ANTIHYPERGLYCEMIC AND ANTIOXIDANT PROPERTIES OF CAFFEIC ACID IN *DB/DB* MICE

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Running Title: Anti-diabetic Effects of Caffeic acid

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The number of text pages: 18

The number of tables: 2

The number of figures: 6

The number of references: 42

The number of words in the Abstract: 215

The number of words in the Introduction: 499

The number of words in the Discussion: 1507

Abbreviations: CAT, catalase; GAPDH, glyceraldehydes-3-phosphate dehydrogenase;

GK, glucokinase; GLUT, glucose transporter; G6Pase, glucose-6-phosphatase; GSH-Px,

glutathione peroxidase; PEPCK, phosphoenolpyruvate carboxykinase; SOD, superoxide

dismutase.

A recommended section: Endocrine and Diabetes

ABSTRACT

This study investigated the blood glucose-lowering effect and antioxidant capacity of caffeic acid in C57BL/KsJ-*db/db* mice. Caffeic acid induced a significant reduction of the blood glucose and glycosylated hemoglobin levels than the control group. The plasma insulin, C-peptide, and leptin levels in caffeic acid group were significantly higher than those of the control group, whereas the plasma glucagon level was lower. Increased plasma insulin by caffeic acid was attributable to an antidegenerative effect on the islets. Caffeic acid also markedly increased glucokinase activity and its mRNA expression and glycogen content, and simultaneously lowered glucose-6-phosphatase and phosphoenolpyruvate carboxykinase activities and their respective mRNA expressions, accompanied by a reduction in the glucose transporter 2 expression in the liver. In contrast to the hepatic glucose transporter 2, adipocyte glucose transporter 4 expression was greater than the control group. Also, caffeic acid significantly increased superoxide dismutase, catalase, and glutathione peroxidase activities and their respective mRNA levels, while lowering the hydrogen peroxide and thiobarbituric acid reactive substances levels in the erythrocyte and liver of *db/db* mice. These results indicate that caffeic acid exhibits a significant potential as an anti-diabetic agent by suppressing a progression of type 2 diabetic states that is suggested by an attenuation of hepatic glucose output and enhancement of adipocyte glucose uptake, insulin secretion, and antioxidant capacity.

INTRODUCTION

Type 2 diabetes is characterized by pancreatic β-cell dysfunction accompanied by insulin resistance. Normal pancreatic β-cells can compensate for the insulin resistance by increasing insulin secretion, however extensive exposure of pancreatic β-cells to high glucose levels causes β-cell dysfunction that is associated with impaired insulin secretion and biosynthesis (Robertson et al., 1992). Insulin resistance contributes to increasing glucose output in the liver and decreasing glucose uptake in adipose tissues (Ferre et al., 1996; Abel et al., 2001). In particular, liver is an insulin-sensitive tissue and plays a major role in maintaining glucose homeostasis by regulating the interaction between the glucose utilization and gluconeogenesis (Ferre et al., 1996). Indeed, resistance to insulin-stimulated glucose transport in adipose tissue is one of the defects in insulin resistance states such as obesity and type 2 diabetes (Abel et al., 2001). Thus, a suitable anti-diabetic agent should improve glucose-induced insulin secretion, hepatic glucose metabolism and peripheral insulin sensitivity.

There is an increasing evidence indicating that oxidative stress produced under hyperglycemia can cause or lead to insulin resistance and diabetes complications (Matsuoka, 1997). Moreover, several studies have shown that antioxidant ameliorates a number of altered physiological and metabolic parameters that occur as a result of type 2 diabetes (Kaneto et al., 1999; Balasubashini et al., 2004). Phenolic compounds, widely distributed in food plants, act as a primary antioxidant and can be helpful for improving or preventing a number of chronic diseases (Scalbert et al., 2005). However, there is a growing interest in several biological properties of phenolic compounds in addition to their antioxidant effects, and the evidence suggests that certain dietary polyphenolic compounds may result in an altered glucose metabolism (Scalbert et al., 2005; Okutan et al., 2005). Among various

phenolic compounds, caffeic acid (3,4-di(OH)-cinnamate), found in many types of fruit and coffee in high concentrations, has exhibited pharmacological antioxidant, anticancer and antimutagenic activities (Okutan et al., 2005). Caffeic acid is known to have an anti-diabetic effect in streptozotocin-induced diabetic rats (Hsu et al., 2000; Cheng et al., 2003; Okutan et al., 2005; Park and Min, 2006). However, there is no available evidence of such effect of caffeic acid in type 2 diabetes or insulin resistance animal model.

The present study was designed to examine the possibility of anti-diabetic effects by caffeic acid in *db/db* mice, a good model for type 2 diabetes that display many of the characteristics of the human disease including hyperphagia, hyperglycemia, insulin resistance, and progressive obesity (Hummel et al., 1966). The initial adaptation to the insulin resistance is one of islet β-cell hyperplasia resulting in marked hyperinsulinemia, but ultimately islets develop β-cell necrosis, insulinopenia, severe hyperglycemia, and weight loss (Orlnd and Permutt, 1987). We evaluated parameters of glucose homeostasis, activities and expressions of gene coding for key insulin-sensitive enzymes regulating hepatic glycolysis and gluconeogenesis, hepatic and adipocyte glucose transporter expression and pancreatic function. This study also investigated the protective effect of caffeic acid on the oxidative damage induced by diabetes and their possible role in ameliorating the development of diabetes.

METHODS

Animals and diets

Twenty male C57BL/KsJ-*db/db* mice were purchased from Jackson Laboratory (Bar Harbor, ME) at 5 wk of age (23 g). They were fed a pelletized commercial chow diet for acclimation from the arrival during 2 wk, then randomly divided into two groups with ten

mice and fed a AIN-76 semisynthetic diet with or without a caffeic acid supplementation (0.02%; 3,4-dihydroxycinnamic acid, 3-(3,4-dihydroxyphenyl)-2-propenoic acid, Sigma, USA) for 5 wk.

All mice were maintained under a controlled light-dark cycle (12:12 h, lights on at 08:00) and constant temperature (24℃). They were given free access to food and distilled water and the food consumption and body weight gain were measured daily and weekly respectively. At the end of the experimental period, all the mice were anesthetized with ketamine following a 12-h fast and blood samples were collected from the inferior vena cava into heparin-coated tube. The blood was centrifuged at 1,000×g for 15 min at 4℃, and plasma and erythrocyte separated. The livers and adipose tissues were removed, washed, weighted, and frozen at -70℃ until analyzed. All procedures were approved by the animal ethics committee of our university.

Blood biomarkers

The blood glucose concentration were measured at 7, 8, 9, 10, 11, and 12 wk of age, following 0, 1, 2, 3, 4, and 5 wk of caffeic acid supplementation, respectively. The blood glucose concentration was measured with whole blood obtained from the tail veins after withholding food for 6 h using a glucose analyzer based on the glucose oxidase method (Glucocard test strip, Arkray, Japan) and the glycated hemoglobin was measured with a analyzer (Roche, Swiss). Plasma insulin (DSL-1600 Insulin RIA kit, Diagnostic Systems Laboratories, USA), C-peptide (C-peptide RIA kit, Diagnostic Systems Laboratories, USA), glucagon (Glucagon RIA kit, Packard, USA) and leptin (Mouse leptin RIA kit, Linco Research, USA) levels were measured based on a radioimmunometric assays.

Hepatic glycogen assay

The hepatic glycogen concentration was determined as previously described by Seifter et al. (1950) with modification.   Briefly, the liver tissue was homogenized in 5 volumes of an 30% (w/v) KOH solution and dissolved at 100℃ for 30 min.   The glycogen was determined by treatment with an anthrone reagent (2 g anthrone/1 L of 95% (v/v) H_2SO_4) and measuring the absorbance at 620 nm.

Enzyme activities

Glucokinase (GK) activity was determined in the hepatic cytosol using a spectrophotometric assay as described by Davidson and Arion (1987) with a slight modification, whereby the formation of glucose-6-phosphate at 37℃ was coupled to its oxidation by glucose-6-phosphate dehydrogenase and NAD^+.   The reaction mixture contained in a final volume of 1 mL, 50 mM sodium Hepes, pH 7.4, 100 mM KCl, 7.5 mM $MgCl_2$, 5 mM ATP, 2.5 mM dithioerythritol, 10 mg/mL albumin, 1 mM NAD^+, 5.5 units of glucose-6-phosphate dehydrogenase (*Leuconostoc mesenteroides*), hepatic cytosol, and 10 mM glucose.   Glucose-6-phosphatase (G6Pase) activity was determined in the hepatic microsome using a spectrophotometric assay according to the method Alegre et al. (1988) with a slight modification, which contained 100 mmol/L sodium Hepes (pH 6.5), 26.5 mmol/L glucose-6-phospate, 1.8 mmol/L EDTA, both previously adjusted to pH 6.5, 2 mmol/L $NADP^+$, 0.6 KIU/L mutarotase, and 6 KIU/L glucose dehydrogenase.

Phosphoenolpyruvate carboxykinase (PEPCK) activity was monitored in the direction of

oxaloacetate synthesis using the spectrophotometric assay developed by Bentle and Lardy

(1976) with a slight modification.   The reaction mixture contained the following in 1 mL

final volume: 50 mM sodium Hepes, pH 6.5, 1 mM IDP, 1 mM $MnCl_2$, 1 mM dithiothreitol,

0.25 mM NADH, 2 mM Phospoenolpyruvate, 50 mM $NaHCO_3$, 7.2 units of malic

dehydrogenase and hepatic cytosol.   Enzyme activity was determined at 25℃ for 2 min by

decrease of absorbance at 340 nm.   Superoxide dismutase (SOD) activity was

spectrophotometrically measured by the inhibition of pyrogallol autoxidation at 420 nm for

10 min according to the method of Marklund and Marklund (1974).   One unit was

determined as the amount of enzyme that inhibited the oxidation of pyrogallol by 50%.

Catalase (CAT) activity was measured using Aebi's (1974) method with a slight modification,

in which the disappearance of hydrogen peroxide was monitored at 240 nm for 5 min using a

spectrophotometer.   Ten microliters of the solution was added to a cuvette containing 2.89

mL of a 50 mM potassium phosphate buffer (pH 7.4), then the reaction was initiated by

adding 0.1 mL of 30 mM H_2O_2 to make a final volume of 3.0 mL at 25℃.   The

decomposition rate of H_2O_2 was measured at 240 nm for 5 min using a spectrophotometer.

Glutathione peroxidase (GSH-Px) activity was measured using the spectrophotometric

assay at 25℃, as described previously by Paglia and Valentine's (1967) method with a slight

modification.   The reaction mixture contained 2.525 mL of a 0.1 M of Tris-HCl (pH 7.2)

buffer, 75 μL of 30 mM glutathione, 100 μL of 6 mM NADPH, and 100 μL of

glutathione reductase (0.24 unit).   One hundred microliters of the solution was added to 2.8

mL of the reaction mixture and incubated at 25℃ for 5 min.   The reaction was initiated by

adding 100 μL of 30 mM H_2O_2 and the absorbance measured at 340 nm for 5 min.   The

protein concentration was measured by the method of Bradford (1976) using BSA as the

standard.   Also, the hemoglobin concentration was estimated in an aliquot of the hemolysate,

using a commercial assay kit (Sigma).

Hydrogen peroxide and lipid peroxidation assay

The hydrogen peroxide levels in erythrocyte and liver were measured by Wolff's

method (1994).   FOX 1 (Ferrus Oxidation with Xylenol orange) reagent was prepared as

following mixture with 100 μM xylenol orange, 250 μM ammonium ferrus sulfate, 100 mM

sorbitol, and 25 mM H_2SO_4.   Fifty microliters of test sample is added to 950 μL FOX 1

reagent, vortexed, and incubated at room temperature for a minimum of 30 min at which

color development is virtually complete.   The absorbance was read at 560 nm and the

standard was linear in the 0~5 μM concentration range.   The erythrocyte and hepatic

thiobarbituric acid-reactive substances (TBARS) concentration, as a marker of lipid peroxide

production, was measured spectrophotometrically by the method of Ohkawa et al (1979).

Northern blot analysis

Total RNA was isolated from the livers by the guanidine thiocyanate-phenol method of

Chomzynski and Sacchi (1987).   The total RNA (20 µg) was separated on a 0.9% agarose

gel containing 2.2 M formaldehyde and transferred to Nytran-Plus membranes (Schleicher &

Schuell, Dassel, Germany).   The membranes were then hybridized with a [^{32}P]-labeled

cDNA probe, washed at room temperature with 2× sodium chloride sodium citrate (SSC)

containing 0.1% SDS followed by two washes at 65℃ with 0.2× SSC containing 0.1% SDS,

and exposed to X-ray film with an intensifying screen at -70℃.   Thereafter, DNA probes

were prepared from the mouse liver RNA using an RT-PCR with following primers: for GK

5'-TTCACCTTCTCCTTCCCTGTAAGGC-3'   and   5'-

TACCAGCTTGAGCAGCACAAGTCG-3' (fragment size: 490 bp, GenBank accession no.:

L38990);   for   G6Pase   5'-AAGACTCCCAGGACTGGTTCATCC-3'   and   5'-

TAGCAGGTAGAATCCAAGCG CG-3' (fragment size: 607 bp, GenBank accession no.:

U00445);   for   PEPCK   5'-TGCTGATCCTGGGCATAACTAACC-3'   and   5'-

TGGGTACTCCTTCTGGAGATTCCC-3' (fragment size: 489 bp, GenBank accession no.:

BC037629);   Cu/Zn   SOD   5'-AGGATTAACTGAAGGCGAGCAT-3'   and   5'-

TCTACAGTTAGCAGGCCAGCA G-3' (fragment size: 410 bp, GenBank accession no.:

M25157);   for   CAT   5'-ACGAGATGGCACACTTTGACAG-3'   and   5'-

TGGGTTTCTCTTCTGGCTATGG-3' (fragment size: 341 bp, GenBank accession no.:

M11670);   for   GSH-Px   5'-AAGGTGCTGCTCATTGAGAATG-3'   and   5'-

CGTCTGGACCTACCAGGAACTT-3' (fragment size: 406 bp, GenBank accession no.:

M21210);   and   for   GAPDH   5'-TTGAAGGGTGGAGCCAAACG-3'   and   5'-

AGTGGGAGTTGCTGTTGAAGTCG-3' (fragment size: 541 bp, GenBank accession no.:

M32599).   The intensities of the mRNA bands were quantified using a Bio Image Whole

Band Analyzer (50S, B.I. System Co., USA) and subsequently normalized based on the intensity of the respective GAPDH mRNA bands.

Western blot analysis

The livers and adipose tissues were prepared according to Nathan et al. (2001) with a slight modification.   Briefly, the tissue was homogenized with a buffer containing 250 mM sucrose, pH 7.4, 10 mM triethanolamin, 10 mM acetic acid, 1 mM EDTA, 1 mM PMSF, 1 mM benzamidine hydrochloride hydrate, and 1 mM DTT.   The homogenates were then centrifuged at 1,000×g for 10 min at 4℃, and the supernatant centrifuged at 100,000×g for 1 h at 4℃ in an ultracentrifuge (Bechman, Fullerton, CA).   The pellet resuspended as a membrane fraction for a GLUT2 and 4 assay.   The protein concentrations were determined using the bicinchoninic acid procedure (Pierce, Rockford, IL).   The proteins were separated by 14% SDS-PAGE gels, transferred to Hybond-ECL nitrocellulose membranes (Amersham, Arlington Heights, IL, USA), blocked and probed with rabbit anti-mouse GLUT2 (1:50,000; Biogenesis, Sandown, NJ, USA) and anti-mouse GLUT4 (1:50,000; Biogenesis, Sandown, NJ, USA) and then with horseradish peroxidase-conjugated anti-rabbit secondary antibody (Amersham, Aylesbury, UK).   The immunoreactive bands were visualized using ECL kit (Upstate, USA) according to the manufacturer's instructions, quantified using a Bio Image Whole Band Analyzer (50S, B.I. System Co., USA).

Pancreas histology and immunohisochemistry

Removed pancreas fixed in 10% (v/v) neutral buffered formalin, processed routinely and embedded in paraffin wax.   Paraffin sections were cut at 4-µm thickness and deparaffinized in xylene for 5 min and rehydrated through the graded ethanol.   The section

were stained with hematoxylin and eosin (H&E) and for immunohistochemistry, rehydrated sections were treated with 3% (v/v) H_2O_2 in methanol for 30 min to block endogenous peroxidase and washed with 0.01 mol/L phosphate buffer for 10 min and then immunostained with the primary antibody, monoclonal mouse anti-insulin. The antigen-antibody complex was visualized by an avidin-biotin peroxidase complex solution using an ABC kit (Vector Laboratories, Burlingame, CA, USA) with 3,3,-diamino benzidine (Zymed Laboratories, San Francisco, CA, USA).

Statistical analysis

All data are presented as the mean ± SE. Statistical analyses were performed using the statistical package for the social science software (SPSS) program. Student's t-test was used to assess the differences between the groups. Statistical significance was considered at $p < 0.05$. Pearson correlation coefficients were calculated to examine the association of the plasma leptin with the blood glucose, plasma insulin, body weight, and adipose tissue weight.

RESULTS

Body weight gain, relative organ weight, and food intake

The body weight of the caffeic acid group increased throughout the experimental period, whereas that of the control group decreased after 3 wk. Thus, the body weight was significantly higher in the caffeic acid group than in the control group at wk 3, 4 and 5 of the experimental period (Fig. 1). Food intakes and relative organ weights were about the same for all groups (data not shown).

Blood glucose and glycosylated hemoglobin levels

All *db/db* mice were diabetic when the experiment began, as indicated by the blood glucose level ($\geq$21.47 mmol/L). Caffeic acid significantly lowered the blood glucose level compared to the control group at wk 3, 4, and 5 of the experimental period (Fig. 1). The glycosylated hemoglobin level was also significantly lower in the caffeic acid group than the control group (Table 1).

Plasma insulin, C-peptide, glucagon and leptin levels

The plasma insulin, C-peptide, and leptin levels of the caffeic acid group were significantly higher than those of the control group, whereas the plasma glucagon level of the caffeic acid group was significantly lower than that of the control group (Table 1). The plasma leptin and blood glucose levels were inversely correlated (r=-0.748, p<0.01) (Fig. 2). In contrast, the plasma leptin level was positively correlated with body weight (r=0.819, p<0.001) and plasma insulin level (r=0.835, p<0.001) (Fig. 2).

Hepatic glucose regulating enzyme activities and glycogen concentration

Caffeic acid significantly elevated hepatic GK activity when compared to the control group by about 28% (Fig. 3). In contrast, G6Pase and PEPCK activities were markedly lower in the caffeic acid group by 29% and 19%, respectively (Fig. 3). The hepatic glycogen concentration was significantly higher in the caffeic acid group (Table 1).

Erythrocyte and hepatic antioxidant enzyme activities, hydrogen peroxide, and lipid peroxidation levels

The erythrocyte SOD, CAT and GSH-Px activities were significantly higher in the caffeic acid group than in the control group (Table 2). Caffeic acid also markedly elevated the hepatic SOD, CAT and GSH-Px activities (Table 2). The hydrogen peroxide levels were significantly lower in the erythrocyte, hepatic cytosolic and mitochondrial fraction from the caffeic acid-supplemented *db/db* mice (Table 2). In addition, the caffeic acid significantly lowered the lipid peroxidation levels in erythrocyte and liver (Table 2).

Hepatic enzyme mRNA expression

The mRNA levels of the hepatic glucose metabolic and antioxidant enzymes were monitored using a Northern blot analysis. As a loading control, the glucose regulating enzyme and antioxidant enzyme mRNA signals were normalized to the GAPDH mRNA signal for each group. The mRNA level of GK was significantly elevated in the caffeic acid-supplemented group than in the control group (Fig. 4). However, the mRNA levels of G6Pase and PEPCK were markedly lower in the *db/db* mice supplemented with caffeic acid (Fig. 4). The mRNA levels of SOD, CAT and GSH-Px were significantly elevated in the caffeic acid group (Fig. 4). Thus, the changes in the glucose metabolic and antioxidant enzymes mRNA expressions were similar to the respective enzyme activities in the liver.

Glucose transporter protein expression

The changes in the hepatic glucose transporter 2 (GLUT2) and adipose tissue glucose transporter 4 (GLUT4) protein expressions were examined by the western blotting analysis (Fig. 5). Caffeic acid significantly lowered the hepatic GLUT2 protein level compared to the control group. In contrast, the expression of GLUT4 protein in adipose tissue was markedly increased in the caffeic acid group.

Histology and immunohisochemistry for pancreas

The control *db/db* mice exhibited islet boundary definition loss and degeneration, whereas *db/db* mice supplemented with caffeic acid preserved islet architecture (Fig. 6). When β-cells were stained with anti-insulin antibodies, caffeic acid-supplemented *db/db* mice exhibited strong staining compared to the control *db/db* mice. (Fig. 6).

DISCUSSION

Caffeic acid significantly lowered the fasting blood glucose level compared to the control *db/db* mice, which is in agreement with previous studies done by others (Hsu et al., 2000; Cheng et al., 2003; Park and Min, 2006).   The level of glycosylated hemoglobin, a well-recognized marker of chronic glycemic control, was also markedly lower in the *db/db* mice supplemented with caffeic acid.   This antihyperglycemic action of caffeic acid is likely associated with a marked enhancement of the GK mRNA expression and activity in the liver. Hepatic GK has a major effect on glucose homeostasis and is a potential target for pharmacological treatment of type 2 diabetes, as evidenced by the fact that liver-specific GK-knockout mice exhibited mild hyperglycemia (Postic et al., 1999) and rats overexpressing GK in the liver had reduced blood glucose (Ferre et al., 1996).   The increase of hepatic GK can cause an increased utilization of the blood glucose for energy production or glycogen storage in the liver (Iynedjian et al., 1988).   This study showed that hepatic glycogen content was significantly higher in the caffeic acid-supplemented group.

A low hepatic GK activity is also reported to favor the release of glucose synthesized by gluconeogenesis into the circulation (Hers and Hue, 1983).   Hepatic gluconeogenesis is also crucial to the maintenance of fasting hyperglycemia and is observed high in *db/db* mice

(Friedman et al., 1997). The G6Pase and PEPCK are the key enzymes that control gluconeogenesis and glucose output from the liver, and their gene expressions were increased in *db/db* mice (Friedman et al., 1997). In the study, caffeic acid caused a marked reduction in the hepatic PEPCK and G6Pase activities and their mRNA levels in *db/db* mice, indicating a decreased hepatic glucose production. Along with this line, hepatic GLUT2 protein expression was also lowered in the caffeic acid group than in the control group. The decrease in GLUT2 expression is known to be related with a decrease in hepatic glucose output (Oka et al., 1990). Based on these results, the caffeic acid seemed to suppress the hepatic glucose output by enhancing hepatic glucose utilization and inhibiting glucose over-production in *db/db* mice.

Hepatic GK, G6Pase and PEPCK activities are reported to be controlled primarily at the level of transcription, being regulated by insulin and glucagon. High insulin levels have been shown to inhibit hepatic glucose production by means of stimulation of GK gene transcription and glycogen synthesis and inhibition of gluconeogenesis (Iynedjian et al., 1988; Friedman et al., 1997). In contrast, glucagon induces an inhibition of GK gene transcription and a stimulation of hepatic PEPCK gene transcription, and even a small increase of glucagon level may induce a relative increase in the gluconeogenesis (Iynedjian et al., 1995; Friedman et al., 1997). In our study, the changes in hepatic glucose regulating enzymes could be partly attributed to insulin and glucagon levels, because plasma insulin level was significantly elevated, whereas plasma glucagon level was lowered in the caffeic acid-supplemented *db/db* mice than in the control *db/db* mice at 12-wk-old.

Plasma insulin levels of *db/db* mice are known to be age-dependent. The initial adaptation to the insulin resistance is one of islet β-cell hyperplasia resulting in marked hyperinsulinemia (Orland and Permutt, 1987). However, when the *db/db* mice reach at 12

to 24-wk-old, islet develops β-cell necrosis, hyperinsulinemia is diminished, and the mice manifest symptoms of insulin deficiency (Orland and Permutt, 1987).   We observed that islet surface area in pancreas is relatively greater in caffeic acid-supplemented *db/db* mice than in the control group.   Caffeic acid also preserved islet and β-cell architecture relatively better compared to the control group.   Moreover, caffeic acid significantly increased the levels of C-peptide that has a longer half-life than insulin and thus may better represent insulin secretion than insulin levels do (Doda, 1996).   Taken together, these data suggest that the plasma insulin level in the *db/db* mice may be gradually declined after reaching the peak point, while caffeic acid is considered to slow the age-dependent insulin decline by a reduction of β-cell mass.   Similar effects of ferulic acid, a phenolic acid, on pancreas of diabetic rats were reported by Balasubashini et al. (2004).

Another possible mechanism by which caffeic acid mediates its anti-diabetic action may be due to enhanced transport of blood glucose to adipose tissue.   In general, glucose transport in liver and adipocytes are regulated by different mechanisms.   Hepatic GLUT2 expression is higher in human and rodent with type 2 diabetes (Friedman et al., 1997), however adipose GLUT4 overexpression is known to alleviate insulin resistance and pancreatic defects in *db/db* mice, resulting in a markedly improved glycemic control (Gibbs et al., 1995).   Conversely, selective elimination of GLUT4 expression in adipose tissue impairs insulin action in liver (Abel et al., 2001).   The present study showed that caffeic acid significantly enhanced the GLUT4 protein expression in adipose tissue compared to the control group.   This result can be supported by Pinent's findings (Pinent et al., 2004) that procyanidins, a polyphenolic compound, increased the amount of insulin-sensitive GLUT4 and stimulated glucose uptake in adipose tissue.   In other words, caffeic acid has a dual

mechanism of action that enhances insulin release from the pancreas and also improves insulin resistance in the liver and adipose tissue.

Insulin also stimulates leptin synthesis and release through the regulation of glucose metabolism in adipocytes (Wabitsch et al., 1996). Leptin enhances insulin action by inhibiting hepatic glucose production (Brazilai et al., 1997). These suggest that low levels of leptin with type 2 diabetes could increase insulin resistance and thereby worsening the condition. Interestingly, the present study exhibited a positive correlation between plasma leptin and insulin levels and body weight (r=0.835, p<0.001; r=0.819, p<0.001) and a reverse association between plasma leptin and blood glucose levels (r=-0.748, p<0.01), as previously reported by others (Wabitsch et al., 1996; Moriya et al., 1999; Considine et al., 1996). Especially, caffeic acid-supplemented *db/db* mice continuously gained body weight throughout the study, although the control *db/db* mice did not gain more after 10 wk of age. Since *db/db* mice stops gaining body weight after 10 wk of age but slowly lose weight along as diabetic phenotype progressed (Orland and Permutt, 1987), it is likely that improvement of hyperglycemia by caffeic acid supplement delays further development of diabetic state and thereby enhances the animal's ability to thrive. Similar result was shown in GLUT4-upregulated *db/db* mice that continue to gain body weight until 15 wk of age and then maintained until at least 35 wk of age (Gibbs et al., 1995).

Antioxidant was previously been recognized as a means to treat diabetes, whose antioxidants such as vitamin E decreases blood glucose levels through improvement of insulin action in type 2 diabetes (Kaneto et al., 1999). In diabetes, reactive oxygen species (ROS) resulted from hyperglycemia cause cell damage (Matsuoka, 1997). Erythrocyte is especially susceptible to oxidative damage resulting from a high concentration of oxygen and hemoglobin (Clemens and Waller, 1987). Liver is also known to undergo free radicals

mediated injury in diabetes and increased ROS is related to the damage of hepatic glucose regulating enzymes (Lelli et al., 2005). Normally, erythrocyte and liver contain enough scavenger such as SOD, CAT, and GSH-Px to protect against free radical injury. However, prolonged exposure of obese-diabetic *db/db* mice to hyperglycemic condition reduces the activities of SOD and CAT (Makar et al., 1995).

From our results, caffeic acid supplement resulted in dramatic increase in the antioxidant enzyme activities and mRNA levels in both erythrocyte and liver compared to the control group. The SOD plays an important role in protecting cells from oxidative damage by converting superoxide radicals into hydrogen peroxide, which is then further metabolized by CAT and GSH-Px, where CAT detoxifies hydrogen peroxide and GSH-Px catalyze the destruction of hydrogen peroxide and lipid hydroperoxide. If the CAT and GSH-Px activity is not sufficiently enhanced to metabolize hydrogen peroxide, this can lead to an increased hydrogen peroxide and TBARS levels (Haron, 1991). As such, a combination of SOD and CAT or GPH-Px may be necessary rather than SOD alone to reduce oxidative stress. It is noteworthy that, in the *db/db* mice supplemented with caffeic acid, the changes of antioxidant enzymes resulted in a decreased hydrogen peroxide levels in erythrocyte and liver compared to the control group. Furthermore, the erythrocyte and hepatic TBARS levels were significantly lower in the caffeic acid group than in the control group, indicating a decreased rate of lipid peroxidation. As a result, enhanced antioxidant enzyme activities in the erythrocyte and liver by caffeic acid may have a protective role against ROS, thereby preventing the formation of hydrogen peroxide and lipid peroxidation. Thus, it seems reasonable that caffeic acid was effective for preventing erythrocyte and hepatic damage.

In conclusion, the data obtained in this study suggest that caffeic acid is an effective anti-diabetic agent via its ability to enhance insulin secretion and to decrease hepatic glucose

output along with the increased level of adipocyte glucose disposal in the type 2 diabetic animals.   Furthermore, it seems likely that caffeic acid is beneficial against oxidative stress, thereby being helpful in preventing or delaying the development of diabetes and its complications.

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FOOTNOTES

LEGENDS FOR FIGURES

Fig. 1. Change in body weight (A) and blood glucose levels (B) for C57BL/KsJ-*db/db* mice supplemented with caffeic acid.   Values are mean±SE (n=10).   *p<0.05 and **p<0.01 vs. control group as determined by student's t-test.

Fig. 2. Correlations between plasma leptin level and blood glucose level (A), plasma insulin level (B), and body weight (C) in C57BL/KsJ-*db/db* mice fed diet supplemented with caffeic acid.

Fig. 3. The activities of hepatic GK, G6Pase, and PEPCK in C57BL/KsJ-*db/db* mice fed diet containing caffeic acid. Values are mean±SE, n=10.   *p<0.05, **p<0.01, ***p<0.001 vs. control group as determined by student's t-test. GK, glucokinase; G6Pase, glucose-6-phosphatase; PEPCK, phosphoenolpyruvate carboxykinase.

Fig. 4. Effects of caffeic acid on mRNA levels of genes encoding regulatory enzymes for glucose metabolism and antioxidant defense in the liver of C57BL/KsJ-*db/db* mice.   A: Quantitative of hepatic glucose metabolic enzymes blot, B: Representative northern blot of hepatic glucose metabolic enzymes, C: Quantitative of hepatic antioxidant enzymes blot, D: Representative northern blot of hepatic antioxidant enzymes.   The results represent mean±SE (n=10).   *p<0.05, **p<0.01, ***p<0.001 vs. control group as determined by student's t-test.   GK, glucokinase; G6Pase, glucose-6-phosphatase; PEPCK, phosphoenolpyruvate carboxykinase; GAPDH, glyceraldehydes-3-phosphate dehydrogenase; SOD, superoxide dismutase; CAT, catalase; GSH-Px, glutathione peroxidase.

Fig. 5. Effects of caffeic acid on protein expression of hepatic GLUT2 and dipocyte GLUT4 in C57BL/KsJ-*db/db* mice.   A: Quantitative of GLUT2 blot, B: Representative western blot of GLUT2, C: Quantitative of GLUT4 blot, D: Representative western blot of GLUT4.   The results represent mean±SE (n=10).   **p<0.01, ***p<0.001 vs. control group as determined by student's t-test.   GLUT2, glucose transporter 2; GLUT4, glucose transporter 4.

Fig. 6. (A) Histological observation of pancreas by hematoxylin and eosin staining. Diabetic *db/db* mice exhibited degenerated islet, however after 5 wk supplement with caffeic acid, it preserved islet architecture. (B) Immunohistochemical staining for insulin.   *Db/db* mice supplemented with caffeic acid exhibited a stronger staining than the control group. Original magnification ×200.

Table 1. Concentrations of plasma insulin, c-peptide, glucagon, leptin, blood glycosylated hemoglobin, and hepatic glycogen in C57BL/KsJ-*db/db* mice fed diet supplemented with caffeic acid

	Control	Caffeic acid
Insulin (pmol/l)	202.10±12.62	328.62±17.04[***]
C-peptide (pmol/l)	199.80±2.35	233.10±2.35[***]
Glucagon (ng/l)	136.64±3.62	98.46±3.39[***]
Leptin (µg/l)	49.10±3.16	77.10±2.78[***]
Glycosylated hemoglobin (%)	13.48±0.11	11.11±0.06[***]
Glycogen (mg/ g liver)	56.15±1.51	70.23±0.48[***]

Values are mean±SE, n=10.

[***]$p < 0.001$ vs. control group as determined by student's t-test

Table 2. The activities of erythrocyte and hepatic antioxidant enzyme and the levels of hydrogen peroxide and TBARS in C57BL/KsJ-*db/db* mice fed diet supplemented with caffeic acid.

	Control	Caffeic acid
Erythrocyte		
SOD (unit/g Hb)	898.28±16.49	1037.96±16.93[***]
CAT (μmol/g Hb /min)	93.40±16.23	143.60±9.57[*]
GSH-Px (μmol/g Hb /min)	28.16±1.86	42.55±2.34[**]
H_2O_2 (μmol/g Hb)	23.52±0.56	21.68±0.09[*]
TBARS (μmol/g Hb)	2.68±0.01	2.27±0.01[**]
Liver		
SOD (unit/mg protein)	7.73±0.57	15.51±0.97[***]
CAT (μmol/mg protein/min)	4.79±0.16	5.82±0.28[*]
GSH-Px (nmol/mg protein/min)	42.22±2.01	57.38±1.80[***]
cH_2O_2 (nmol/mg protein)	7.77±0.32	5.17±0.35[**]
mH_2O_2 (nmol/mg protein)	66.32±2.15	52.10±2.26[**]
TBARS (nmol/ g liver)	4.88±0.33	2.49±0.42[**]

Values are mean±SE, n=10.

[*]$p < 0.05$, [**]$p < 0.01$, [***]$p < 0.001$ vs. control group as determined by student's t-test

cH_2O_2, cytosolic H_2O_2 ; mH_2O_2, mitochondria H_2O_2; Hb, hemoglobin.

Figure 1
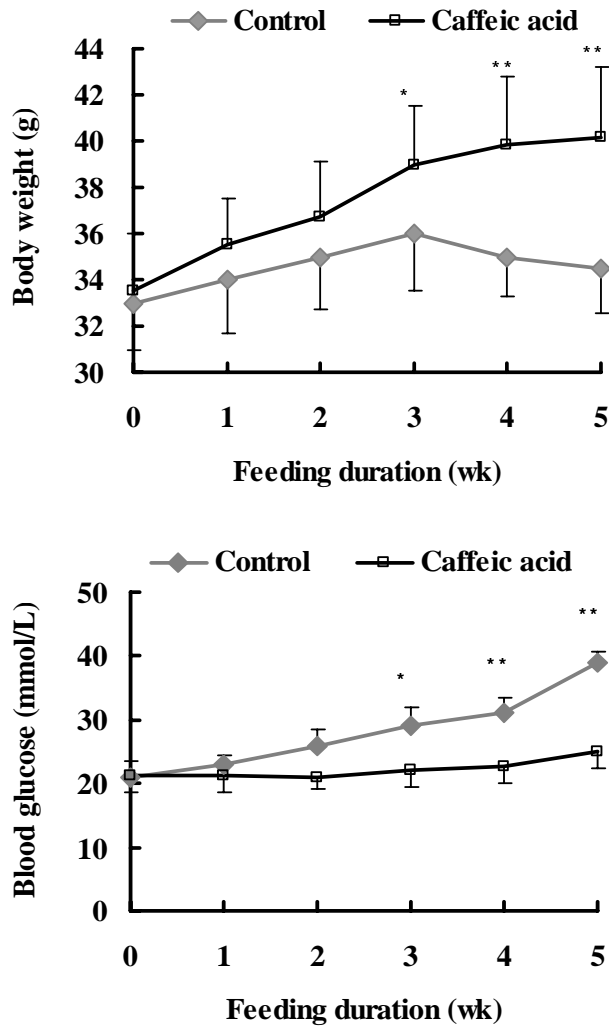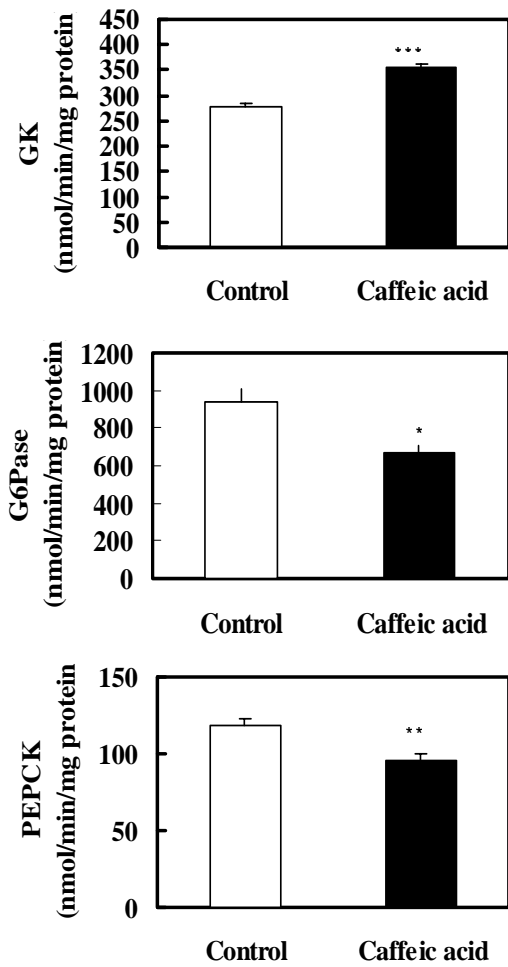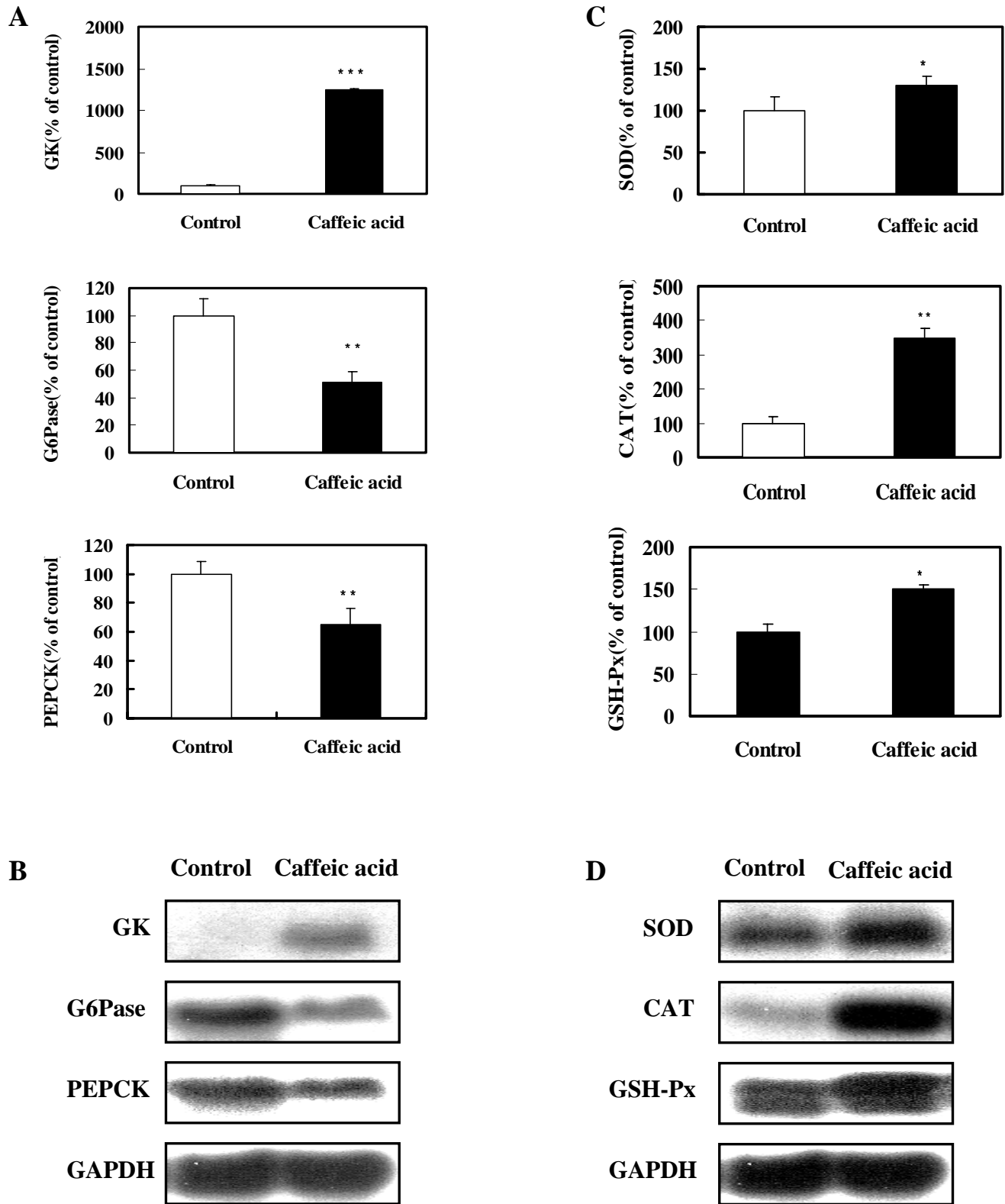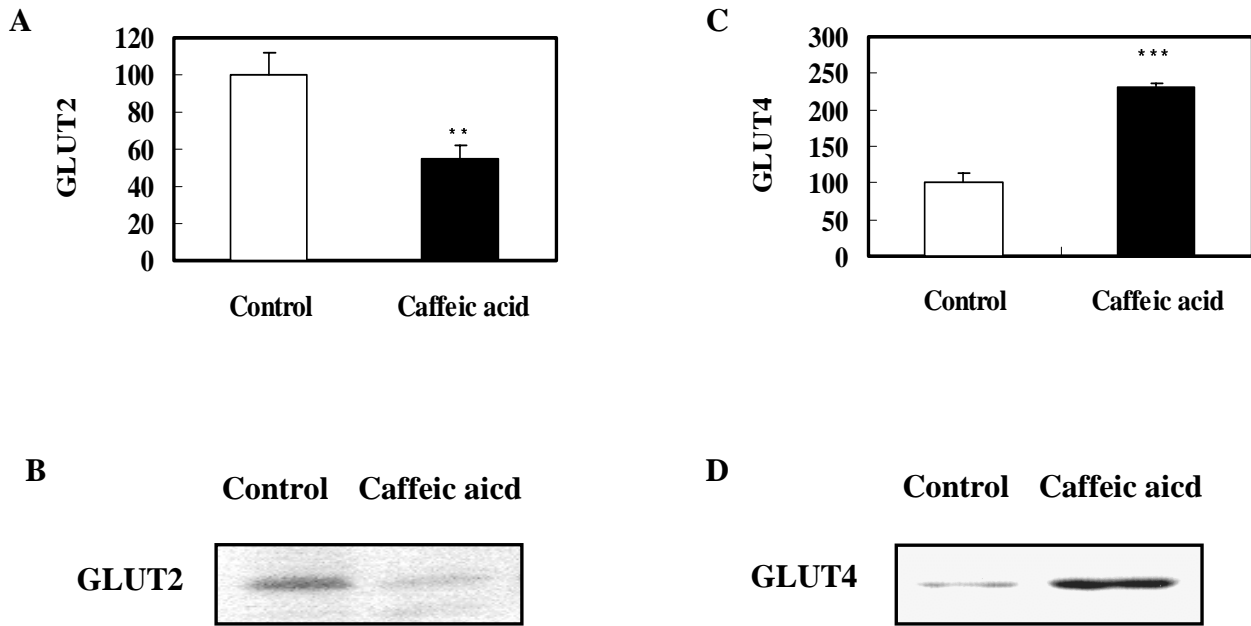
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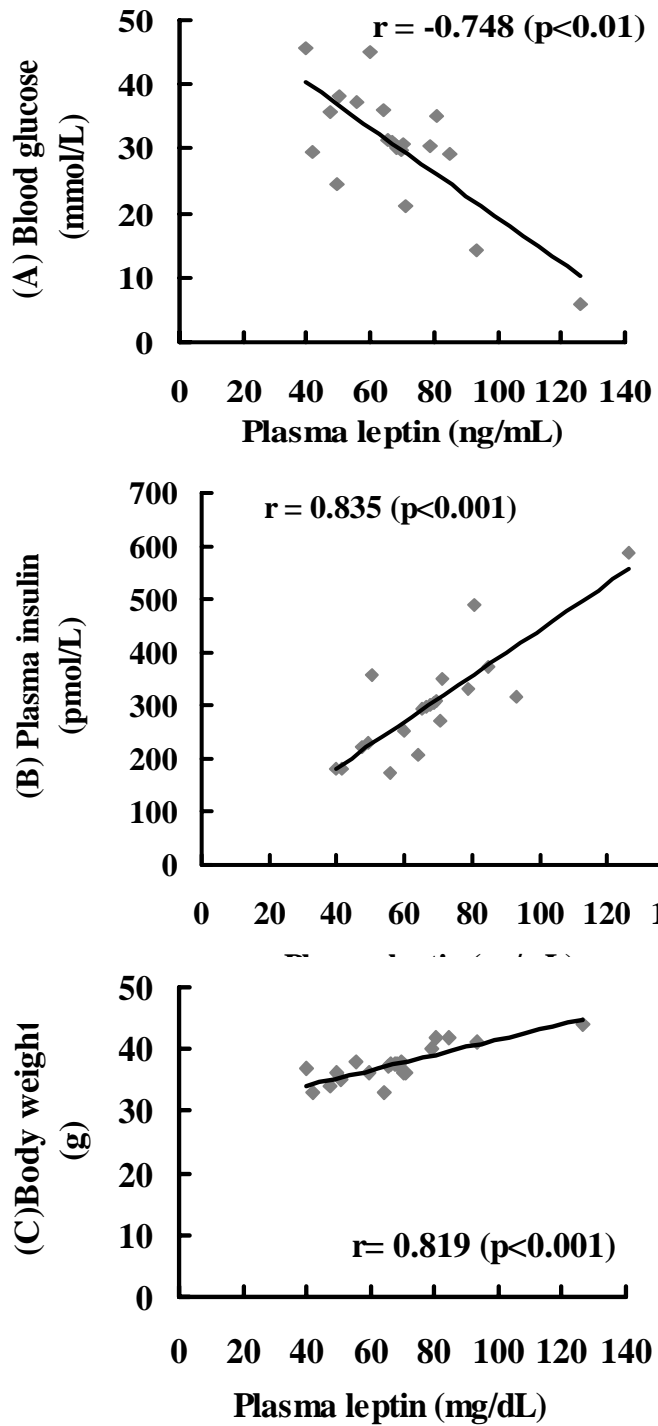
Figure 3

Figure 4

Figure 5

A

C

B

D

Figure 6