these abnormalities.

Table 2. Summary of hemodynamic measurements in control, STZ-induced diabetic rats, and STZ+ANT or GLB-treated rats

	Control ($n=4$)	STZ ($n=5$)	STZ+ANT (same-day, $n=5$)	STZ+ANT (post, $n=5$)	STZ+GLB (post, $n=5$)
LVSP (mm/Hg)	132.4$\pm$13.7	97.5$\pm$6.4*	122.3$\pm$13.0†	128.08$\pm$12.4†	123.7$\pm$7.4†
LVEDP (mmHg/sec)	16.2$\pm$1.3	12.6$\pm$1.0**	14.6$\pm$0.6†	14.9$\pm$0.8†	6.5$\pm$1.1††
HR (bpm)	269.9$\pm$17.6	257.2$\pm$38.4	261.2$\pm$15.8	257.4$\pm$7.2	278.6$\pm$25.3
$+dp/dt$ (mmHg/s)	4670.7$\pm$360.6	3202.2$\pm$218.7**	4533.1$\pm$227.1††	4491.4$\pm$98.4††	4627.4$\pm$456.5††
$-dp/dt$ (mmHg/s)	$-$4317.3$\pm$584.3	$-$2396.1$\pm$216.5**	$-$3780.8$\pm$386.6††	$-$3991.9$\pm$258.4††	$-$4035.95$\pm$452.2††
Systolic blood pressure (mmHg)	127.8$\pm$15.8	94.9$\pm$7.0*	118.0$\pm$14.1	125.1$\pm$13.2†	123.9$\pm$15.4†
DBP (mmHg)	97.1$\pm$9.4	69.3$\pm$9.7*	90.5$\pm$9.1	93.1$\pm$10.3†	89.4$\pm$8.4
Mean arterial pressure (mmHg/s)	107.3$\pm$11.4	77.8$\pm$8.7*	99.7$\pm$10.8	103.7$\pm$10.9†	100.8$\pm$3.5†

Data are presented as means$\pm$SD of three independent experiments (significance compared with control $^{*}p<0.01$ and $^{**}p<0.01$; significance compared with STZ-treated rats, $^{\dagger}p<0.05$ or $^{\dagger\dagger}p<0.01$).

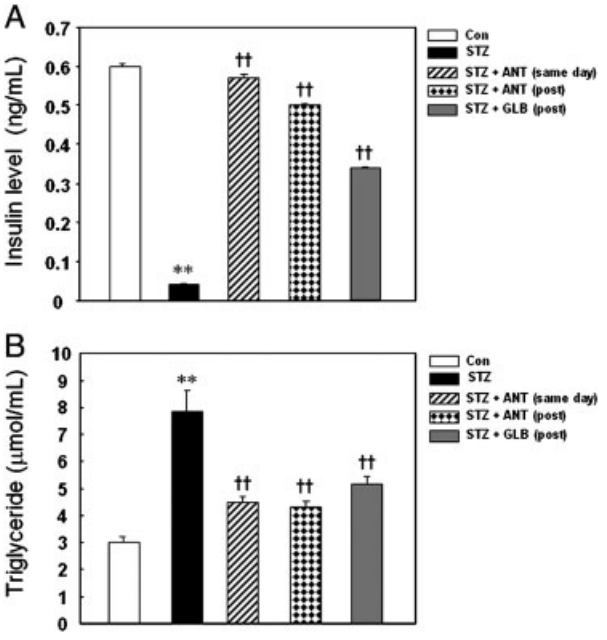

Figure 2. The effect of ANT on insulin (A) and triglyceride (B) concentrations in serum from STZ-induced diabetic rats. Serum was collected on the 30th day of the experiment, and insulin levels and triglyceride concentrations were determined by commercial kits as described in Section 2. Data are presented as means ± SD of three independent experiments (significance compared with control, $^{**}p<0.01$; significance compared with STZ-treated rats, $^{\dagger}p<0.05$ or $^{\dagger\dagger}p<0.01$).

3.4 The effect of ANT on GLUT4 expression in skeletal muscle and heart tissue from STZ-induced diabetic rats

It is known that glucose uptake is controlled by GLUT4 in the plasma membrane, and that GLUT4 translocation to the membrane seems to depend on insulin-mediated signaling pathways. Thus, we investigated whether the hypoglycemic effect of ANT involves the regulation of myocardial and skeletal GLUT4 translocation and subsequent glucose uptake in STZ-induced diabetes. The STZ treatment of rats leads to a marked reduction of GLUT4 expression in the membrane fraction of skeletal muscles and heart tissues (Figs. 3A and B). ANT or glibenclamide significantly influenced the STZ-reduced GLUT4 expression in membrane fractions. These results showed that both drugs effectively increase GLUT4 translocation and glucose uptake, although ANT seem to provoke a much stronger effect than does glibenclamide (clearly shown in heart tissues).

3.5 Stimulation of IRβ phosphorylation by ANT in STZ-induced diabetic rats

Next, we tested whether ANT can act as IR activators with insulin-mimetic activity by analyzing their effect on

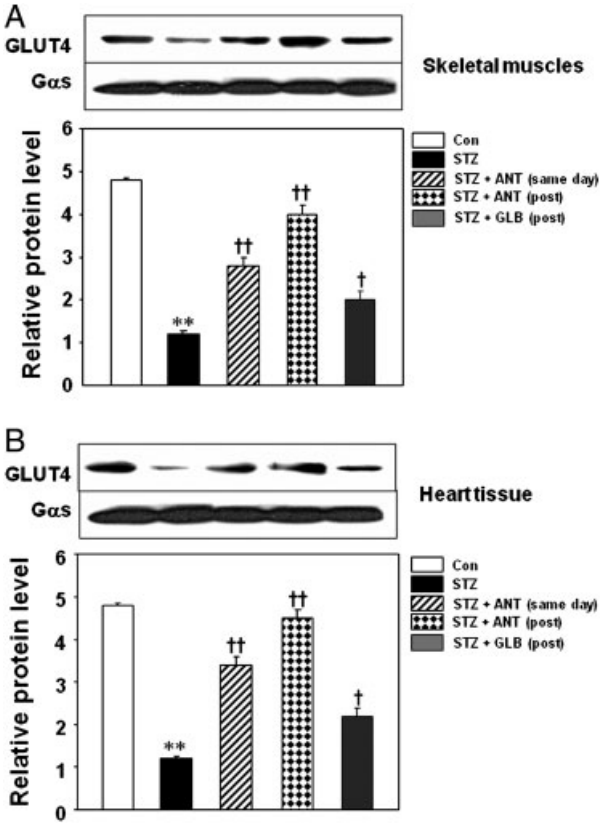

Figure 3. ANT increase the expression of GLUT4 in skeletal and heart tissues in STZ-induced diabetic rats. Membrane fractions were extracted as described in Section 2, and GLUT4 protein levels were determined in skeletal (A) and heart (B) tissues. Data are presented as means ± SD of three independent experiments (significance compared with control, $^{**}p<0.01$; significance compared with STZ-treated rats, $^{\dagger}p<0.05$ or $^{\dagger\dagger}p<0.01$) (GaS, guanine nucleotide-binding protein G(s), α subunit (Adenylate cyclase-stimulating G α protein), a loading control for membrane fractionation).

IRβ phosphorylation in heart tissues and skeletal muscles. Figure 4 shows that IR phosphorylation is significantly decreased in STZ-treated rats, whereas ANT or glibenclamide co-treated rats presented higher tyrosine kinase activity of the IRβ subunit. Interestingly, the effect of ANT on phosphorylation of IRβ in skeletal muscles is stronger than that of glibenclamide for both the same-day and the post-treatment groups; in contrast, these two compounds demonstrate similar effectiveness in heart tissue. This result suggests that ANT work as IR activators, which additionally may explain their hypoglycemic properties.

3.6 Protective effect of ANT against STZ-induced apoptosis in the pancreas

In order to determine the protective effects of ANT against STZ-induced apoptotic cell death of pancreatic tissues, the

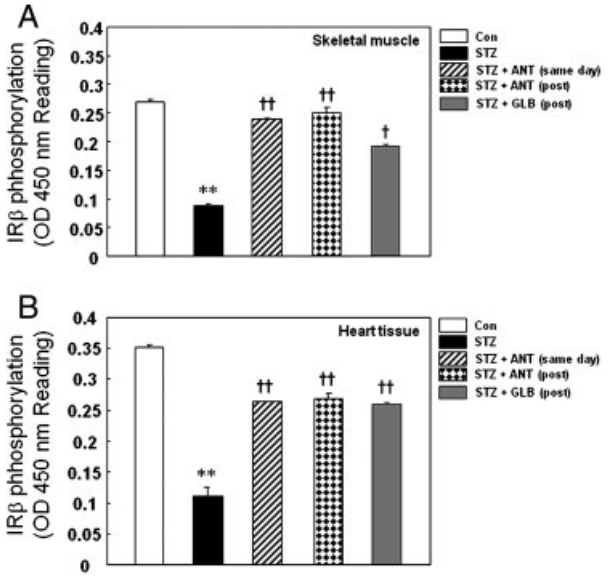

Figure 4. Administration of ANT restores IRβ phosphorylation decreased in STZ-induced diabetic condition. After the rats were treated with ANT for 30 days, they were anesthetized and skeletal (A) and heart (B) tissues were removed. Membrane fractionations were performed according to the manufacture's protocol, and the level of IRβ tyrosine kinase phosphorylation was determined in the protein extracts. Data are presented from three independent experiments (significance compared with control, $^{**}p<0.01$; significance compared with STZ-treated rats, $^{\dagger}p<0.05$ or $^{\dagger\dagger}p<0.01$).

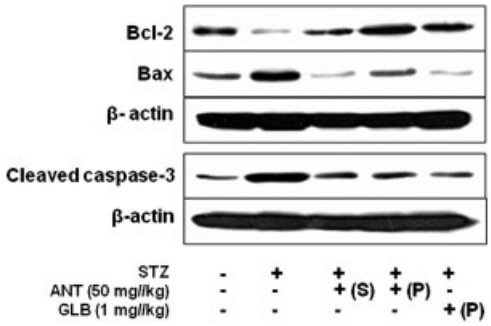

Figure 5. ANT protect the pancreas of STZ-induced diabetic rats from apoptosis through the inhibition of caspase 3 cleavage and proapoptotic protein Bax, and the up-regulation of antiapoptotic protein Bcl-2. On the 30th day of the experiments, rats were sacrificed and pancreatic tissues were removed and homogenized. Total proteins were extracted as described in Section 2. The levels of Bcl-2, Bax, and cleaved caspase-3 were determined by Western blot analysis. These data were confirmed by three independent experiments (ANT (S), anthocyanins same-day treatment; ANT (P), anthocyanins post-treatment; and GLB (P), glibenclamide post-treatment).

levels of apoptosis-associated proteins, such as Bcl-2, Bax, and cleaved caspase-3, were detected by Western blot analysis. Figure 5 clearly shows that 30 days after STZ treatment, ANT exert a protective effect against STZ-induced apoptosis in pancreatic tissue (including β-islet cells) through the up-regulation of Bcl-2 and down-regulation of Bax protein. In addition, ANT down-regulated the cleavage of caspase-3. Moreover, a TUNEL assay also showed that STZ induced a significant induction of apoptotic cell death in pancreatic tissue, which was effectively inhibited by ANT (Fig. 6). Figures 5 and 6 show that the protective effects of ANT are at the very least as significant as those of glibenclamide in post-treatment groups.

3.7 Protective effect of ANT against oxidative stress induced by STZ in rat

Figure 7A shows the levels of malondialdehyde (MDA) in the serum from control and experimental animals in each group. The MDA levels were greatly increased in STZ-induced diabetic rats compared with normal rats. The treatment of STZ-induced diabetic rats with ANT or glibenclamide for 30 days resulted in a marked decrease in MDA levels. Interestingly, ANT treatment on the same day as STZ showed a greater potential to reduce MDA levels than did glibenclamide or ANT given 5 days after STZ treatment. In Figs. 7B and C, the activities of enzymatic antioxidants (SOD and CAT) in the serum from STZ-induced diabetic rats were significantly decreased compared with normal rats. ANT treatment increased SOD and CAT activities, and the effect of ANT was stronger than that of glibenclamide. The STZ-mediated decrease of SOD and CAT activities was also more effectively recovered by ANT treatment on the same day as STZ. These results reveal that the anti-oxidant potential of ANT against STZ-induced oxidative stress is greater than that of glibenclamide.

4 Discussion

DM is the most common endocrine disorder, and is an important health problem worldwide. DM is characterized by chronic hyperglycemia with disturbances of carbohydrate, fat, and protein metabolism resulting from defects in insulin secretion, insulin action, or both. Chronic hyperglycemia causes complications linked to diabetes, such as heart disease, retinopathy, kidney disease, and neuropathy [29]. Thus, strict control of the blood glucose level is considered essential in order to delay and/or prevent the development of diabetic complications. In addition to hyperglycemia, diabetic patients also commonly suffer from hyperlipidemia, which can lead to increased atherosclerosis and incidence of heart disease [30]. DM is associated with a specific cardiomyopathy, in which left ventricular dysfunctions present as the earliest manifestation of diabetic cardiomyopathy [31]. Moreover, heart dysfunctions observed in diabetic patients are believed to be caused by a progression of diabetic nephropathy [32]. In this study, we have clearly demonstrated that ANT markedly decrease blood glucose levels in STZ-induced diabetic rats and prevent the

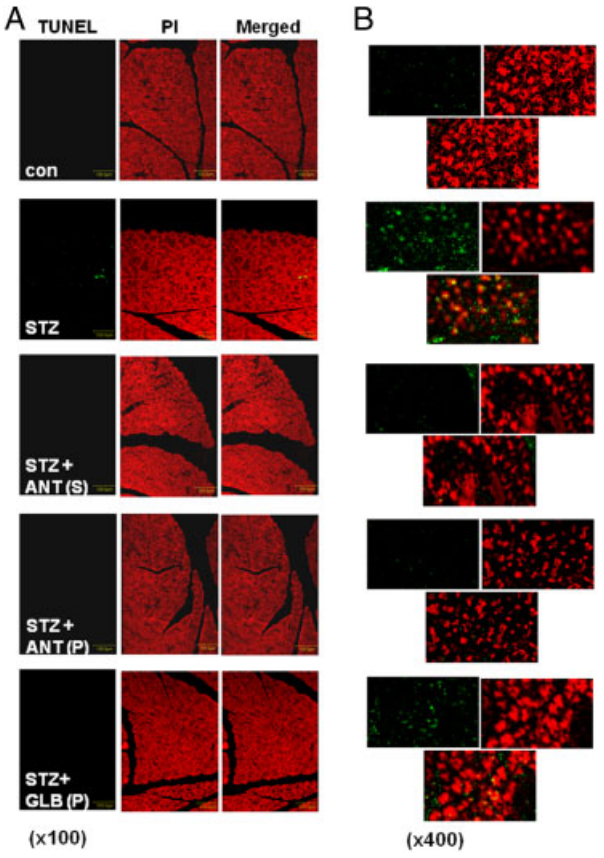

Figure 6. ANT protect pancreatic tissue (including β-islet cells) from STZ-induced apoptotic cell death, as shown by a TUNEL assay. Representative micrographs of TUNEL staining of pancreatic tissues are shown ((A) × 100 magnification; (B) × 400 magnification). ANT exhibited marked protective effects compared with glibenclamide and decreased the positive nuclei (green) in pancreatic tissues (including β-islet cells). Data were confirmed by three independent experiments.

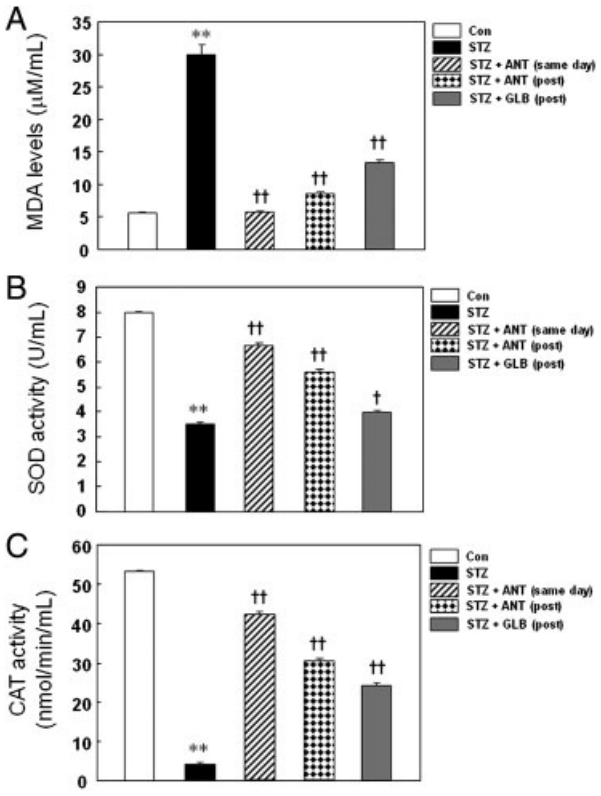

Figure 7. Administration of ANT prevents changes in the levels of MDA, SOD, and CAT in STZ-induced diabetic rats. MDA (A), SOD (B), and CAT (C) levels were measured in serum extracted by cardiac punctures on the 30th day of the experiment. Data are presented as means ± SD of three independent experiments (significance compared with control, $^{**}p<0.01$; significance compared with STZ-treated rats, $^{\dagger}p<0.05$ or $^{\dagger\dagger}p<0.01$).

decrease in body weight typically seen after the onset of diabetes. We have also demonstrated that ANT (50 mg/kg) significantly improve LVEDP and $\pm dp/dt$ in diabetic rats, thus suggesting that they can prevent the development of macrovascular complications in diabetic patients. As the efficacy of flavonoids such as ANT is dose dependent, 100 or 200 mg/kg ANT would have the protective effect on STZ-induced diabetic rat. However, in our previous studies, ANT showed the significant preventive effects from 50 mg/kg against various oxidative stresses [23, 33]. Thus, we used only one dose (50 mg/kg) to show the protective effect of ANT on STZ-induced diabetic rat.

A major metabolic defect associated with T2DM is the failure of peripheral tissues in the body to properly utilized glucose, thereby resulting in chronic hyperglycemia. GLUT4, the major insulin-dependent transporter, is predominantly present in skeletal muscles, myocardium, and adipose tissue, and plays an important regulatory role in whole-body glucose homeostasis [34–36]. Severe insulin resistance and glucose intolerance appear at an early age in muscle-GLUT4 knockout mice [37]. Conversely, the up-regulation of GLUT4 due to treatment with the anti-diabetic agent troglitazone is related to an improvement in insulin resistance in type 2 diabetic rats [38]. Thus, increasing the expression of GLUT4 may contribute to enhanced insulin sensitivity. STZ injection decreases GLUT4 expression in skeletal muscles and heart tissues, whereas administration of ANT and glibenclamide to diabetic rats restores GLUT4 translocation to the plasma membranes for the uptake of glucose. Taken together, the data reveal that ANT evoke anti-hyperglycemic effects *via* the improvement of GLUT4 translocation in the membranes of skeletal and heart tissues.

The IR is a tetrameric protein consisting of two identical extracellular α-subunits and two identical transmembrane β-subunits with intracellular tyrosine kinase activity [39, 40]. The binding of insulin to the α-subunits leads to a conformational change and stimulation of the receptor kinase activity *via* auto-phosphorylation of tyrosine residues in the β-subunits [39–41]. This study has shown that the administration of ANT leads to a higher level of IR auto-phosphorylation. These observations suggest that IRβ is the site

of action of ANT, and provides a possible explanation for the hypoglycemic effect of ANT as IR activators in the treatment of diabetes.

Blood glucose levels are mainly controlled by insulin secretion from pancreatic β cells. STZ destroys pancreatic β cells by different mechanisms, including DNA damage by alkylation, depletion of NAD^+, and generation of reactive oxygen species (ROS) and nitrogen species. The generation of ROS and subsequent increase of local oxidative stress, DNA methylation, and protein modification are suggested as the pathophysiological mechanisms of STZ-induced diabetes. Thus, antioxidants were considered to be promising agents against STZ-induced diabetes since they can diminish oxidative stress by inhibiting ROS generation and lipid peroxidation [42]. ROS react with lipids and cause peroxidative changes that result in elevated lipid peroxidation. The increase in lipid peroxidation might be indicative of a decrease in enzymatic and nonenzymatic antioxidants defense mechanism. Thus, we examined if ANT might prevent STZ-induced oxidative stress and further apoptotic cell death. Western blot analysis revealed that treatment with ANT prevents STZ-induced apoptotic cell death in pancreatic tissues (including β-islet cells) through the regulation of pro-apoptotic proteins, such as Bax, and anti-apoptotic proteins, such as Bcl-2, and the cleavage of caspase-3 (Fig. 5). In addition, a TUNEL assay shows that ANT prevent STZ-induced apoptotic cell death. Of course, our data do not directly provide evidence that ANT protect pancreatic β-islet cells from apoptosis by STZ; however, it is commonly known that detecting apoptotic cell death in β-islet cells is difficult [43]. Therefore, we attempted to demonstrate the protective effect of ANT on STZ-induced apoptosis using pancreatic tissues consisting of β-islet cells. In addition, the treatment of STZ-induced diabetic rats with ANT resulted in a marked decrease in the MDA levels increased by STZ. Apart from the nonenzymatic antioxidants, enzymatic antioxidants, such as SOD and CAT, play an important role in protecting cells from oxidative damage. SOD is an enzymatic antioxidant that catalyzes the conversion of a superoxide radical to hydrogen peroxide (not a free radical itself, but a reactive molecule) and molecular oxygen. Another enzymatic antioxidant, CAT, catalyzes the reduction of hydrogen peroxides and protects the tissues against reactive hydroxyl radicals. Decreased activities of enzymatic antioxidants such as SOD and CAT have been well documented in STZ-induced diabetic rats [44]. In this investigation, the low levels of activity of both of these enzymes registered in diabetic rats indicated diabetes-induced stress. ANT administration to diabetic animals improved both SOD and CAT activities substantially, reflecting the antioxidant potency of these drugs.

In conclusion, the results of this study demonstrate that (i) the administration of ANT demonstrates anti-hyperglycemic effects, which control blood glucose levels and restored body weight in diabetic conditions; (ii) hemodynamic parameters are significantly improved by ANT in both groups (*i.e.* same-day and post-treatment), thereby suggesting that ANT may, in a long term, prevent the development of diabetic complications such as nephropathy, retinopathy, or heart dysfunction; (iii) ANT increase the concentration of insulin in diabetic rats; (iv) ANT restore GLUT4 expression in the plasma membranes of skeletal and heart tissues, thereby inducing glucose uptake in diabetic conditions; (v) the administration of ANT results in the activation of IRβ tyrosine kinases, suggesting that ANT may be IR activators in the treatment of diabetes; (vi) ANT protect pancreatic tissue including β-islet cells against apoptosis induced by STZ through the regulation of the Bax/Bcl-2 ratio and caspase-3 activity, and thus, prevent the loss of islet viability and functionality; and (vii) ANT restore antioxidant-defense mechanisms, thus protecting tissues from oxidative damage in the diabetic state. When we treated rats with ANT before the STZ injection (same-day treatment) to examine if ANT would prevent onset of diabetes, although ANT could not prevent the onset of STZ-induced diabetic condition, interestingly, pretreatment of ANT (same-day treatment) had a more marked effect on insulin production and a protective effect of STZ-induced oxidative stress. Furthermore, when we compared these effects of ANT with those of a clinically used anti-diabetic agent (glibenclamide), ANT given either on the same day or after STZ treatment showed better effects than does glibenclamide.

To our knowledge, this is the first report related to the anti-hyperglycemic, anti-hyperlipidemic, and antioxidant properties of ANT obtained from black soybean seed coats. Black soybeans have long been popularly utilized as a food and medicinal material with a low price. Some drugs are used for the therapy of diabetes and its associated complications; however, there has been little evidence that food factors themselves are directly beneficial in the diabetic state. Therefore, we suggest the possibility that ANT from black soybean seed coats may be used to prevent the development of diabetes, or furthermore modulate T2DM and its complications.

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