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Antihyperglycemic Effect of a Caffeamide Derivative, KS370G, in Normal and Diabetic Mice

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The antihyperglycemic actions of caffeamide derivatives, especially KS370G, in normal ICR, streptozotocin-induced diabetic (T1DM) and diet-induced diabetic (T2DM) mice were investigated in this study. Oral administration of the compound decreased the plasma glucose levels in both normal and diabetic mice, and appeared to be in a dose-dependent manner in normal and diet-induced type 2 diabetic mice. It was found that KS370G could stimulate the release of insulin in both normal and T2DM mice, and a dose of 1 mg per kg KS370G could significantly attenuate the increase of plasma glucose induced by an intraperitoneal glucose challenge test in normal and diabetic mice. Similar treatment with KS370G significantly increased glycogen content in both liver and skeletal muscle. Hence, the hypoglycemic effect of KS370G in normal and diabetic mice could be attributed to the stimulation of insulin release and the increase of glucose utilization.

KEYWORDS: Caffeamide; KS370G; diabetic mice; T1DM; T2DM; antihyperglycemia

INTRODUCTION

Lifestyle patterns in industrialized societies comprise an increasing availability and ingestion of high-caloric food in the prevalence of sedentary living, and these factors are emerging as the fundamental causes of fast-spreading diabetes. Diabetes mellitus (DM) is a metabolic disease with hyperglycemia and usually accompanied by many complications. The incidence of acute myocardial infarction and hypertrophic cardiomyopathy are pretty high in the population of DM disease (*1*). Therefore, an attempt was devoted to find new antidiabetic agents with cardiovascular protective activity. Many naturally occurring compounds, such as resveratrol, quercetin, theaflavin, berberine, curcumin and CAPE (caffeic acid phenethyl ester), are structurally similar to polyphenol, which significantly accelerates AMPK phosphorylation and is successfully employed in the prevention and treatment of a variety of diseases (*2*), including suppressing hepatic gluconeogenesis, stimulating glucose uptake, antihyperglycemia and antiobesity effects (*3*−*10*). CAPE is one of the major components of honeybee propolis and appears to exhibit antioxidant (*11*), anti-inflammatory (*12*), proapoptotic (*13*), antiviral (*14*), and immunomodulatory properties (*15*). Lee et al. reported that AMPK and Akt are involved in CAPE-induced glucose uptake in the muscle cells (*7*), suggesting that CAPE might have some metabolic and antidiabetic effects via accelerating AMPK phosphorylation. The present study was to find out whether KS370G, a derivative of CAPE, possessed hypoglycemic activities in normal ICR mice, streptozotocin (STZ)-induced diabetic mice and diet-induced diabetic mice, the latter two representing insulin-dependent DM (IDDM) and non-insulin-dependent DM (NIDDM) animal models, respectively.

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MATERIALS AND METHODS

Chemicals. Compounds were obtained from the following method of amide binding coupling to prepare these compounds (**Figure 1**). A solution of benzotriazol-1-yloxytris(dimethylamino)phosphonium hexafluorophosphate (BOP) (1.2 equiv) in dichloromethane (CH_2Cl_2) (5 mL) was added to a mixture of caffeic acid (100 mg), R−NH_2 (1.2 equiv) and triethylamine (Et_3N) (0.08 mL) in dimethylformamide (DMF) (1.0 mL). The mixture was stirred at 0 °C for 30 min, and then stirred at room temperature for 12 h. This reaction mixture was evaporated under vacuum, and the residue was partitioned between ethyl acetate (AcOEt) and H_2O. Successively, the AcOEt layer was washed with 3 N aqueous HCl and 10% $NaHCO_3$(aq), dried over $MgSO_4$ and concentrated in a vacuum. The residue was further purified by column chromatography with eluting solution (CH_2Cl_2−AcOEt 1:1, v/v) on silica gel (70−230 and 230−400 mesh, Merck 7734). The final products (82−88% yield) were recrystallized from AcOEt to obtain pure crystals. ^1H and ^{13}C NMR spectra were recorded on a Bruker Avance 500 spectrometer. Electron impact mass spectra (EIMS) were determined on a Finnigan TSQ-46C mass spectrometer. IR spectra were recorded on a Nicolet Magna-IR 550 spectrophotometer.

KS368A: solid; mp 125−126 °C. IR ν_{max} (cm^{-1}): 3423, 1646, 1593, 1546, 1467, 1360, 1255, 1138, 1020, 970, 857, 817. ^1H NMR (CD_3COCD_3, 500 MHz): δ 2.77 (2H, t, J = 6.8 Hz), 3.51 (2H, q, J = 6.8 Hz), 3.76 (3H, s), 3.78 (3H, s), 6.51 (1H, d, J = 15.6 Hz), 6.73 (1H, dd, J = 8.0, 2.0 Hz), 6.81 (1H, d, J = 8.0 Hz), 6.81 (1H, d, J = 8.0 Hz), 6.84 (1H, d, J = 2.0 Hz), 6.91 (1H, dd, J = 8.0, 2.0 Hz), 7.02 (1H, d, J = 2.0 Hz), 7.24 (1H, br s, -NH), 7.45 (1H, d, J = 15.6 Hz), 8.19 (1H, s, −OH), 8.38 (1H, s, −OH). EI-MS m/z (%): 343 (M^+, 10), 163 (100), 151 (16).

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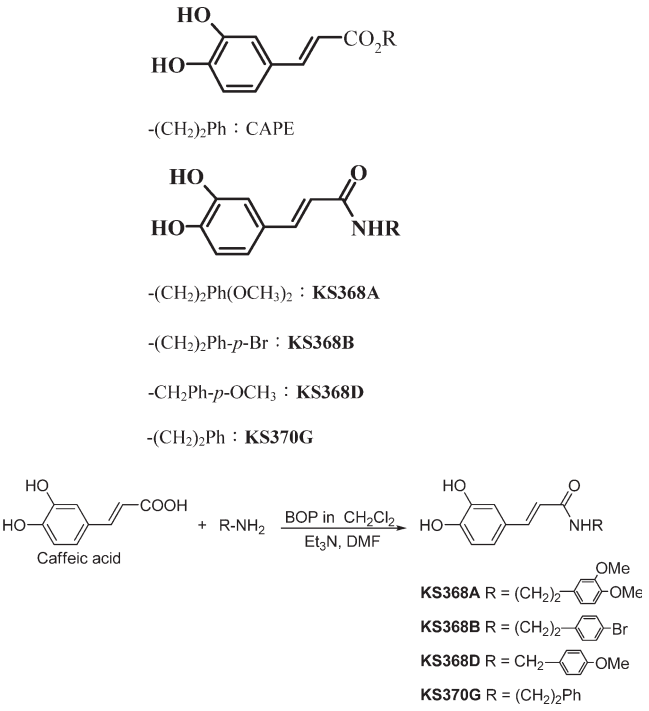

Figure 1. The chemical structures of CAPE and caffeamide derivatives used in the present study.

KS368B: solid; mp 198−199 °C. IR ν_{max} (cm^{-1}): 3317, 1653, 1593, 1527, 1487, 1441, 1367, 1285, 1195, 1116, 1016, 988, 811. ^1H NMR (CD_3COCD_3, 500 MHz): δ 2.83 (2H, t, J = 6.6 Hz), 3.54 (2H, q, J = 6.6 Hz), 6.40 (1H, d, J = 16.0 Hz), 6.82 (1H, d, J = 8.0 Hz), 6.92 (1H, dd, J = 8.0, 1.8 Hz), 7.05 (1H, d, J = 1.8 Hz), 7.21 (2H, d, J = 8.0 Hz), 7.34 (1H, br s, −NH), 7.42 (1H, d, J = 16.0 Hz), 7.44 (2H, d, J = 8.0 Hz), 8.13 (1H, s, −OH), 8.33 (1H, s, −OH). EI-MS m/z (%): 361 (M^+, 8), 207 (15), 178 (29), 163 (100), 89 (24).

KS368D: solid; mp 170−171 °C. IR ν_{max} (cm^{-1}): 3283, 1653, 1613, 1520, 1447, 1374, 1116, 1009, 850. ^1H NMR (CD_3COCD_3, 500 MHz): δ 3.75 (3H, s), 4.44 (2H, d, J = 6.2 Hz), 6.49 (1H, d, J = 15.8 Hz), 6.81−6.94 (4H, m), 7.07 (1H, d, J = 1.6 Hz), 7.25 (2H, d, J = 8.8 Hz), 7.45 (1H, d, J = 15.8 Hz), 7.59 (1H, br s, −NH), 8.17 (1H, s, −OH), 8.38 (1H, s, −OH). EI-MS m/z (%): 299 (M^+, 7), 163 (100).

KS370G: solid; mp 148−149 °C. IR ν_{max} (cm^{-1}): 3288, 1642, 1591, 1523, 1361, 1279, 1036, 975, 849. ^1H NMR (CD_3COCD_3, 500 MHz): δ 2.84 (2H, t, J = 6.8 Hz), 3.53 (2H, q, J = 6.8 Hz), 6.43 (1H, d, J = 15.2 Hz), 6.83 (1H, d, J = 8.1 Hz), 6.92 (1H, dd, J = 8.1, 1.8 Hz), 7.07 (1H, d, J = 1.8 Hz), 7.15−7.30 (5H, m), 7.35 (1H, br s, −NH), 7.43 (1H, d, J = 15.2 Hz), 8.20 (1H, s, −OH), 8.42 (1H, s, −OH). EI-MS m/z (%): 283 (M^+, 17), 178 (22), 163 (100).

Animals. 4-Week-old male ICR mice were acquired from BioLasco Taiwan Co., Ltd. and maintained at National Taiwan University College of Medicine Experimental Animal Center, in a temperature- and humidity-controlled (22 ± 1 °C and 60 ± 5%) environment with a strict 12 h light−dark cycle and given free access to food and water. After the acclimatizing period (3 days), mice with fasting plasma glucose levels higher than 130 mg dL^{-1} or lower than 70 mg dL^{-1} were excluded. Type 1 diabetic mice were induced by modifying the previous method (*16*). In brief, an intraperitoneal injection of STZ (Sigma Chemical Co.; St. Louis, MO) at 150 mg kg^{-1} dissolved in 1% citrate buffer was performed in 4-week-old mice fasted for 48 h. Mice with plasma glucose level of 350 mg dL^{-1} or greater were considered as type 1 diabetic. Type 2 diabetic mice were induced by high-fat and high-fructose diet according to previous method (*17*) and our previous study. After 4 weeks of feeding, mice with fasting plasma glucose levels of 150 mg dL^{-1} or greater were considered as type 2 diabetic. The investigation followed the University guidelines for the use of animals in experimental studies and conformed to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH publication no. 85-23, revised 1996). The animal experiments were approved by the IACUC of National Taiwan University (IACUC No. 20070004).

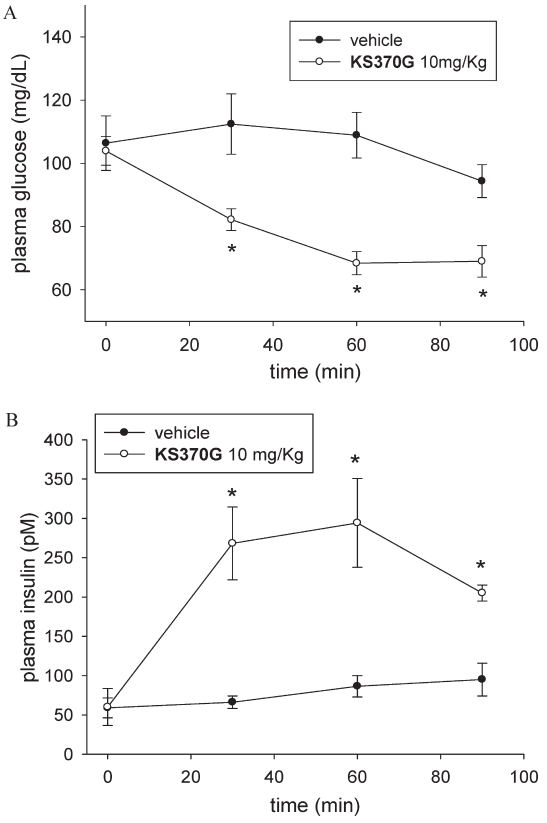

Figure 2. The relationship of the time and (**A**) hypoglycemic and (**B**) insulin secretagogue activity of KS370G (10 mg/kg, po) in normal mice. Data are mean ± SEM (n = 4). Asterisks indicate significant difference compared with the basal level before drug treatment (*P < 0.05 by one-way ANOVA with Dunnett's post-hoc test).

Drug Preparation and Administration. KS368A, KS368B, KS368D and KS370G were added into drinking water to appropriate concentrations. A volume of about 0.05 mL of drug solution was given to mice by oral gavage. 0.05 mL of drinking water was given by oral gavage as vehicle. Glibenclamide and insulin were dissolved in sterile normal saline for intraperitoneal injection as positive controls.

Blood Sampling. Mice were anesthetized with pentobarbital (80 mg/kg, intraperitoneal, Sigma), and blood was withdrawn from the orbital venous plexus using a heparinized capillary tube. Blood samples were centrifuged at 13000 rpm for 5 min, and plasmas were placed on ice or −20 °C until assay (*18*). To evaluate the hypoglycemic activity and stimulation of insulin release in normal and diabetic mice, caffeamide derivatives including KS370G, vehicle and drugs were given to anesthetized mice after an overnight fast. Blood was withdrawn from the orbital venous plexus using a heparinized capillary tube before and at 60 min after drug administration for plasma glucose or insulin assay. In the hypoglycemic activity of caffeamide derivatives assay, the average value of plasma glucose level before drug administration were normalized to be 100, and plasma glucose levels in each group at 60 min after drug administration were calculated.

Determination of Plasma Glucose. An aliquot of plasma was added to Glucose Kit Reagent (Biosystems S.A., Barcelona, Spain) and incubated at 37 °C for 5 min. The concentration of plasma glucose was then estimated via an analyzer (Biosystems 330, Barcelona, Spain) with samples run in duplicate (*19*).

Measurement of Serum Insulin Levels. Determination of serum insulin concentration adopted ELISA (Mammalian Insulin ELISA; Mercodia AB, Uppsala, Sweden) (*20*).

Intraperitoneal Glucose Tolerance Test (IPGTT). An intraperitoneal glucose tolerance test (IPGTT) was performed according to the

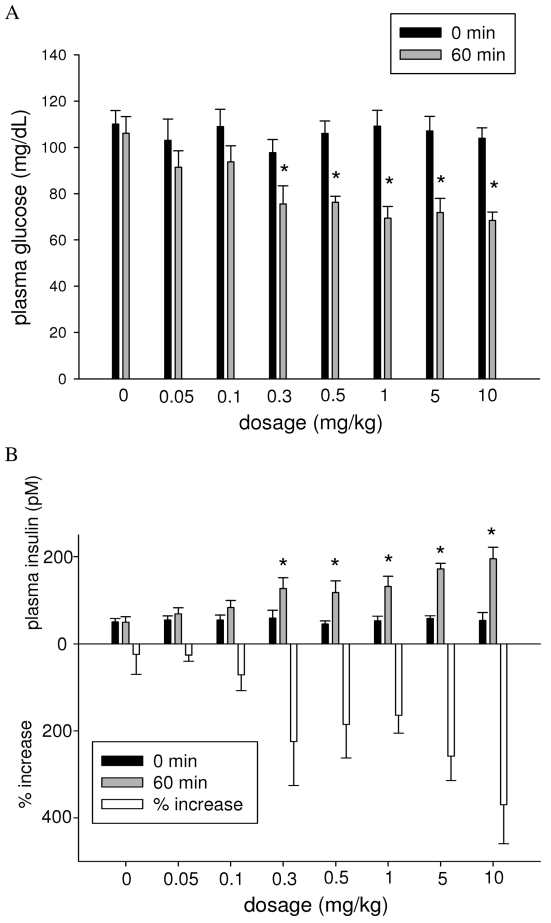

Figure 3. The relationship of the dose and (**A**) hypoglycemic and (**B**) insulin secretagogue activity of KS370G in normal mice. Data are mean ± SEM (n = 8). Asterisks indicate significant difference compared with the basal level before drug treatment (*P < 0.05 by one-way ANOVA with Dunnett's post-hoc test).

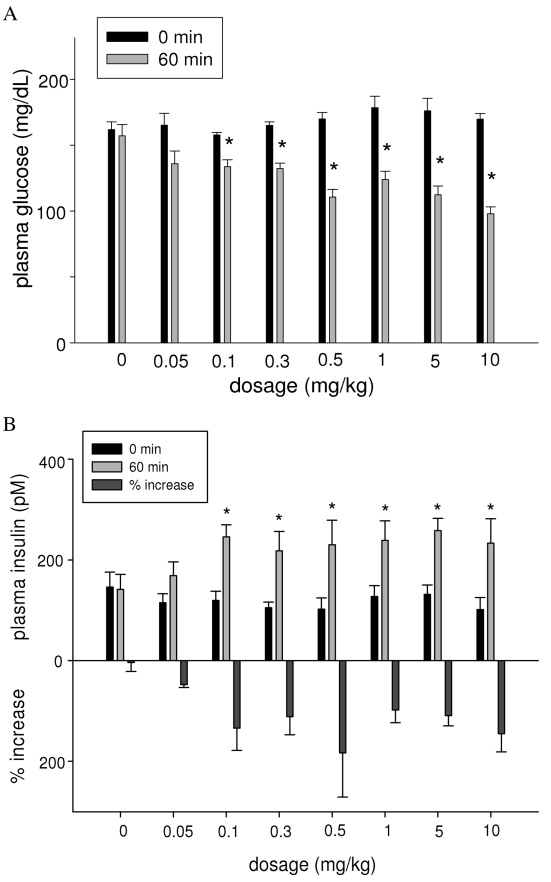

Figure 4. The relationship of the dose and (**A**) hypoglycemic and (**B**) insulin secretagogue activity of KS370G in diet-induced-diabetic mice. Data are mean ± SEM (n = 8). Asterisks indicate significant difference compared with the basal level before drug treatment (*P < 0.05 by one-way ANOVA with Dunnett's post-hoc test).

method described previously (*21*). Briefly, the basal plasma glucose concentration was obtained from samples taken from the orbital venous plexus of ICR mice under anesthesia with sodium pentobarbital (80.0 mg/kg, ip) before the IPGTT. KS370G at indicated dosages or the same volume of vehicle was given to mice orally. After 10 min, blood samples from the orbital venous plexus were drawn and indicated as 0 min. Then, a glucose dose of 2.0 g/kg was injected intraperitoneally. Blood samples from the orbital venous plexus were drawn at 30, 60, 120, and 150 min following the glucose injection for the measurement of the plasma glucose concentrations. Mice were maintained under anesthesia by pentobarbital throughout the procedure.

Glycogen Content Assay. Mice were divided into three groups: vehicle, KS370G and insulin. Vehicle (0.05 mL) and KS370G (10 mg/kg) were given by oral gavage, and insulin (1 IU/kg) was given by intraperitoneal injection. About 1 h after drug administration, mice were sacrificed. The liver and soleus muscle were removed for the glycogen content assay. About 50 mg of tissue sample was dissolved in 1 N KOH at 70 °C for 30 min. Dissolved homogenate was neutralized by glacial acetic acid and incubated overnight in acetate buffer (0.3 M sodium acetate, pH 4.8, Mallinckrodt Baker, Xalostoc, Estado de México, Mexico) containing amyloglucosidase (Sigma, St. Louis, MO). Samples were then analyzed by measuring glucosyl units using the Trinder reaction. The reaction mixture was neutralized with 1 N NaOH (*22*). The averages of glycogen content of vehicle group were normalized to be 100, and glycogen contents in each group were calculated. Glycogen synthesis could be estimated by compared the differences between KS370G/insulin and vehicle groups.

Statistical Analysis. Results of plasma glucose lowering activity were calculated as percentage decrease of the initial value according to formula: $(G_i - G_t)/G_i \times 100\%$; G_i was the initial glucose concentration, and G_t was the plasma glucose concentration after treatment with testing agents. Data were represented as the mean ± SEM for the number (n) of animals in the group as indicated in the figures. Statistical analysis was performed by one-way analysis of variance (ANOVA) with Dunnett's post-hoc test. $P < 0.05$ was regarded as statistically significant.

RESULTS AND DISCUSSION

We found that oral administration of KS370G (**Figure 1**), a caffeamide derivative, could lower plasma glucose concentration in normal and diabetic ICR mice in a dose-dependent manner, and the maximum effect occurred at 60 min after drug administration (**Figure 2**). KS370G significantly decreased the blood glucose at the doses ranging from 0.3 to 1.0 mg/kg in normal ICR mice (**Figure 3**) and diet-induced-diabetic mice (**Figure 4**). However, other caffeamide derivatives had slight (KS368A) or no (KS368B and KS368D) hypoglycemic activities (**Table 1**). In STZ-diabetic mice, the plasma glucose lowering activity of KS370G was found at doses higher than 1.0 mg/kg (**Figure 5**). The plasma glucose lowering activity of KS370G was compared with the effect of insulin (0.5 IU/kg, ip) and glibenclamide (10 mg/kg, ip) in normal, diet-induced-diabetic, and STZ-diabetic mice. Insulin (0.5 IU/kg, ip) decreased the plasma glucose by 50.4 ± 7.8% in normal mice, 33.7 ± 10.8% in diet-induced-diabetic mice, and 28.8 ± 6.8% in STZ-diabetic mice. Glibenclamide (10 mg/kg, ip) decreased the plasma glucose by 27.2 ± 2.9% in normal mice and 23.8 ± 3.0% in diet-induced-diabetic mice, and did not decrease the plasma glucose significantly in STZ-diabetic mice.

Table 1. The Hypoglycemic Activity of Caffeamide Derivatives in Normal Mice[a]

	plasma glucose (expressed as % of that before treatment)	
treatment	before treatment	after treatment
vehicle	100.0 ± 5.3	96.3 ± 6.6
KS368A (10 mg kg^{-1})	100.0 ± 5.1	83.8 ± 5.0[b]
KS368B (10 mg kg^{-1})	100.0 ± 6.7	80.5 ± 10.2
KS368D (10 mg kg^{-1})	100.0 ± 7.1	95.6 ± 8.6
KS370G (10 mg kg^{-1})	100.0 ± 4.4	65.8 ± 3.5[b]

[a] Values expressed as mean ± SEM from six animals in each group. [b] $P < 0.05$ vs before treatment.

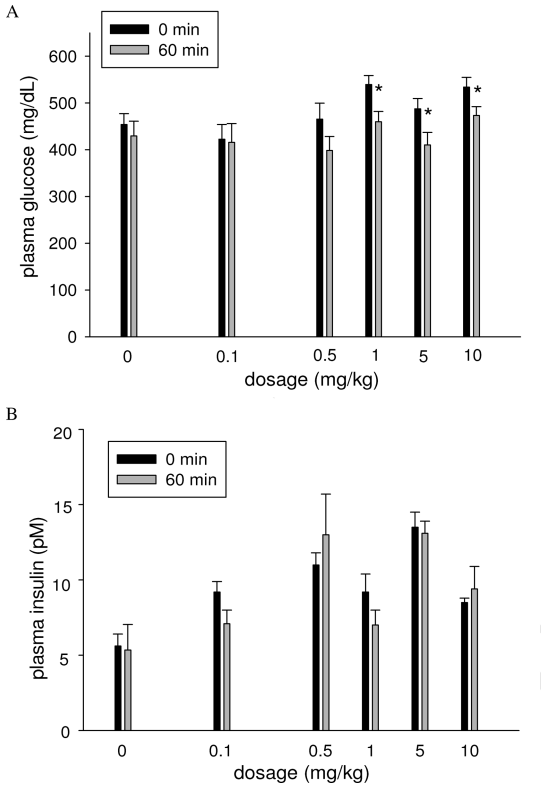

Figure 5. The relationship of the dose and (**A**) hypoglycemic and (**B**) insulin secretagogue activity of KS370G in STZ-diabetic mice. Data are mean ± SEM (n = 8). Asterisks indicate significant difference compared with the basal level before drug treatment (*$P < 0.05$ by one-way ANOVA with Dunnett's post-hoc test).

Table 2. Stimulation of Insulin Release in Normal and Diabetic Mice by KS370G[a]

	plasma insulin (pmol L^{-1})	
treatment	before treatment	after treatment
Normal Mice		
vehicle	50.2 ± 8.1	49.7 ± 12.2
KS370G (10 mg kg^{-1})	53.4 ± 18.2	195.4 ± 26.1[b]
glibenclamide (10 mg kg^{-1})	49.1 ± 19.4	205.8 ± 31.4[b]
Diet-Induced-Diabetic Mice (T2DM)		
vehicle	140.1 ± 29.8	141.7 ± 29.4
KS370G (10 mg kg^{-1})	101.2 ± 24.2	233.4 ± 48.4[b]
glibenclamide (10 mg kg^{-1})	150.0 ± 17.3	258.6 ± 50.7[b]
STZ Mice (T1DM)		
vehicle	9.2 ± 0.7	7.0 ± 0.9
KS370G (10 mg kg^{-1})	8.5 ± 0.3	9.4 ± 1.5
glibenclamide (10 mg kg^{-1})	10.8 ± 1.1	11.2 ± 0.9

[a] Values expressed as mean ± SEM from eight animals in each group. [b] $P < 0.05$ vs before treatment.

Table 3. Effect of KS370G and Insulin on Glycogen Content in Liver and Skeletal Muscle from Normal and Diabetic Mice[a]

	glycogen content (expressed as % of vehicle)	
treatment	liver	skeletal muscle
Normal Mice		
vehicle	100.0 ± 4.4	100.0 ± 15.1
KS370G	310.2 ± 35.9[a]	168.9 ± 15.0[b]
insulin	249.6 ± 19.5[a]	147.6 ± 8.7[b]
Diet-Induced-Diabetic Mice (T2DM)		
vehicle	100.0 ± 4.7	100.0 ± 3.8
KS370G	130.1 ± 10.2[a]	109.4 ± 8.8
insulin	122.6 ± 5.6[a]	108.3 ± 11.4
STZ Mice (T1DM)		
vehicle	100.0 ± 8.1	100.0 ± 4.7
KS370G	145.0 ± 11.3[a]	101.2 ± 4.2
insulin	197.0 ± 11.0[a]	160.0 ± 5.8[b]

[a] Values expressed as mean ± SEM from six animals in each group. The average value of glycogen content of vehicle-treated mice in each group was seen as 100. [b] $P < 0.05$ vs animal treated with vehicle only.

KS370G decreased the plasma glucose by 19.1 ± 5.6% (0.3 mg/kg) to 38.3 ± 2.7% (10 mg/kg) in normal mice, 16.6 ± 3.1% (0.05 mg/kg) to 42.4 ± 2.3% (10 mg/kg) in diet-induced-diabetic mice, and about 15% (1, 5, and 10 mg/kg) in STZ-diabetic mice. Blood insulin levels in normal and NIDDM mice were significantly increased by KS370G from 53.4 ± 18.2 to 195.4 ± 26.1 pmol L^{-1} and 101.2 ± 24.2 to 233.4 ± 48.4 pmol L^{-1}, respectively (n = 8, $P < 0.05$) (**Table 2**). The alteration of plasma insulin levels associated with the plasma glucose lowering effect of KS370G was mainly examined in diet-induced-diabetic mice and compared with the glibenclamide (10 mg/kg, ip), which was known as insulin secretagogue (*23*, *24*). At 10.0 mg/kg, KS370G increased plasma insulin to a level comparable to that induced by glibenclamide (**Table 2**). In STZ-diabetic mice, KS370G did not alter the insulin level (**Table 2**) but significantly decreased plasma glucose level (**Figure 5**). **Table 3** shows that KS370G could increase the glycogen synthesis in either normal or diabetic mice, especially in the liver. The effect of KS370G on the glucose utilization was further verified by IPGTT test, which shows that KS370G markedly accelerated the glucose uptake and utilization into peripheral tissues after ip infusion with glucose (**Figure 6**). The result indicates that KS370G possesses antihyperglycemic action through insulin-dependent and insulin-independent mechanism. Since KS368A, KS368B and KS368D have weak or no antihyperglycemic effect, the electron donating substituted group on the phenyl group of the amide might abate the bioactivities of these synthetic polyphenolic compounds. Many of the naturally occurring compounds, which significantly accelerate AMPK phosphorylation, are structurally similar to polyphenols. In the previous study, CAPE was found to stimulate glucose uptake in differentiated L6 rat myoblast cells and activate AMPK. In addition, the inhibition of AMPK blocked CAPE-induced glucose uptake, and CAPE activated the Akt pathway in a PI3K-dependent manner (*7*). Curcumin was also found to exert an antidifferentiation effect through AMPK-PPAR-γ in 3T3-L1 adipocytes and an antiproliferatory effect through

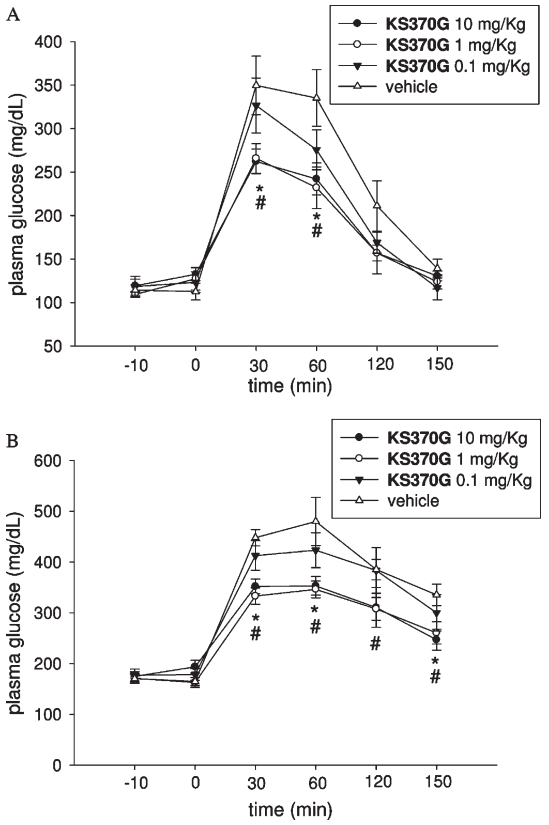

Figure 6. Effect of KS370G on plasma glucose in (**A**) normal ICR mice and (**B**) diet-induced-diabetic mice receiving an intraperitoneal glucose tolerance test (IPGTT). IPGTT was performed by an injection of glucose at 2 g/kg 10 min after oral administration with KS370G at indicated dosages or vehicle. The blood samples were obtained at the indicated points before (at 0 min) and after glucose injection. Asterisk and pound symbol indicate significant difference in plasma glucose between vehicle and KS370G treated groups checked at the same time point (*$P < 0.05$ KS370G 1 mg/kg vs vehicle, #$P < 0.05$ KS370G 10 mg/kg vs vehicle, $n = 8$, by one-way ANOVA with Dunnett's post-hoc test).

AMPK-COX-2 in cancer cells (*25*). Whether these caffeamide derivatives, especially KS370G, have effects similar to CAPE or curcumin and the detailed mechanisms of antihyperglycemic activities remain to be clarified. Further investigations are needed to determine how caffeamides and natural compounds such as CAPE and other polyphenols activate AMPK and whether the AMPK activating activities of these compounds contribute to their biological activities in disease control.

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