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Complete List of Authors:	Huang, Chun-Fa; China Medical University, Graduate Institute of Chinese Medical Science Chen, Ya-Wen; China Medical University Yang, Ching-Yao; National Taiwan University Hospital Lin, Hui-Yi; China Medical University Way, Tzong-Der; china medical university, Department of Biological Science and Technology Chiang, Wenchang; National Taiwan University Liu, Shing; National Taiwan University, Institute of Toxicology, College of Medicine

Extract of Lotus Leaf (*Nelumbo nucifera*) and Its Active Constituent Catechin with Insulin Secretagogue Activity

Chun Fa Huang,[†,⊥] Ya Wen Chen,[‡,⊥] Ching Yao Yang,[§] Hui Yi Lin,[|] Tzong Der Way,[¶] Wenchang Chiang,[+] and Shing Hwa Liu[#,‖,*]

[†]Graduate Institute of Chinese Medical Science, School of Chinese Medicine, College of Chinese Medicine, China Medical University, Taichung, Taiwan

[‡]Department of Physiology and Graduate Institute of Basic Medical Science, College of Medicine, China Medical University, Taichung, Taiwan

[§]Department of Surgery, National Taiwan University Hospital, Taipei, Taiwan

[|]School of Pharmacy, College of Pharmacy, China Medical University, Taichung, Taiwan

[¶]Department of Biological Science and Technology, College of Life Sciences, China Medical University, Taichung, Taiwan

[+]Graduate Institute of Food Science and Technology, National Taiwan University, Taipei, Taiwan

[#]Institute of Toxicology, College of Medicine, National Taiwan University, Taipei, Taiwan

[‖]Department of Urology, National Taiwan University Hospital, Taipei, Taiwan

[⊥]These authors contributed equally to this work.

* Author to whom correspondence: Shing-Hwa Liu, Ph.D., Institute of Toxicology, College of Medicine, National Taiwan University, No. 1, Section 1, Jen-Ai Road, Taipei, 10051, TAIWAN. Fax: 886-2-23410217; E-mail: shinghwaliu@ntu.edu.tw

Abstract

The effect of lotus leaf (*Nelumbo nucifera* Gaertn.) on diabetes is unclear. We hypothesized that lotus leaf can regulate insulin secretion and blood glucose levels. The *in vitro* and *in vivo* effects of lotus leaf methanolic extract (NNE) on insulin secretion and hyperglycemia were investigated. NNE increased insulin secretion from β-cells (HIT-T15) and human islets. NNE enhanced the intracellular calcium levels in β-cells. NNE could also enhance phosphorylation of extracellular signal-regulated protein kinases (ERK)1/2 and protein kinase C (PKC), which could be reversed by a PKC inhibitor. The *in vivo* studies showed that NNE possesses the ability to regulate blood glucose levels in fasted normal mice and high-fat-diet-induced diabetic mice. Furthermore, the *in vitro* and *in vivo* effects of the active constituents of NNE, quercetin and catechin, on glucose-induced insulin secretion and blood glucose regulation were evaluated. Quercetin did not affect insulin secretion, but catechin significantly and dose-dependently enhanced insulin secretion. Orally administered catechin significantly reversed the glucose intolerance in high-fat-diet-induced diabetic mice. These findings suggest that NNE and its active constituent catechin are useful in the control of hyperglycemia in non-insulin-dependent diabetes mellitus through their action as insulin secretagogues.

Keyword: *Nelumbo nucifera* Gaertn. (NNE); Diabetes mellitus; extracellular signal-regulated protein kinases 1/2; protein kinase C; Catechin

Introduction

The prevalence of diabetes mellitus is increasing worldwide and the disease is becoming a serious threat to health in all parts of the world (*1*). It has been estimated that the total number of people with diabetes is projected to rise from 171 million in 2000 to 366 million in 2030 (*1*). The major complication associated with long-term diabetic patients is hyperglycemia, which may secondarily cause deranged insulin secretion from pancreatic β-cells and/or insulin action in peripheral tissues as well as diabetic microvascular complications (nephropathy, retinopathy, and neuropathy) (*2*). Diabetic patients require pharmacological intervention to achieve optimal glycemic control and prevent diabetic complications as well as improve their quality of life (*3*). Hypoglycemic agents can be used either alone or in combination with other hypoglycemic agents or insulin (*4*). Treatment options for patients with type-2 diabetes are quite diverse, including the use of insulin sensitizers, α-glucosidase inhibitors, and β-cell secretagogues (*4*). However, Krentz and Bailey (*5*) have reported that both sulfonylureas and biguanides could not significantly alter the rate of progression of hyperglycemia in patients with type-2 diabetes. Furthermore, conventional secretagogues, which are effective in increasing insulin secretion, might be associated with abnormal side effects, including hypoglycemia and pancreatic β-cell death (*6*). Therefore, there is an urgent need for more effective, advantageous, and safe antihyperglycemic agents.

The lotus plant (*Nelumbo nucifera* Gaertn., Nymphaeaceae) is an aquatic perennial plant that is widely cultivated in eastern Asia and India. Lotus is an agricultural crop that is cultivated for food and drink. *Nelumbo nucifera* is also a folk medicine that is traditionally used for dispersing summer heat. Numerous studies have shown that *Nelumbo nucifera* possesses pharmacologic and physiologic activities,

including hepatoprotective (*7*), antioxidant (*8*), antidiarrheal (9), antiviral (*10*), immunomodulatory (*11*), and antiobesity (*12*) effects. The leaves of *Nelumbo nucifera* contain several flavonoids and alkaloids (*13*). Recently, Lin and colleagues showed that lotus leaves have potential benefits for human health because of their antioxidant activities (*13*). They further indicated that the antioxidant capacity of lotus leaves is related to their flavonoid content (*13*). Moreover, Mukherjee and colleagues (*14*) showed that *Nelumbo nucifera* rhizome extract is capable of reducing hyperglycemia in streptozotocin-treated diabetic rats. However, the effects of *Nelumbo nucifera* leaves on hyperglycemia or type-2 diabetes remain unclear. In a preliminary study, we found that the methanolic extract of *Nelumbo nucifera* leaf (NNE) is capable of enhancing insulin secretion by pancreatic β-cells. Therefore, we hypothesized that NNE possesses the ability to promote insulin secretion and regulate blood glucose levels. In this study, we investigated the *in vitro* effects and possible mechanisms of NNE and some of its active constituents on insulin secretion in pancreatic β-cells and isolated human islets and tested whether NNE regulates blood glucose levels after starch loading in fasted mice and ameliorates the altered blood glucose regulation in high-fat-diet-induced diabetic mice.

Materials and Methods

Plant Materials and Extraction. The plant materials and extracts were obtained as described previously (*13*). In brief, the leaves of *N. nucifera* were purchased from a local farmer in Tainan, Taiwan. After the leaves were harvested, they were dried at ambient temperature, blended into a powder form, and then screened through a 20-mesh sieve. The dried powders were stored at 4°C before use. The dried powders of lotus leaves were extracted 3 times with methanol at 100°C for 1 h. The methanolic extract was collected and concentrated to dryness under conditions of reduced

pressure. In addition, the pure compounds (+)-catechin and quercetin dehydrate were purchased from Sigma (St. Louis, MO, USA).

Cell Culture. Hamster pancreatic β-cell-derived HIT-T15 cells (CRL-1777; ATCC) were used for *in vitro* experiments as described previously (*15*). The cells were cultured in a humidified chamber with a 5% CO_2-95% air mixture at 37°C and maintained in RPMI 1640 medium (Gibco BRL, Life Technologies, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin and containing 11.1 mM glucose.

Human Islet Purification. Human islets of Langerhans were isolated by collagenase digestion from rounding non-tumor pancreatic tissue as described previously (*16*). Pancreatic tissue was obtained from patients with benign pancreatic tumors after their written informed consent was obtained. This experiment was approved by the Research Ethics Committee at the National Taiwan University Hospital. After enzyme digestion at 37°C, the islet cells were obtained at an islet gradient of 1.069–1.096. The islet number was counted by staining samples with dithizone and expressed as the number of islets equivalent to 150 μm in diameter. The islet equivalent of quality (IEQ) was calculated and 75–150 μm (1 IEQ = 150 μm) were used. The islet cells were cultured in CMRL1066 medium containing 5.5 mM glucose supplemented with 10% fetal bovine serum, 1% penicillin/streptomycin/amphotericin B, 2 mM L-glutamate, 25 mM HEPES, and ITS Premix at 37°C in an atmosphere of 95% air/5% CO_2.

Insulin Secretion. Experiments investigating insulin secretion in HIT-T15 cells were performed as described previously (*15*). Cells were cultured in cell culture media and incubated for 4 h under conditions of 5% CO_2-95% air mixture at 37°C, and studies of insulin secretion in islets were performed in Krebs Ringer buffer (KRB). All experimental agents were mixed together in experimental solutions (media or KRB)

and then added to the cells at the start of the experimental incubation. To measure the amount of insulin secreted, aliquots of samples were collected from the plasma or experimental solutions at indicated time points and subjected to insulin antiserum immunoassay according to the manufacturer's instructions (Mercodia AB, Uppsala, Sweden). In some experiments, cells were preincubated in Ca^{2+}- and Mg^{2+}-free KRB containing 2.8 mM D-glucose and 2 mg/ml BSA (experimental solution) for 1 h before the start of experiments. Cells were subsequently washed with experimental solution and supplemented with the agent of interest in the presence of 1.2 mM $CaCl_2$. The aliquots of samples were collected from each well and subjected to insulin antiserum immunoassays.

Intracellular Calcium Measurements with Fluo-3. Intracellular calcium was monitored by the calcium indicator fluo-3 by measuring the fluorescence signal at 530 nm by using a 488 nm excitation wavelength as described previously (*16*). HIT-T15 cells (2×10^5 cells) were loaded with 5 μM fluo-3/acetoxymethyl ester (fluo-3/AM, Sigma) in anhydrous dimethyl sulfoxide and incubated for 30 min at 37°C in the dark. The final dimethyl sulfoxide concentration was less than 0.1% and had no effect on the basal intracellular calcium level. Fluorescence intensities were measured using the FACScan flow cytometry device (Becton Dickinson, Franklin Lakes, NJ, USA). In some experiments, after 30 min of incubation of fluo-3/AM at 37°C in the dark, the islets were washed twice, and images were captured by Leica DMIL inverted microscope equipped with a cooled CCD camera (RT KE™, Diagnostic Instruments, Sterling Heights, MI, USA).

Western Blotting. The experiments were performed as described previously (*15*). Fifty micrograms of protein of each cell lysate was subjected to electrophoresis on 10% SDS-polyacrylamide gels. The samples were then electroblotted on polyvinylidene difluoride membranes. The membrane was blocked for 1 h in PBST

(PBS, 0.01% Tween-20) containing 5% nonfat dry milk and then incubated with anti-anti-extracellular signal-regulated protein kinases (ERK)1/2, anti-phospho ERK1/2, alpha-tubulin (Santa Cruz Biochemicals, Santa Cruz, CA, USA), and anti-phospho protein kinase C antibodies (PKC, New England BioLabs, Ipswich, MA, USA). After the membranes were washed in PBST, the respective secondary antibodies conjugated to horseradish peroxidase were applied for 1 h. The antibody-reactive bands were identified by enhanced chemiluminescence reagents (Amersham Pharmacia Biotech, Piscataway, NJ, USA) and exposed on Kodak radiographic film.

Animals. Male ICR mice (6 weeks old) were purchased from the Animal Center of the College of Medicine, National Taiwan University, Taipei, Taiwan. Protocols were approved by the Institutional Animal Care and Use Committee (IACUC), and the care and use of laboratory animals were conducted in accordance with the guidelines of the Animal Research Committee of College of Medicine, National Taiwan University. The mice were housed (7 per cage) under standard laboratory conditions at a constant temperature (23°C ± 2°C), 50% ± 20% relative humidity with 12-h light and dark cycles. After 3 days of acclimatization, the mice had free access to either standard rodent chow (fat content 12% kcal) or a high-fat diet (TestDiet, Richmond, IN, USA; fat content 60% kcal) based on lard for a period of 12 weeks.

***In Vivo* Experiments in Fasted Normal Mice.** The experiments were performed as described previously (*16*). Male ICR mice (6 weeks old) were fasted overnight before the experiments. The mice were orally administered 100 mg/kg NNE for 2 h. Thereafter, starch was orally given to fasted mice at a dose of 1 g/kg. Blood samples obtained from the orbital sinus were drawn before and 15, 45, 75, and 105 min after starch challenge. Blood glucose levels were determined using the SURESTEP blood glucose meter (Lifescan, Milpitas, CA, USA).

Oral Glucose Tolerance and Insulin Tolerance Tests. The oral glucose tolerance test was performed as described previously (*16*). The mice were administered NNE for 2 h. The control mice or high-fat-diet-induced diabetic mice with or without drug treatment received an oral glucose challenge (1 g/kg). Blood samples were collected before and 15, 45, 75, and 105 min after delivery of the glucose load. Blood glucose levels were determined using the SURESTEP blood glucose meter. Moreover, the insulin tolerance test was performed in mice after an 8-h fast, and insulin (1.0 U/kg) was administered by intraperitoneal injection. The mice were orally administered NNE for 2 weeks. Blood samples were collected from the orbital sinus of each mouse at 0, 30, and 60 min after the insulin injection. Blood glucose levels were determined using the SURESTEP blood glucose meter.

Statistical Analysis. The values are presented as mean ± SEM. Statistical significance was evaluated using paired Student's t-test. When more than 1 group was compared with 1 control, significance was evaluated according to one-way analysis of variance (ANOVA); Duncan's post-hoc test was applied to identify group differences. Probability values < 0.05 were considered significant.

Results

Effect of NNE on insulin secretion in HIT-T15 cells and isolated human islets

To understand whether NNE could affect insulin secretion in pancreatic β-cells, we investigated the *in vitro* effect of NNE (25–150 μg/ml) on insulin secretion. Exposure of β-cell-derived HIT-T15 cells to NNE for 24 h did not affect cell viability (data not shown). The measurement of insulin secretion from HIT-T15 cells after exposure to NNE for 4 h showed that NNE significantly enhanced insulin secretion in a dose-dependent manner (Figure 1A). In addition, we examined whether insulin secretion by isolated human islets could also be regulated by NNE. As shown in

Figure 1B, NNE (25–150 μg/ml) significantly enhanced glucose (20 mM)-stimulated insulin secretion by isolated human islets in a dose-dependent manner.

The role played by calcium in NNE-enhanced insulin secretion

Calcium influx was associated with insulin secretion in islet β-cells (*16*). We next investigated whether NNE could affect intracellular calcium levels in HIT-T15 cells and isolated islets. HIT-T15 cells treated with NNE (50–150 μg/ml) for 30 min had significantly increased intracellular calcium levels as determined by fluo-3 fluorescence (Fig. 2A). Similarly, the result of fluo-3 fluorescence staining showed that NNE (150 μg/ml) could also increase intracellular calcium levels in isolated human islets (Fig. 2B).

Furthermore, exposure to NNE or vehicle in the absence of extracellular Ca^{2+} caused a small increase in insulin secretion by HIT-T15 cells in the presence of a substimulatory concentration of glucose (2.8 mM). However, the addition of extracellular Ca^{2+} (1.2 mM) to the medium evoked a much higher degree of glucose (2.8 mM)-stimulated insulin secretion by HIT-T15 cells treated with NNE (50 μg/ml) for 30 min than that evoked by the vehicle control (Fig. 3).

Involvement of PKC and ERK1/2 in NNE-triggered responses in HIT-T15 cells

It has been shown that Ca^{2+} can regulate PKC and ERK1/2 signals in β-cells (*17, 18*). Therefore, to further evaluate the cellular responses triggered by NNE, the phosphorylation of PKC and ERK1/2 in HIT-T15 cells was determined. As shown in Figure 4, phosphorylation of PKC (pan) and ERK1/2 was enhanced in HIT-T15 cells treated with NNE (50 μg/ml) for 30 min to 2 h. However, Akt phosphorylation in HIT-T15 cells was not affected by NNE (50 μg/ml) (Fig. 4A). RO320432 (a potent PKC inhibitor, 5 μM) effectively inhibited NNE-enhanced PKC and ERK1/2 phosphorylation in HIT-T15 cells (Fig. 4B). Moreover, PD98059 (a specific ERK/ mitogen-activated protein kinase (MAPK) inhibitor, 10 μM) effectively inhibited the

phosphorylation of ERK1/2 triggered by NNE; however, NNE-enhanced PKC phosphorylation was not inhibited by PD98059 (Fig. 4C). In addition, RO320432 and PD98059 could also inhibit Ca^{2+}-stimulated insulin secretion in β-cells (Fig. 3). These results imply that NNE enhances insulin secretion through a Ca^{2+}-activated PKC-regulated ERK1/2 signaling pathway.

In vivo effect of NNE on blood glucose regulation

Next, the *in vivo* effect of NNE on blood glucose regulation was investigated in fasted normal mice and high-fat-diet-induced diabetic mice. As shown in Figure 5A, NNE (100 mg/kg) was orally administered for 2 h before starch loading in fasted normal mice. NNE had a hypoglycemic effect in fasted mice after starch loading. A significant increase in plasma insulin levels was also shown in mice treated with NNE for 2 h (Fig. 5B). Moreover, in the oral glucose tolerance test, marked glucose intolerance was exhibited by high-fat-diet-induced diabetic mice as compared with age-matched controls, and this condition could be significantly reversed by oral administration of NNE (100 mg/kg) for 2 h (Fig. 6A). On the other hand, after the administration of 100 mg/kg NNE for 2 weeks, insulin sensitivity was markedly improved in high-fat-diet-induced diabetic mice as determined by the insulin tolerance test (Fig. 6B).

Effects of some active constituents of NNE on insulin secretion in HIT-T15 cells and blood glucose regulation in animal models

Recently, Lin and colleagues (*13*) isolated several flavonoids from NNE. Among these compounds, quercetin showed the highest antioxidant activity, and catechin possessed moderately potent antioxidant activity against LDL ox idation. Therefore, we next evaluated the effects of quercetin and catechin on glucose-induced insulin secretion in HIT-T15 cells. As shown in Figure 7, the effects of quercetin and catechin on glucose-induced insulin secretion in HIT-T15 cells were

evaluated. Unexpectedly, quercetin (0.1–10 μM) did not affect insulin secretion, but catechin (0.1–10 μM) significantly and dose-dependently enhanced insulin secretion.

The *in vivo* analysis showed that catechin has a hypoglycemic effect in fasted mice after starch loading when catechin (100 mg/kg) was orally administered for 2 h before starch loading in fasted normal mice (Fig. 8A). Moreover, oral administration of catechin (100 mg/kg) could also significantly reverse the glucose intolerance in high-fat-diet-induced diabetic mice (Fig. 8B).

Discussion

In this study, in keeping with the hypothesis, we showed for the first time that lotus leaf extract enhances insulin secretion and regulates blood glucose level *in vitro* and *in vivo*. The findings also indicate that lotus leaf extract significantly enhances insulin secretion in β-cells via a Ca^{2+}-activated PKC-regulated ERK1/2 signaling pathway.

Type-2 diabetes is one of the fastest growing problems of public health worldwide. Postprandial glycemia is now well known as the major determinant of glycemic control in type-2 diabetes (*4*). It has been shown that therapy targeted at postprandial glucose improves glucose control and reduces the progression of atherosclerosis and cardiovascular events in patients with type-2 diabetes. Some chemically synthesized short-acting insulin secretagogues have been shown to improve postprandial hyperglycemia after major meals (*19*). In the present study, we found that NNE is capable of enhancing glucose-induced insulin secretion from cultured β-cells and isolated human islets. In the *in vivo* experiments, postprandial hyperglycemia was observed in fasted normal mice, and the glucose intolerance observed in high-fat-diet-induced diabetic mice could be significantly reversed by oral administration of NNE for 2 h. This *in vivo* study indicates that NNE exerts rapid or

short-term effects on blood glucose regulation. In addition, insulin resistance was observed in high-fat-diet-induced diabetic mice. After treatment with NNE for 2 weeks, insulin sensitivity was markedly improved in high-fat-diet-induced diabetic mice as determined by insulin tolerance tests, indicating that NNE exerts a long-term effect on blood glucose regulation via improvement of insulin resistance.

Previous research showed that the activation of voltage-dependent calcium channels and the increased intracellular Ca^{2+} levels play important roles in glucose-stimulated insulin secretion (*20*). Another study also showed that enhanced ATP/ADP ratios cause closure of K_{ATP} channels and cell depolarization and subsequently activate L-type-Ca^{2+} channels and increase cytosolic free Ca^{2+} concentrations. The increased intracellular Ca^{2+} levels triggered the fusion of insulin-containing vesicles with the cell membrane and induced exocytosis of insulin (*20*). The increased intracellular calcium levels could also be related to membrane-associated PKC activation in rat islet cells (*17*). PKCs, especially conventional PKCs (α, β, and γ isoforms), were activated in a Ca^{2+}-dependent manner. It has also been shown that PKC plays an important role in insulin secretion (*17*). Moreover, ERK1/2, a serine/threonine kinase of the MAPK family, could be activated by glucose, insulin, glucagon-like peptide 1 (GLP-1), KCl, and phorbol esters (*21*). ERK1/2 has also been shown to be regulated by Ca^{2+} entry through L-type voltage-gated Ca^{2+} channels in MIN6 cells (*18*), a β-cell type. It has further been shown that PKC is involved in the regulation of the response of ERK/MAPA to glucose in MIN6 cells (*22*). A PI3K/Akt-dependent signaling pathway has also been shown to exist in β-cells, and it might function to restrain glucose-induced insulin secretion from β-cells (*23*). In contrast, the JNK pathway plays a crucial role in the progression of pancreatic β-cell dysfunction and insulin resistance (*24*). However, Burns and colleagues have shown that the p38/MAPK cascade is not required for the

stimulation of insulin secretion from rat islets (*25*). In the present work, we found that lotus leaf extract enhances Ca^{2+} entry and insulin secretion in HIT-T15 cells and primary human islets. Increasing concentrations of extracellular Ca^{2+} evoked a much higher stimulation of insulin secretion in NNE-treated β-cells. Furthermore, NNE activated the phosphorylation of PKC and ERK1/2 in cultured β-cells, and this could be reversed by a PKC inhibitor (RO320432), but an ERK inhibitor (PD98059) did not affect the phosphorylation of PKC. In addition, Akt phosphorylation was not affected by NNE treatment in β-cells. These results indicate that a Ca^{2+}-activated PKC-regulated ERK1/2 signaling pathway is involved in the NNE-enhanced insulin secretion in β-cells.

Flavonoids are a group of phenolic compounds that are extensively distributed in fruits, vegetables, flowers, and leaves. The beneficial effects of dietary flavonoids are possibly attributable to their antioxidant, anti-inflammatory, and antimicrobial activities (*26*). Recently, 7 flavonoids were isolated from NNE by Lin et al. (*13*). The contents of these 7 compounds in NNE are as follows: catechin, 14.5 ± 2 mg/g; quercetin, 4.6 ± 0.4 mg/g; quercetin-3-*O*-glucopyranoside, 42.1 ± 1.8 mg/g; quercetin-3-*O*-glucuronide, 70.3 ± 2.7 mg/g; quercetin-3-*O*-galactopyranoside, 4.2 ± 0.7 mg/g; kaempferol-3-*O*-glucopyranoside, 8.5 ± 1.4 mg/g; and myricetin-3-*O*-glucopyranoside, 5.0 ± 1.2 mg/g. Among these compounds, quercetin is present at the lowest level in NNE but exerts the most potent antioxidant effects against LDL oxidation, and catechin is present at the third highest level among these 7 compounds and shows moderately potent antioxidant activity against LDL oxidation (*13*). Dietary catechins have been suggested to be effective in delaying the progression of diabetes and the associated oxidative stress (*27*). Crespy and Williamson have shown that long-term feeding of tea catechins could be beneficial for

the suppression of high-fat-diet-induced obesity through modulation of lipid metabolism and could have a beneficial effect against lipid and glucose metabolism disorders implicated in type-2 diabetes (*28*). In the present study, we further evaluated the *in vitro* and *in vivo* effects of quercetin and catechin on glucose-induced insulin secretion and blood glucose regulation. Unexpectedly, quercetin did not affect insulin secretion in cultured β-cells, but catechin significantly and dose-dependently enhanced insulin secretion. The animal studies showed that catechin has a hypoglycemic effect in fasted mice after starch loading and could significantly reverse glucose intolerance in high-fat-diet-induced diabetic mice. These findings supported that NNE and its active constituent catechin are useful in the control of hyperglycemia in non-insulin-dependent diabetes mellitus because of their action as insulin secretagogues. However, in addition to the effects of catechin and quercetin, the effects of other constituents in NNE on insulin secretion and blood glucose regulation also need to be clarified in the future.

One known class of insulin secretagogues is sulfonylurea agents (ex. glimepiride). When a sulfonylurea agent added, ATP-dependent potassium channels close; therefore, the efflux of potassium is inhibited, causing membrane depolarization. In addition, voltage-gated calcium channels are opened, and calcium influx leads to insulin release (*29*). Moreover, new insulin secretagogues that target the incretin gut hormone GLP-1 are now available. Oral incretin enhancers that act as antagonists of the enzyme DPP-4 (dipeptidylpeptidase-4), which inactivates natural GLP-1, could stimulate insulin secretion (*30*). In the present study, NNE acted as an insulin secretagogue that is capable of enhancing insulin secretion in β-cells through a Ca^{2+}-activated PKC-regulated ERK1/2 signaling pathway and may be effective in controlling diabetes.

In conclusion, in this study, we found that lotus leaf extract enhances insulin

secretion and regulates blood glucose level *in vitro* and *in vivo*. NNE was capable of enhancing insulin secretion in β-cells, which may occur through a Ca^{2+}-activated PKC-regulated ERK1/2 signaling pathway. Moreover, catechin, the active constituent of NNE, significantly and dose-dependently enhanced insulin secretion. The results of *in vivo* studies in fasted normal mice and high-fat-diet-induced diabetic mice indicate that lotus leaf extract and catechin possess the ability to regulate blood glucose level and improve postprandial hyperglycemia under diabetic conditions.

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References

1. Wild, S.; Roglic, G.; Green, A.; Sicree, R.; King, H. Global prevalence of diabetes: estimates for the year 2000 and projections for 2030. *Diabetes Care.* **2004**, *27*, 1047-1053.
2. Nair, M. Diabetes mellitus, part 1: physiology and complications. *Br. J. Nurs.* **2007**, *16*, 184-188.
3. Lasker, R. D. The diabetes control and complications trial. Implications for policy and practice. *N. Engl. J. Med.* **1993**, *329*, 1035-1036.
4. Cheng, A. Y.; Fantus, I. G. Oral antihyperglycemic therapy for type 2 diabetes mellitus. *Cmaj.* **2005**, *172*, 213-226.
5. Krentz, A. J.; Bailey, C. J. Oral antidiabetic agents: current role in type 2

diabetes mellitus. *Drugs* **2005**, *65*, 385-411.

6. Giorgino, F.; Laviola, L.; Leonardini, A.; Natalicchio, A. GLP-1: a new approach for type 2 diabetes therapy. *Diabetes. Res. Clin. Pract.* **2006**, *74*, S152-S155.
7. Sohn, D. H.; Kim, Y. C.; Oh, S. H.; Park, E. J.; Li, X.; Lee, B. H. Hepatoprotective and free radical scavenging effects of Nelumbo nucifera. *Phytomedicine* **2003**, *10*, 165-169.
8. Ling, Z. Q.; Xie, B. J.; Yang, E. L. Isolation, characterization, and determination of antioxidative activity of oligomeric procyanidins from the seedpod of Nelumbo nucifera Gaertn. *J. Agric. Food. Chem.* **2005**, *53*, 2441-2445.
9. Talukder, M. J.; Nessa, J. Effect of Nelumbo nucifera rhizome extract on the gastrointestinal tract of rat. *Bangladesh. Med. Res. Counc. Bull.* **1998**, *24*, 6-9.
10. Kuo, Y. C.; Lin, Y. L.; Liu, C. P.; Tsai, W. J. Herpes simplex virus type 1 propagation in HeLa cells interrupted by Nelumbo nucifera. *J. Biomed. Sci.* **2005**, *12*, 1021-1034.
11. Liu, C. P.; Tsai, W. J.; Lin, Y. L.; Liao, J. F.; Chen, C. F.; Kuo, Y. C. The extracts from Nelumbo Nucifera suppress cell cycle progression, cytokine genes expression, and cell proliferation in human peripheral blood mononuclear cells. *Life. Sci.* **2004**, *75*, 699-716.
12. Ono, Y.; Hattori, E.; Fukaya, Y.; Imai, S.; Ohizumi, Y. Anti-obesity effect of Nelumbo nucifera leaves extract in mice and rats. *J. Ethnopharmacol.* **2006**, *106*, 238-244.
13. Lin, H. Y.; Kuo, Y. H.; Lin, Y. L.; Chiang, W. Antioxidative effect and active components from leaves of Lotus (Nelumbo nucifera). *J. Agric. Food. Chem.* **2009**, *57*, 6623-6629.
14. Mukherjee, P. K.; Saha, K.; Das, J.; Pal, M.; Saha, B. P. Studies on the anti-inflammatory activity of rhizomes of Nelumbo nucifera. *Planta. Med.* **1997**,

63, 367-369.

15. Chen, Y. W.; Huang, C. F.; Tsai, K. S.; Yang, R. S.; Yen, C. C.; Yang, C. Y.; Lin-Shiau, S. Y.; Liu, S. H. The role of phosphoinositide 3-kinase/Akt signaling in low-dose mercury-induced mouse pancreatic beta-cell dysfunction in vitro and in vivo. *Diabetes* **2006**, *55*, 1614-1624.
16. Leu, Y.L.; Chen, Y.W.; Yang, C.Y.; Huang, C.F.; Lin, G.H.; Tsai, K.S.; Yang, R.S.; Liu, S.H. Extract isolated from Angelica hirsutiflora with insulin secretagogue activity. *J. Ethnopharmacol.* **2009**, *123*, 208-212.
17. Wang, J.; Chakravarthy, B. R.; Morley, P.; Whitfield, J. F.; Durkin, J. P.; Begin-Heick, N. Glucose, potassium, and CCK-8 induce increases in membrane-associated PKC activity that correspond to increases in $[Ca^{2+}]i$ in islet cells from neonatal rats. *Cell. Signal.* **1996**, *8*, 305-311.
18. Benes, C.; Roisin, M. P.; Van Tan, H.; Creuzet, C.; Miyazaki, J.; Fagard, R. Rapid activation and nuclear translocation of mitogen-activated protein kinases in response to physiological concentration of glucose in the MIN6 pancreatic beta cell line. *J. Biol. Chem.* **1998**, *273*, 15507-15513.
19. Schmitz, O.; Lund, S.; Andersen, P. H.; Jonler, M.; Porksen, N. Optimizing insulin secretagogue therapy in patients with type 2 diabetes: a randomized double-blind study with repaglinide. *Diabetes Care.* **2002**, *25*, 342-346.
20. Rorsman, P. The pancreatic beta-cell as a fuel sensor: an electrophysiologist's viewpoint. *Diabetologia* **1997**, *40*, 487-495.
21. Cheng, H.; Straub, S. G.; Sharp, G. W. Inhibitory role of Src family tyrosine kinases on Ca^{2+}-dependent insulin release. *Am. J. Physiol. Endocrinol. Metab.* **2007**, *292*, E845-E852.
22. Benes, C.; Poitout, V.; Marie, J. C.; Martin-Perez, J.; Roisin, M. P.; Fagard, R. Mode of regulation of the extracellular signal-regulated kinases in the pancreatic

beta-cell line MIN6 and their implication in the regulation of insulin gene transcription. *Biochem. J.* **1999**, *340*, 219-225.

23. Zawalich, W.S.; Zawalich, K.C. A link between insulin resistance and hyperinsulinemia: inhibitors of phosphatidylinositol 3-kinase augment glucoseinduced insulin secretion from islets of lean, but not obese, rats. *Endocrinology* **2000**, *141*, 3287-3295.
24. Kaneto, H.; Nakatani, Y.; Kawamori, D.; Miyatsuka, T.; Matsuoka, T. Involvement of Oxidative Stress and the JNK Pathway in Glucose Toxicity. *Rev. Diabet. Stud.* **2004**, *1*, 165-174.
25. Burns, C.J.; Howell, S.L.; Jones, P.M.; Persaud, S.J. The p38 mitogen-activated protein kinase cascade is not required for the stimulation of insulin secretion from rat islets of Langerhans. Mol Cell Endocrinol. **1999**, *148*, 29-35.
26. Tripoli, E.; Guardia, M. L.; Giammanco, S.; Majo, D. D.; Giammanco, M. Citrus flavonoids: molecular structure, biological activity and nutritional properties: a review. *Food. Chem*. **2007**, *104*, 466–479.
27. Igarashi, K.; Honma, K.; Yoshinari, O.; Nanjo, F.; Hara, Y. Effects of dietary catechins on glucose tolerance, blood pressure and oxidative status in Goto-Kakizaki rats. *J. Nutr. Sci. Vitaminol.* (Tokyo) **2007**, *53*, 496-500.
28. Crespy, V.; Williamson, G. A review of the health effects of green tea catechins in in vivo animal models. *J. Nutr.* **2004**, *134*, 3431S-3440S.
29. Briscoe, V.J.; Griffith, M.L.; Davis, S.N. The role of glimepiride in the treatment of type 2 diabetes mellitus. *Expert. Opin. Drug Metab. Toxicol.* **2010**, *6*, 225-235.
30. Scheen, A.J. New therapeutic approaches in type 2 diabetes. Acta Clin. Belg. **2008** , *63*, 402-407.

Figure Legends

Figure 1. Effects of lotus leaf extract on the glucose-induced insulin secretion in pancreatic β-cell derived HIT-T15 cells and human islets. Cells (A) or islets (B) were treated with lotus leaf extract (NNE, 25-150 μg/ml) for 4 h under 20 mM glucose condition. All data are presented as means±SEM for four independent experiments with triplicate determinations. *P<0.05 as compared with control.

Figure 2. Elevation of intracellular Ca^{2+} levels by lotus leaf extract in HIT-T15 cells and human islets. A, HIT-T15 cells were treated with lotus leaf extract (NNE, 50-150 μg/ml) for 30 min. Intracellular Ca^{2+} levels in HIT-T15 cells were determined by flow cytometry using fluo-3/AM as described in "Materials and Methods". Data are presented as means±SEM for four independent experiments with triplicate determinations. *P<0.05 as compared with control. B, Fluorescence of fluo-3/AM in human islets at 30 min after lotus leaf extract (NNE, 150 μg/ml) treatment (a: control, b: NNE treatment; left panel: transmitted light images, right panel: fluorescence images). Results shown are representative of three independent experiments.

Figure 3. Elevation of extracellular Ca^{2+} concentration stimulates insulin secretion in HIT-T15 cells treated with lotus leaf extract. Cells were incubated with a buffered salt solution in the absence of extracellular Ca^{2+} with or without lotus leaf extract (NNE, 50 μg/ml) in the presence or absence of RO320432 (5 μM) and PD98059 (10 μM) for 10 min to establish a basal insulin secretion after which the cells were incubated with a buffered salt solution in the presence of extracellular Ca^{2+} 1.2 mM with or without lotus leaf extract. The aliquots of samples were collected at indicated time-point from experimental solution and subjected to insulin antiserum immunoassay. Data are presented as means±SEM for three independent experiments with triplicate

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Figure 4. Effects of lotus leaf extract on phosphorylations of PKC, ERK1/2, and Akt in HIT-T15 cells. Cells were treated with lotus leaf extract (NNE, 50 μg/ml) for 0.5-2 h (A). In some experiments, cells were treated with lotus leaf extract (NNE, 50 μg/ml) in the presence or absence of 5 μM RO320432 (B) or 10 μM PD98059 (C) for 1 h. The phosphorylations of PKC (pan), ERK1/2, and Akt were detected by western blotting. Results shown are representative of three independent experiments.

Figure 5. Effects of lotus leaf extract on the regulations of blood glucose and insulin in fasted normal mice. Lotus leaf extract (NNE, 100 mg/kg) was orally administered 2 h before starch (1 g/kg) loading in fasted normal mice. Blood glucose levels were detected in mice before and 15-105 min after starch loading (A). In some experiments, fasted normal mice were treated with NNE (100 mg/kg) for 1 and 2 h, and then plasma insulin levels were determined in NNE-treated fasting mice 15 min after starch loading (B). All data are presented as means±SEM (n=8 in each group). *P<0.05 as compared with control.

Figure 6. Effects of lotus leaf extract on blood glucose regulation in high fat diet-induced diabetic mice. Lotus leaf extract (NNE, 100 mg/kg) was orally administered 2 h before glucose (1 g/kg) loading (A, glucose tolerance test) or insulin (1.0 U/kg) injection (B, insulin tolerance test) in high-fat diet (HFD)-induced diabetic mice. Blood glucose levels were detected in mice before and 15-105 min after glucose loading or 15-60 min after insulin injection. All data are presented as means±SEM (n=8 in each group). *P<0.05 as compared with control. # P<0.05 as compared with HFD group.

Figure 7. Effects of catechin and quercetin on glucose-induced insulin secretion in HIT-T15 cells. Cells were treated with quercetin and catechin (0.1-10 μM) for 4 h under 20 mM glucose condition. All data are presented as means±SEM for four

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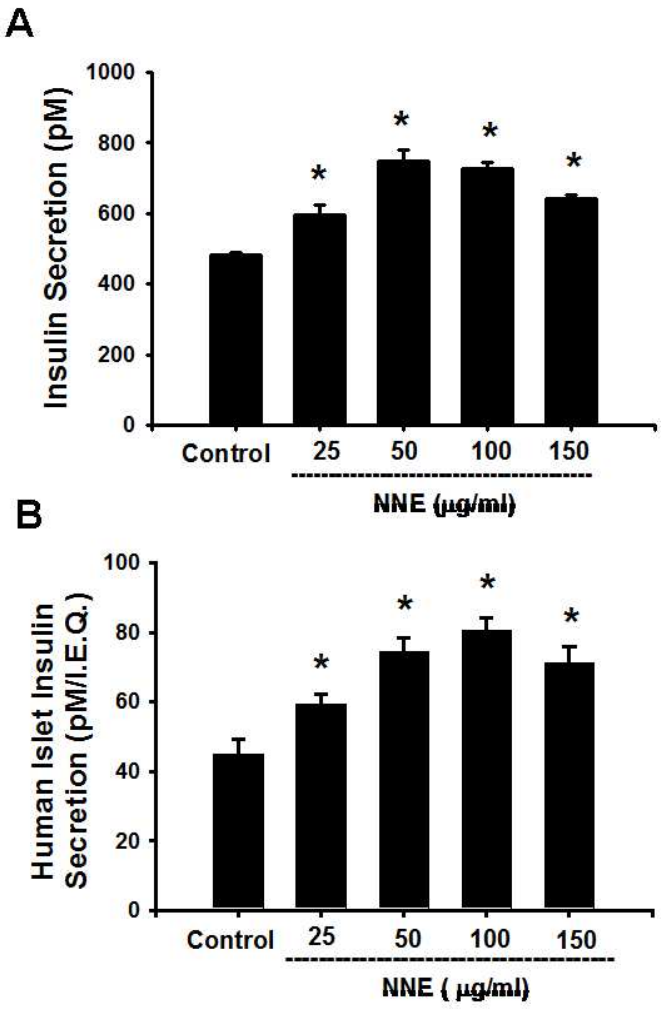
Figure 8. Effects of catechin on blood glucose regulation in fasted normal mice and high fat diet-induced diabetic mice. Catechin (100 mg/kg) was orally administered 2 h before starch (1 g/kg) loading in fasted normal mice (A) or glucose (1 g/kg) loading in high-fat diet (HFD)-induced diabetic mice (B). Blood glucose levels were detected in mice before and 15-105 min after starch or glucose loading. All data are presented as means±SEM (n=8 in each group). *P<0.05 as compared with control.



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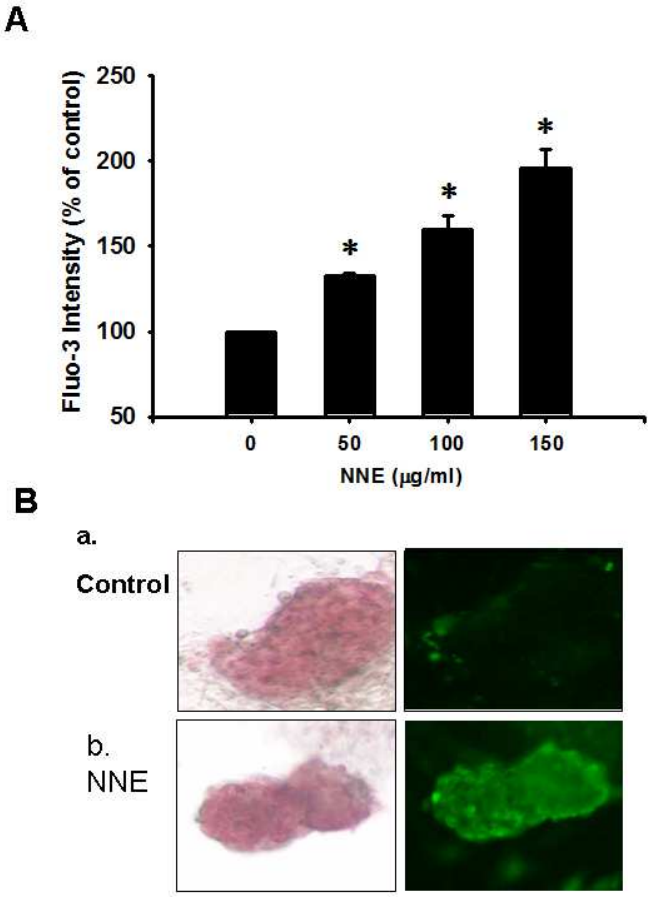

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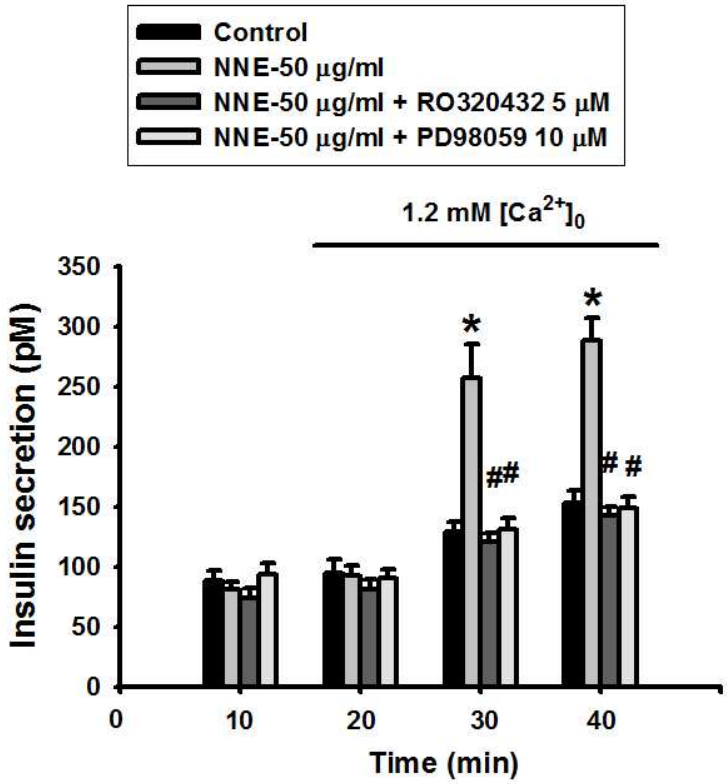

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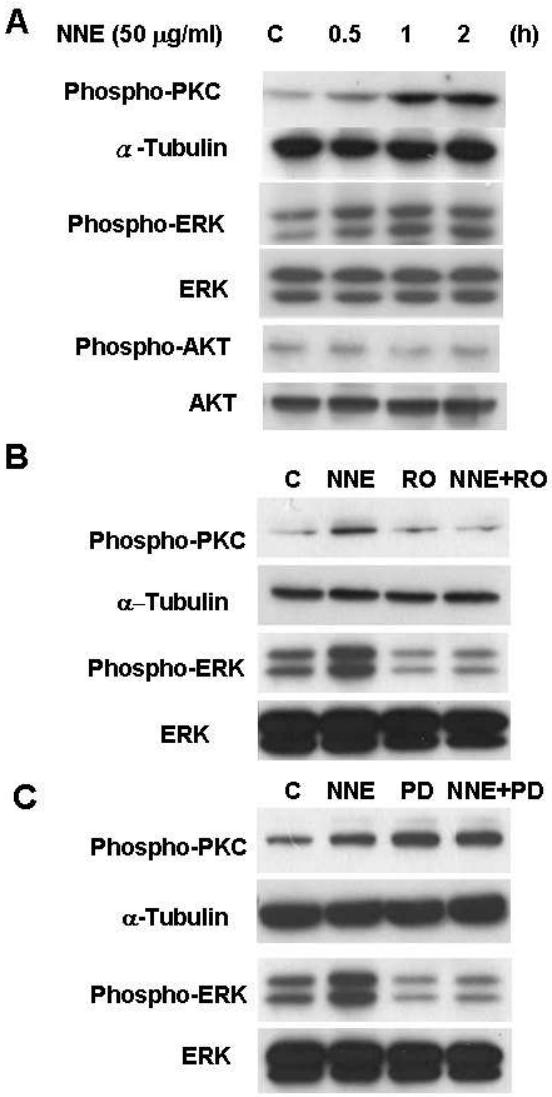

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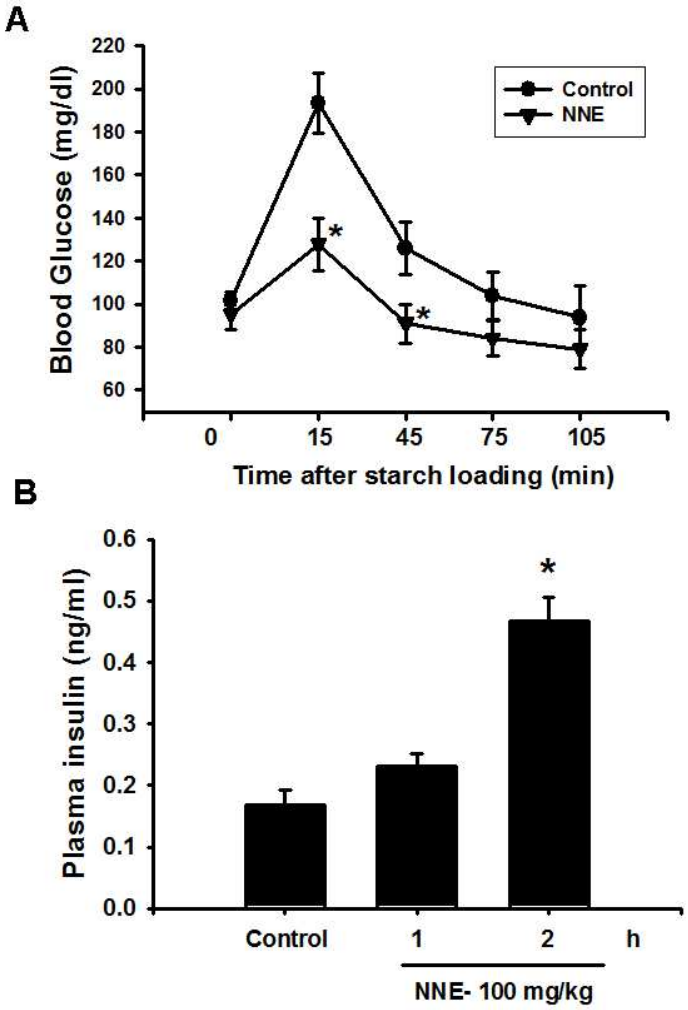

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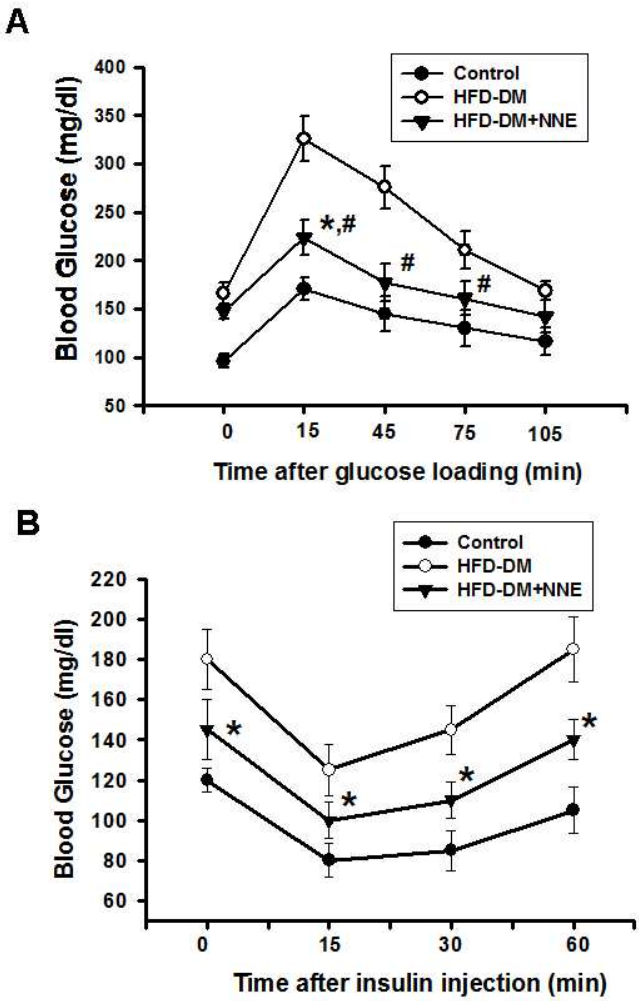

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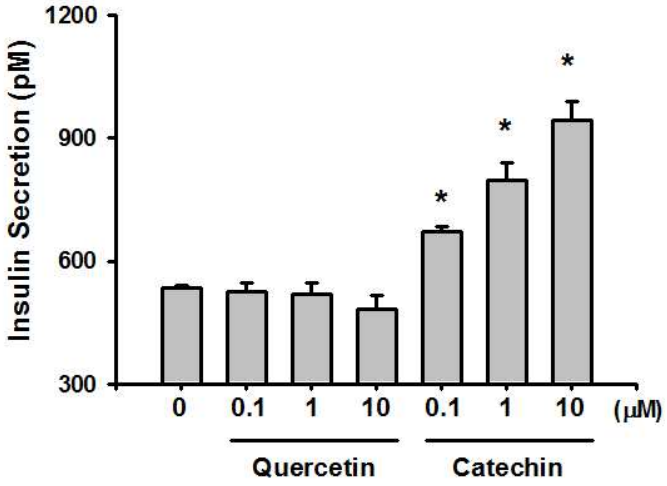

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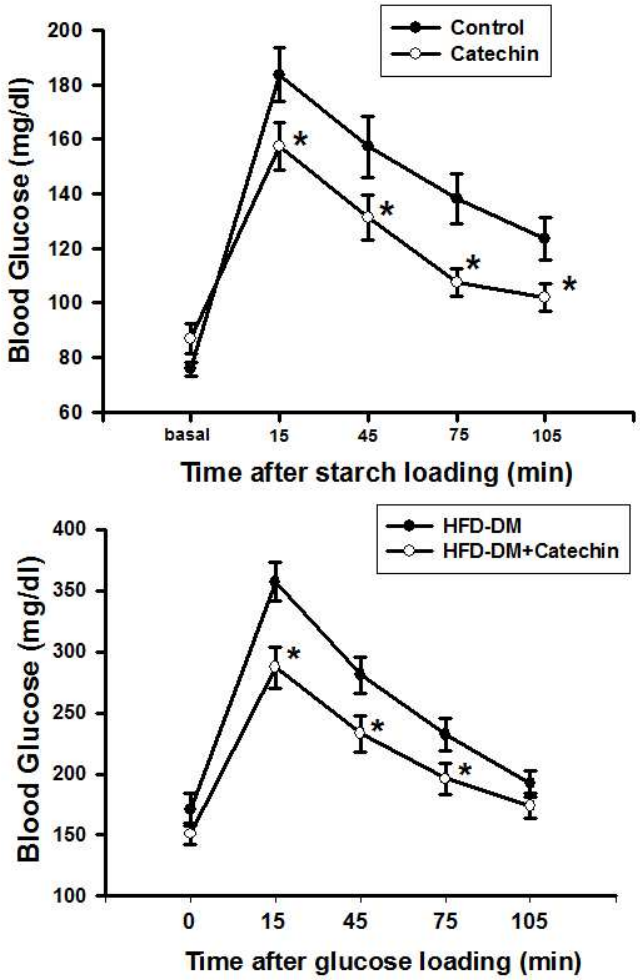

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