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Original Article

Metabolic fate of excessive glucose in fibroblast cells in a diabetic setting

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ABSTRACT — Glucose is important for energy; however, excessive daily intake of sugar may act as a toxin inducing the body to become overweight or obese. High blood glucose level reduces secretion of insulin, and glucose toxicity worsens insulin resistance. We investigated the metabolic fate of excess glucose by changing glucose levels in MRC-5 fibroblasts. Uptake of glucose into fibroblasts, the first stage of glucose metabolism, was measured. Treatment of fibroblasts under diabetic conditions led to rapid glucose incorporation. Glucose was absorbed into the cell almost constantly and reached excessive levels, and its metabolism was assessed by $^{14}CO_2$ output from [U-^{14}C] D-glucose, the glucose metabolism end product. When fibroblasts were cultured in the presence of high glucose levels, CO_2 production decreased significantly in comparison with normal glucose conditions. Glucose metabolism in the diabetic setting was not accompanied by an increase in glucose uptake. Diabetic patients exercise tight glycemic control to avert disorders from such glucose toxicity. Pyruvate dehydrogenase (PDH) activity is reduced in diabetes; therefore, we investigated the influence of thiamine on PDH activity and intracellular glucose concentration in fibroblast cells exposed to diabetic conditions. Thiamine reversed high glucose-induced PDH inhibition and prevented glucose accumulation. These results, taken together with those of our previous report, suggest that thiamine partially plays a role in modifying the metabolic fate of glucose and reducing glucose toxicity.

Key words: Glucose toxicity, Diabetes, Glucose metabolism, MRC-5 fibroblasts, PDH activity, Thiamine

INTRODUCTION

Glucose is a simple monosaccharide sugar found in the blood and required for energy. It is the only fuel normally used by the brain. In diabetes, the body is unable to monitor blood glucose levels either due to low or no insulin in type I diabetes or due to insulin resistance in type II diabetes. Therefore, people with diabetes need to carefully monitor their own blood glucose levels by changes to diet and medication.

Diabetes, sometimes called "sugar disease", is not always caused by glucose, but glucose plays a major role in most diabetic complications (Singh *et al*., 2001; Hammes *et al*., 2003; Brownlee, 2005; Beltramo *et al*., 2008). The excessive sugar in doughnuts, cakes, and other sweets is converted to blood glucose that is deposited directly into fat cells; glucose not used for energy is stored as triglycerides and fatty acids. Although glucose is important for energy, excessive daily sugar intake may act as a toxin inducing the body to become overweight or obese. Glucose is ubiquitous and carbohydrates are foods consumed everywhere, stealthily infiltrating our body systems, and slowly but surely destroying molecules, cells, and tissues.

The study of the glucose metabolic pathway has been ongoing for a long time and the property and enzyme group being discussed. However, relatively few studies have addressed the metabolic fate of glucose in the cell. In an experiment using pancreatic beta cells, the production of citric acid, malic acid, and carbonic acid does not increase proportionally with increase in extracellular glucose level (Heimberg *et al*., 1993; Schuit *et al*., 1997).

In this study, we investigated the metabolic fate of excess glucose by mimicking glucose levels in insulin secretion disorder or insulin resistance, which increase in type II diabetes mellitus. Insulin is a protein produced

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by the beta cells of the pancreas. Its role is to transport sugar (glucose) from the blood into the cells of the body. To establish a diabetic setting in *in vitro* experiments, no insulin was added to the culture medium. It is known that fibroblasts indicate if an insulin-independent glucose transporter (GLUT) has developed. In this study, to exclude the influence of insulin, *in vitro* experiments were performed using fibroblast cells. Uptake of glucose into the cells, which was the first stage of glucose metabolism, and production of carbon dioxide (CO_2), the glucose metabolic end product, were examined in a systematic circulating hyperglycemic state.

Next, we focused on the effect of high-glucose conditions on pyruvate dehydrogenase (PDH) in fibroblast cells. It has been reported that PDH activity is reduced in diabetic patients (Curto *et al*., 1997; Brownlee, 2005). We also investigated the influence of thiamine on PDH activity in fibroblast cells exposed to a diabetic setting. Thiamine (vitamin B_1) is a relatively simple compound consisting of a pyrimidine and a thiazole ring. It is naturally present in most foods as free thiamine. It is essential for production of energy from glycemic carbohydrates in foods. We tried to determine if thiamine affects the metabolism fate of glucose or if thiamine supplementation overcomes glucose toxicity.

MATERIALS AND METHODS

Chemicals and reagents

MRC-5 cells were supplied by DS Pharma Biomedical (Osaka, Japan). 2-Deoxy-D-glucose was purchased from Wako Pure Chemical Industries (Osaka, Japan). 2-Deoxy-D-glucose [1-^{14}C], radioactive glucose analogs, with specific activity 53 mBq/mmol, was purchased from Moravek Biochemical (Brea, CA, USA). [2-^{14}C] Pyruvate (15 MBq/mmol) and hyamine hydroxide 10-X were purchased from Perkin Elmer (Osaka, Japan). MEM and no-glucose medium were obtained from Invitrogen (San Diego, CA, USA). A solution of penicillin/streptomycin was purchased from MP Biomedicals (Solon, OH, USA). Thiamine hydrochloride was supplied by Sigma (St. Louis, MO, USA). Amplex® Red glucose assay kit was purchased from Molecular Probes (Paisley, UK). All other chemicals were of the highest purity available (Kishida Chemical Co., Ltd., Osaka, Japan).

Glucose uptake measurements

MRC-5 fibroblasts were grown in 24-well plates in MEM with 10% FBS, 100 U/mL penicillin, and 100 µg/mL streptomycin. After cells reached confluence, the medium was replaced with glucose-free medium for 24 hr. Cells were then rinsed in phosphate-buffered saline (PBS), and glucose uptake was assessed with 5-20 mM 2-deoxy-D-glucose [1-^{14}C] (1.96 MBq/mmol) for 5 min at 37°C. The reaction was terminated by placing the plates on ice and adding ice-cold PBS. After washing with PBS, the cells were lysed in 0.2 mL of 1 M NaOH per well. Trace activities were evaluated with a liquid scintillation counter (Perkin Elmer).

Measurement of glucose metabolism

MRC-5 fibroblasts were grown in 6-cm^2 dishes to confluence in MEM at 5.5 mM glucose. The culture medium was discarded, and attached cells were washed twice with PBS. Cells were then incubated for 2 hr at 37°C in the indicated concentrations of D-glucose [U-^{14}C]. Glucose metabolism was measured by production of CO_2 from ^{14}C-labeled D-glucose tracers. Cellular metabolism was stopped by addition of perchloric acid. Hyamine hydroxide 10X was used to capture the produced $^{14}CO_2$. The $^{14}CO_2$ production was measured by liquid scintillation counting.

Measurement of PDH activity

MRC-5 cells were washed with PBS, and a cell suspension was obtained by trypsinization. The cell concentration after centrifugal separation at 1,500 g for 5 min was 6×10^6 per 100 µL of suspension. The suspension was added to Triton X-100 and again subjected to centrifugal separation at 1,500 g for 5 min. The supernatant was used to measure PDH activity. To determine the active PDH fraction, the assay mixture contained the following in a final volume of 97.5 µL: 50 mM HEPES (pH 7.4), 2 mM $MgCl_2$, 3.35 mM NAD^+, 0.4 mM CoA, 10 mM L-carnitine, 0.5 mM DTT, 0.08 mM EGTA, 2.5 mM NaF, 0.8 U of carnitine acetyltransferase, and the cell suspension. The reaction was initiated with 2.5 µL of [2-^{14}C] pyruvate (specific radioactivity 15 MBq/mmol). PDH activity was calculated from the specific radioactivity of [^{14}C] acetylcarnitine and expressed in dpm/10^3 cells.

Determination of intracellular glucose levels

The intracellular glucose concentration in MRC-5 cells was evaluated by Amplex® Red glucose assay kit, a sensitive one-step fluorometric method for detecting glucose. Cells were washed twice with PBS, harvested by trypsinization, solubilized in Triton X-100, and centrifuged at 11,000 g at room temperature for 10 min. The clear upper aqueous layer was used to measure glucose concentration according to the instruction manual. Briefly, 50 µL of the reaction solution containing with 10 mM Amplex® Red, 10 U/mL horseradish peroxidase, 100 U/mL glucose oxi-

dase, and 50 mM sodium phosphate buffer, pH 7.4, were added to 50 µL of cell lysate and incubated in the dark for 30 min at room temperature. The absorbance was then measured at 545 nm using a microplate reader. Intracellular glucose concentration was determined from a standard curve generated using various concentrations of glucose up to 100 µM.

Statistical analyses

Data are expressed as means ± S.E. Statistical analyses of the data from multiple groups were performed using ANOVA followed by Scheffe's F tests. $P < 0.05$ was considered statistically significant.

RESULTS

Incorporation of glucose into MRC-5 fibroblasts

It has been reported that insulin-independent GLUT1 mainly develops as a carrier involved in uptake of glucose by fibroblasts. In this experiment, insulin was not added to the medium, so as to mimic insulin secretion disorder or insulin resistance increase in type II diabetes mellitus. Thus, the uptake of glucose in MRC-5 cells passed through insulin-independent GLUT1. At an extracellular glucose concentration of 5 mM, glucose was rapidly absorbed by cells and was absorbed almost constantly at subsequent excessive glucose levels (Fig. 1).

Glucose flows into the cell in response to extracellular glucose concentration, and glucose uptake at the cell membrane is not a rate-determining step in glucose metabolism. 2-Deoxy-D-glucose penetrates the cell and is phosphorylated, but the tracer is not metabolized. Thus, production of the CO_2 metabolic end product was measured to determine the subsequent metabolic fate of glucose after uptake.

Glucose metabolism in MRC-5 fibroblasts

The MRC-5 cells achieved confluence in MEM medium containing 5.5 mM glucose. Next, 5, 10, 15, 20, and 25 mM D-glucose [U-^{14}C] were added, and CO_2 generated by glucose oxidation was measured as $^{14}CO_2$ output from [U-^{14}C] D-glucose. The quantity of generated CO_2 gas was evaluated with an increment between each concentration. The amount of generated CO_2 gas increased with increasing extracellular glucose concentration (Fig. 2); however, this increase decreased above 15 mM D-glucose (Table 1). MRC-5 cells were maintained for 3 days at various concentrations of glucose and then incubated for 3 hr in the presence of 16.7 mM glucose. The CO_2-producing number of MRC-5 cells cultured in 25 mM glucose under diabetic conditions was significant-

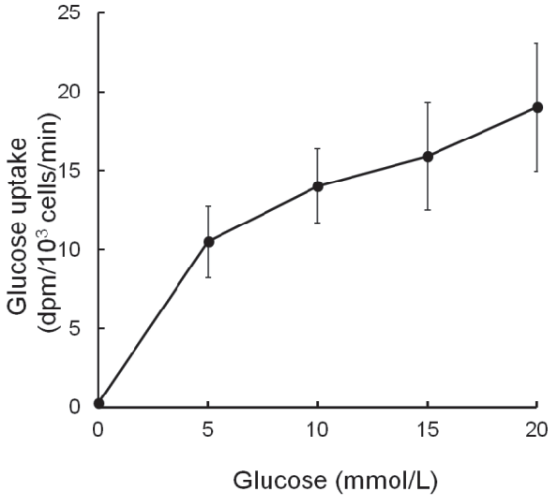

Fig. 1. Glucose uptake in MRC-5 fibroblasts measured as glucose residues from 2-deoxy-D-glucose [1-^{14}C]. MRC-5 cells were grown in 24-well plates, equilibrated in glucose-free medium for 24 hr, and then incubated with 2-deoxy-D-glucose [1-^{14}C] at 5, 10, 15 and 20 mM for 5 min. After these treatments, 2-deoxy-D-glucose [1-^{14}C] uptake was measured. Results are expressed as average disintegrations per minute (dpm) of 2-deoxy-D-glucose absorbed by cells in 1 min ± S.E. of the mean of three assays.

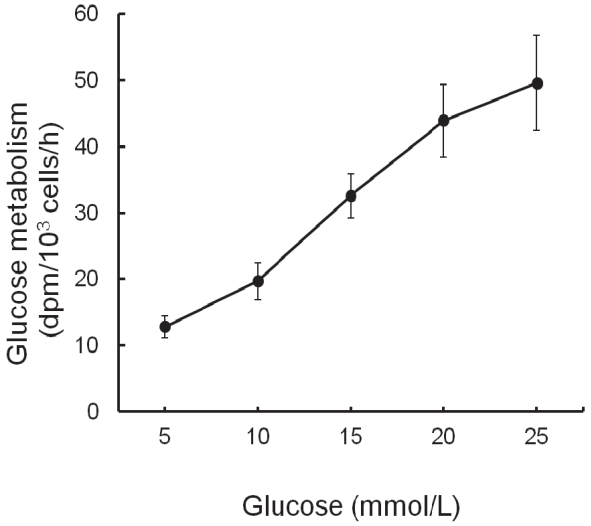

Fig. 2. Glucose metabolism in MRC-5 fibroblasts measured as $^{14}CO_2$ from D-glucose [U-^{14}C]. MRC-5 cells were grown in 6-cm^2 dishes to confluence in culture medium and then incubated with D-glucose [U-^{14}C] at 5, 10, 15, 20 and 25 mM for 2 hr. Glucose metabolism was measured as the production of CO_2 from D-glucose [U-^{14}C]. Results are expressed as average disintegrations per minute (dpm) of $^{14}CO_2$ production in 1 hr ± S.E. of the mean of four assays.

Table 1. Increment of metabolism in intervals of respective glucose concentrations.

Intervals of concentration (mM)	Increment of metabolism (dpm/10^3cells/hr)
5-10	6.9
10-15	12.9
15-20	11.4
20-25	5.7

ly lower than that of cells cultured in 5.5 mM glucose under normal glucose conditions (Fig. 3).

Effect of thiamine on PDH inhibition caused by high glucose conditions in MRC-5 fibroblasts

It has been reported that PDH activity is reduced in diabetic patients (Curto *et al*., 1997; Brownlee, 2005). Therefore, we investigated whether thiamine affected PDH activity in a diabetic setting. PDH activity was markedly lower under high than under normal glucose conditions (Fig. 4). In *in vitro* experiments under diabetic conditions, we continuously incubated MRC-5 fibroblasts in a medium containing 40 mM glucose for 3 days and measured PDH activity. Treatment of MRC-5 cells with high concentrations of glucose markedly reduced PDH activity. Thiamine dramatically reversed the PDH inhibition induced by diabetic conditions in MRC-5 fibroblasts (Fig. 5A).

Intracellular glucose concentration in MRC-5 fibroblasts after exposure to high glucose as in a diabetic setting

As shown in Fig. 5B, exposure to continuous high glucose for 3 days significantly increased intracellular glucose concentration. The addition of thiamine at 100 μM, a concentration that reversed high glucose-induced PDH inhibition, significantly reduced the accumulation of glucose in MRC-5 cells (Fig. 5B).

DISCUSSION

A so-called "obesity tax" has recently been proposed in a certain country, exemplifying the modern global recognition of obesity as a serious threat to human health. Research toward pathological elucidation and means of prevention of lifestyle-related diseases will contribute not only to the containment of spiraling healthcare costs but also to health enhancement. It is conceivable that the incidence of obesity will increase in future, owing to excessive nutritional intake and inadequate physical activity. It has been reported that low-carbohydrate diets are effective for achieving weight loss and reducing the risks of cardiovascular disease (Hession *et al*., 2009). Reducing the dietary glycemic load could improve all health issues.

Obesity often leads to diabetes, increasing the risk of diabetic complications. Only in situations where an individual consumes too many calories — hypercaloric con-

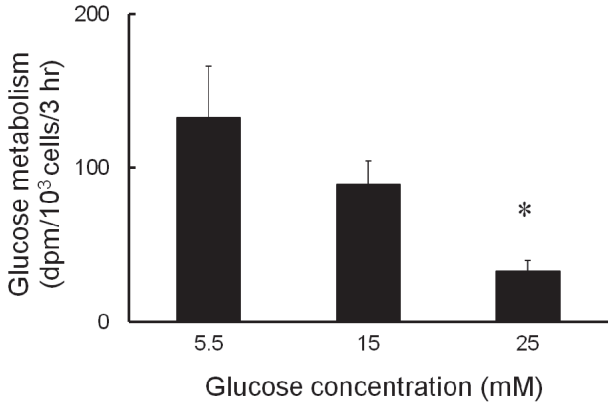

Fig. 3. Glucose metabolism in MRC-5 fibroblasts measured as $^{14}CO_2$ from D-glucose [U-^{14}C]. MRC-5 cells were maintained at various concentrations of glucose for 72 hr, and then incubated for 3 hr in the presence of 16.7 mM D-glucose [U-^{14}C]. Glucose metabolism was assessed by CO_2 production. Results are expressed as average disintegrations per min (dpm) of $^{14}CO_2$ production in 3 hr ± S.E. of the mean of four assays. *$P < 0.05$, compared with 5.5 mM glucose.

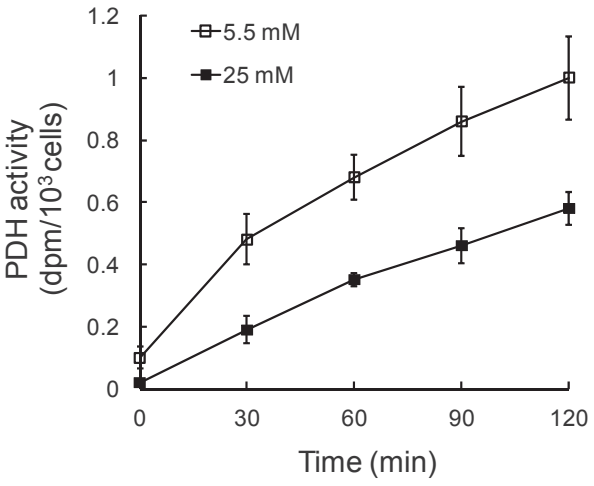

Fig. 4. Effect of glucose exposure on PDH activity measured as [2-^{14}C] acetyl-CoA from pyruvate [2-^{14}C] in MRC-5 fibroblasts. MRC-5 cells were cultured for 72 hr at 5.5 and 25 mM glucose, as described under Experimental Procedure. Results are means ± S.E. of three experiments (25 mM) or six experiments (5.5 mM).

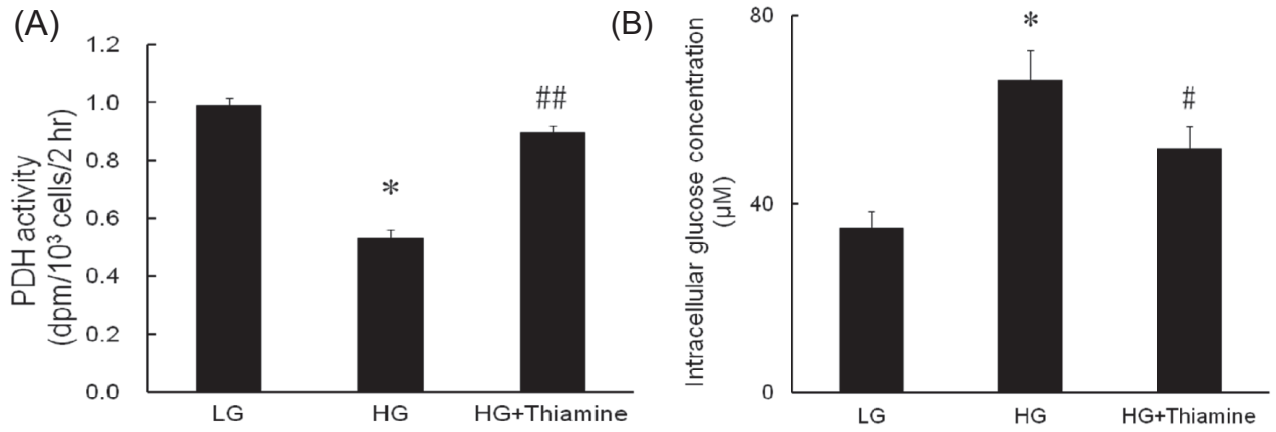

Fig. 5. Effects of thiamine on PDH activity (A) and intracellular glucose concentration (B) in MRC-5 fibroblasts exposed to high glucose. MRC-5 cells were cultured for 72 hr in the presence of low glucose (LG: 5.5 mM), high glucose (HG: 40 mM), or HG with thiamine (100 μM). Results are means ± S.E. of six experiments (A) or four experiments (B). $^{*}P < 0.001$, compared with LG. $^{\#\#}P < 0.001$, and $^{\#}P < 0.01$, compared with HG.

ditions — and is obese, fat leads to insulin resistance. In insulin-resistant individuals, the result is an increase of daylong concentrations of glucose. With persistent hyperglycemia, glucose exceeding the metabolic capacity of the major glycolysis pathway overflows into minor pathways. Various metabolites from the promoted alternative pathways have been suggested to play roles in the pathogenic mechanisms of diabetic complications (Brownlee, 2005; Muoio and Newgard, 2008).

Cultured fibroblasts were used to conduct an experiment investigating glucose metabolism in terms of intracellular glucose uptake and glucose oxidation. The amount of CO_2 production at various glucose concentrations was used to evaluate glucose oxidation in acute-phase diabetes, and the CO_2 production of cells cultured over 3 days at various glucose concentrations was used to evaluate glucose oxidation in chronic-phase diabetes. These experiments established that glucose uptake is dependent on extracellular glucose concentration, and that the production of CO_2 slows as extracellular glucose concentration increases. CO_2 production decreased in cells cultured for 72 hr at a high glucose concentration, even though the extracellular glucose concentration was constant. This indicates that in cells under high glucose conditions, glucose uptake and oxidation become decoupled. Thus, it was established that these cells did not produce CO_2 in proportion with the intracellular uptake of glucose. The same phenomenon has also been reported in pancreatic beta cells, suggesting that PDH, which plays an important role in the tricarboxylic acid cycle, is involved in this decoupling of glucose uptake and oxidation (Schuit *et al*., 1997).

The next step was to measure PDH activity to investigate the influence of diabetic condition and thiamine on PDH activity and intracellular glucose concentration. It was observed that PDH activity decreased in cells cultured at a high glucose concentration. In contrast, culturing cells in the presence of thiamine inhibited the decrease in PDH activity and reduced intracellular glucose concentration. PDH phosphorylation caused by increased fatty acid metabolism is known to reduce PDH activity in diabetes (Holness, 2003). This experiment suggests that PDH, which uses thiamine as a coenzyme, may be a target enzyme for preventing the onset of diabetes as well as the development of diabetic complications.

Patients with diabetes have been reported to be deficient in thiamine (Jermendy, 2006). It is considered that the administration of thiamine to patients with hyperglycemia may reduce glucose flow into the minor pathways of glucose metabolism via activation of glucose oxidation, and thereby ameliorate pathological changes due to obesity and diabetes. Thiamine intake is expected to exert preventive effects against not only diabetic complications but also obesity, through the improvement of carbohydrate and lipid metabolism, and eventually to lead to the prevention of lifestyle-related diseases. For this reason, research in this field has been pursued.

Glycemic control is a description of overall blood glucose control. A desirable level of glycemic control depends on the ability of a person with diabetes to keep glucose levels as close to normal as possible. Attaining good glycemic control is the critical. Tight glycemic con-

trol is the key to decreasing risks for many severe short-term diabetic complications, which experts believe are avoidable.

However, it is questionable whether the progression of diabetic complications can be prevented solely by blood glucose control, as targeted by current diabetes treatment. According to a 1998 survey of clinical studies, no appreciable difference in the incidence of diabetes complications was noted between intensive and observational treatment groups in some reports, indicating that strict control of blood glucose level is not sufficient to prevent diabetic complications (Pontiroli and Folli, 1998). In comparison with blood glucose levels, intracellular glucose concentration and glucose metabolites are presumably more closely associated with the onset and progression of diabetes and its associated complications. It is speculated that the intracellular glucose level, rather than blood glucose level is deeply involved in the development and progression of diabetes as well as its long-term complications. In this study, thiamine supplementation affected PDH activity and intracellular glucose concentration. Thiamine moderated glucose toxicity, which is considered to be responsible for the discrepancy between glucose uptake and oxidation in the glucose metabolism.

We intend to elucidate the molecular mechanism underlying the effects of improved glucose metabolism on the pathologies of obesity and diabetic complications by focused research on protein glycosylation via the activation of the hexosamine synthetic pathway, which may be caused by disordered glucose metabolism (Kohda *et al*., 2008, 2009, 2010). Our ultimate research goal is to elucidate the pathologies of and establish means for preventing diabetic complications and obesity (Tanaka *et al*., 2010; Kohda *et al*., 2012). To achieve optimal outcomes, treatment requires a multi-pronged approach. Because weight loss and the control of abnormal insulin, glucose, and lipid levels can take time, herbal and nutritional supplements will be initially required.

Conflict of interest---- The authors declare that there is no conflict of interest.

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