Cucurbita ficifolia Bouché fruit acts as an insulin secretagogue in RINm5F cells

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Abstract

Diabetes mellitus (DM) is an important global public health problem. Despite the availability of drug therapy, patients have continued using plants with anti-diabetic properties as alternative treatments for diabetes. Cucurbita ficifolia Bouché (C. ficifolia) is a plant cultivated in Mexico for its edible fruit that is medicinally used to control type 2 DM (T2D). The hypoglycemic effect of C. ficifolia has been demonstrated in different experimental models and in T2D patients. However, no studies have determined the mechanism of action of this hypoglycemic effect. The aim of the present investigation was to determine if the hypoglycemic action of C. ficifolia and D-chiro-Inositol (DCI; a compound found in the fruit of C. ficifolia) occurs through an increase in the production of insulin. An aqueous extract of this fruit was obtained and standardized by its content of DCI, the principal hypoglycemic compound of the fruit. To study the mechanism of hypoglycemic activity of this extract and DCI alone, RINm5F cells were exposed to different concentrations of both the extract and DCI, and the production of insulin and Kir6.2 channels were measured. The mRNA expression levels of insulin and Kir6.2 were found to be increased in cells that were treated with DCI and the C. ficifolia aqueous extract. This effect suggests a mechanism of action that involves both the expression and secretion of insulin. This research maintains the interest in developing new nutraceuticals from C. ficifolia fruits for use in the treatment of T2D.

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Abbreviations

DM, diabetes mellitus; T2D, type 2 diabetes mellitus; SUR1, sulfonylurea receptor 1; DCI, D-chiro-inositol;

Keywords

Type 2 diabetes, insulin, Kir6.2, Cucurbita ficifolia Bouché, D-chiro-inositol.
Introduction

Type 2 diabetes mellitus (T2D) is a set of metabolic disorders characterized by increased levels of blood glucose (hyperglycemia) due to defects in insulin production and secretion by the pancreatic β cells (1-4).

One of the key factors in the secretion of insulin by pancreatic β cells is the ATP-dependent K⁺ channels (K⁺\textsubscript{ATP}). These channels are formed by two tetramers of the subfamily Kir6.2 and the sulfonylurea receptor 1 (SUR1) (5). Both Kir6.2 and SUR1 have the ability to induce changes in membrane electrical excitability. This change leads to calcium channel opening and an increase in cytoplasmic calcium concentration in beta cells, which triggers the mobilization and release of insulin (6). Sulfonylureas are anti-diabetic agents that act by releasing endogenous insulin following binding to SUR1 (7,8).

Despite the widespread use of antidiabetic drugs, more than 70% of the world population uses medicinal plants as the only available alternative for the treatment of their diabetes-related health problems (9). Furthermore, many medicinal plants have been reported to have “anti-diabetic” properties (10-12). Cucurbita ficifolia Bouché (C. ficifolia, Cucurbitaceae) is cultivated in Mexico for its edible fruit (13). It is an annual monocoeous plant, which is grown primarily in the states of Mexico, Hidalgo, Puebla and Veracruz. C. ficifolia is commonly known as “chilacayote”, and its immature fruit is used for preparing different dishes. The mature fruit of C. ficifolia is used to make a traditional candy (14). In the states of Mexico and Morelos, the annual production of C. ficifolia is 4,706 tons and the harvested area is 300 hectares (15).

C. ficifolia fruits are also medicinally used to control type 2 DM (T2D) and their hypoglycemic effect has been demonstrated in different experimental models and in T2D patients. D-chiro-inositol (DCI) has been proposed to be the active component in C. ficifolia (16). DCI, originally discovered as a mediator of intracellular insulin action, has been shown to accelerate the dephosphorylation of the glycogen synthase and pyruvate dehydrogenase, two regulatory enzymes of the glucose oxidation pathways (14,17). Clinical studies have demonstrated a linear relationship between body DCI deficiency and the degree of insulin resistance in subjects with T2D. In addition, the administration of DCI to diabetic rats and rhesus monkeys improved glucose utilization and insulin action (17). In streptozotocin-induced diabetic rats, daily administration of C. ficifolia fruit and DCI over a 30-day period reduced glyceria and increased the levels of liver glycerin, total hemoglobin and insulin (16). Although both C. ficifolia fruit extracts and DCI might be used as coadjuvants in DM control (18,19), there are no studies that explain the mechanisms of action involved in their hypoglycemic effect. This is very important because the juice of the fruit has been studied in Mexico as a hypoglycemic agent in experimental animal models and in diabetic patients (19).

Therefore, the aim of this work was to determine if the hypoglycemic effect of the C. ficifolia fruit extracts and DCI may be explained by a rise in the expression levels of insulin and Kir6.2 in RINmF5 cells.

Material and methods

Plant material

Fresh mature fruits of C. ficifolia with a diameter of 18–20 cm and an approximate weight of 4 kg were gathered in the Acolman municipality, State of Mexico during April of 2011. The endocarp, free of seeds, was cut into thin slices and placed in a container at room temperature with constant aeration for dehydration. The dried material was ground in an electrical Wiley mill, using a grid of 1 mm diameter (14). This material (100 g) was macerated with water (1 L) for 72 h in a laminar flow hood. The aqueous phase was filtered and centrifuged at 805 x g to obtain a precipitate, which was separated and freeze-dried (LABCONCO) (yield = 5%) (19). Quantification of the DCI content in the C. ficifolia extract was performed using high performance liquid chromatography (HPLC Waters 2695 separation module) as reported by Roman-Ramos et al. (2012) (14). This resulted in an extract with a concentration of 3.3 mg of DCI/g of extract. A pure DCI standard was obtained from Sigma-Aldrich.

RINmF5 cell culture

RINmF5, which is an insulin-producing cell line derived from a pancreatic islet tumor, cells were commercially acquired from the American Type Culture Collection (ATCC). RINmF5 cells were grown in a monolayer culture using RPMI 1640 medium (11.1 mM glucose) ( Gibco™), supplemented with 10% fetal bovine serum (ATCC), 2 mM 1-glutamine, 1 mM sodium pyruvate and 2 μg L⁻¹ of gentamycin (In vitrotec). The cells were grown at 37°C in disposable plastic bottles (Nunc™) and in a humidified atmosphere of 5% CO₂/95% air (20–22). The medium was replaced twice a week.

MTT assay

Cell viability, following treatment with both the extract of C. ficifolia fruit and DCI alone, was measured using the 3-(4,5-dimethylthiazole-2-yl)-2,5-dihenyltetrazolium bromide (MTT, sigma) assay, according to Mosmann (23). The assay measures the conversion of MTT to insoluble
formazan by the dehydrogenase enzyme activity of the intact cells; this assay is widely used for studies of cytotoxicity, viability and proliferation. The amount of the formazan produced is directly proportional to the number of living cells (22). The RINm5F cells were seeded into 96-well microplates at a semi-confluent density (5000 cells/well). After 24 h, the medium was replaced with complete medium containing concentrations of DCI ranging from 0.1 to 50 μM in the aqueous extract of C. ficifolia fruit or the equivalent concentrations of DCI alone (Sigma). The cells were treated for 24 h. They were then washed with phosphate-buffered saline (PBS), pH 7.4, and a solution of 0.1 mg mL⁻¹ MTT in PBS (pH 7.5) was added. The cells were incubated for 3 h at 37 °C, followed by washing with PBS. Then, 200 μL of 40 mM HCl (prepared in isopropanol) was added to each well for 15 min to solubilize the produced formazan. The OD was read at 570 nm (23). The data were expressed as the percentage of viable cells following treatment with the aqueous extract of C. ficifolia and DCI compared to the control cells.

Because the treatments with the aqueous extract of C. ficifolia fruit were based on its DCI content, we chose the equivalent concentrations for the DCI alone treatments of the RINm5F cells and quantify the insulin and Kir6.2 mRNA expression levels. Thus, the choice of the concentrations to treat the cells with the aqueous extract of C. ficifolia fruit and with DCI alone (0.25 μM of DCI in both cases) preceded the extraction of total RNA from the treated cells.

RNA isolation, reverse-transcriptase polymerase chain reaction and real-time PCR

For the extraction of total RNA, cells were seeded at 1 x 10⁶ cells (RINm5f) per treatment in 6-well culture dishes and incubated for 24 h. Both the aqueous extract of C. ficifolia fruit and the DCI treatments were performed at a concentration of 0.25 μM and over 24 h. After treatment, the cells were washed with 1 mL ice-cold PBS and solubilized with 1 mL of TRIzol® reagent (Invitrogen). RNA was treated with chloroform, centrifuged at 12,235 x g for 15 min at 4 °C and precipitated with ethanol as described by Chomczynski (21, 25). RNA was extracted and redissolved in diethylpyrocarbonate treated water and the OD at 260 nm was measured to determine the RNA concentration. cDNA was synthesized using 2 μg total RNA by reverse transcriptase PCR using the lmProm II reverse transcription system (Promega). The cDNA was amplified by an enzyme DNA polymerase kit, the DNA master plus SYBR Green 1 PCR MasterMix (Applied Biosystems) (20) for the following genes: Insulin NM_09129.3 (5’-TGCCAGGCTTTTGCAAAAC-3’), Kir6.2 NM_031358.3 (5’-GTACAGATCTTGGTGCGT-3’) and β-actin NM_031144.3 (5’-GTGGGTATGGTCAGAGA-3’), which was used as a control.

![Figure 1. Function of C. ficifolia fruit extract and DCI in RINm5F cells.](image-url) Cells were treated with the aqueous extract of C. ficifolia fruit and DCI at concentrations ranging from 0.1 to 50 μM (x-axis). The Y-axis shows the percentage of activity in the treated cells. A concentration of 0.25 μM was chosen for treating the cells (n=6). *Significantly different compared to control (p<0.05).
Statistical analysis

Data are presented as the mean ± standard error of the mean (S.E.M.). Significant differences among the treatments were determined by an analysis of variance using the Tukey-Kramer Multiple Comparison post-hoc test (p<0.05).

Results and Discussion

Fig. 1 shows the percentage of dehydrogenase functionality in the RINm5F cells treated with different concentrations of both the aqueous extract of C. ficifolia fruit and DCI. At a concentration of 1 μM of DCI in the aqueous extract of C. ficifolia fruit, a 20% cell functionality decrease compared to the control group (p<0.05) was observed; this effect was indicative of a concentration-dependent cytotoxic effect. A DCI alone concentration of 0.25 μM showed a retention of 98% of the dehydrogenase functionality of the cell compared to the control group. DCI alone did not show a cytotoxic effect at any of the concentrations examined. Therefore, a concentration of 0.25 μM DCI was chosen to examine insulin and Kir6.2 gene expression following treatment with the aqueous extract of C. ficifolia fruit and DCI alone.

A statistically significant increase in insulin mRNA expression (a 5-fold increase) compared with the control group was observed after treatment with the aqueous extract of C. ficifolia fruit and with DCI alone in the RINm5F cells (Figure 2). Therefore, the hypoglycemic effect of C. ficifolia may be explained by an increase in the expression of the insulin gene. Various studies that focused on gene therapy for diabetes have proposed the use of agents with a strong relationship between the stimulation of insulin gene expression and its secretion (26). Ultra-structural analysis of beta cells has shown reduced insulin granules in human D2T. These survival and functional changes are accompanied by modifications of beta cells gene and protein expression (27). Finding agents that regulate insulin gene expression to give an advantage to beta cells holds considerable promise and could provide the beta cells the necessary flexibility to adapt to the changing environment in DT2. If C. ficifolia exerts any effect on the regulation of gene expression of insulin, it would be a viable therapeutic agent for the treatment of T2D.

Studies in rat beta cells have associated the effect of glybenclamide on insulin mRNA expression with an increase in the cytoplasmic concentration of [Ca²⁺] after 24 h treatment (28). Therefore, the aqueous extract of C. ficifolia fruit could stimulate insulin gene expression through an increase in cytoplasm [Ca²⁺]. Although our results are in agreement with other studies in which C. ficifolia fruit extracts and DCI have shown an effect on insulin secretion (16), other studies are required that measure insulin secretion and the cytoplasmic concentration of [Ca²⁺]. Nevertheless, in our studies, glybenclamide (24 h, 4 μM) did not cause an increase in the expression of insulin mRNA as has been reported in previous studies (28). This is likely due to the difference in the treatment concentrations (24 h, 0.5 μM).

ATP-sensitive potassium channels (K⁺ATP) in pancreatic beta cells contain SUR1 and the inwardly rectifying potassium channel (Kir) 6.2 subunits. SUR1 and the Kir6.2 protein assemble into a potassium channel and play a key role in the regulation of insulin secretion (29). We evaluated the effect of the aqueous extract of C. ficifolia fruit and DCI on Kir6.2 mRNA expression. Figure 3 shows that in RINm5F cells, the aqueous extract of C. ficifolia fruit and DCI significantly increased Kir6.2 mRNA expression (greater than 100-fold) compared with the control group. This is the first report of the effect of DCI and the aqueous extract of C. ficifolia fruit on Kir6.2 mRNA expression. The K⁺ATP channel plays an important role in insulin secretion, and this investigation opens the possibility that in the hypoglycemic effect of DCI and C. ficifolia is involved the expression of both insulin and DCI. In other studies, the expression of Kir6.2 has been positively correlated with cellular protein content (30). Thus, the increase in Kir6.2 mRNA by the aqueous extract of C. ficifolia fruit and DCI should indicate an increase in the protein content, which would be crucial for the secretion of insulin. Other studies are required to confirm the association among Kir6.2 expression, protein content and insulin secretion.

Notably, the mechanism of action of glibenclamide involves a block of the K⁺ATP channel by binding to SUR1 in

Figure 2. Insulin mRNA gene expression in RINm5F cells.
Cells were treated with the aqueous extract of C. ficifolia fruit and DCI at a concentration of 0.25 μM for 24 h. The aqueous extract of C. ficifolia fruit and DCI induced insulin gene expression, which was increased 5-fold (n=6). *Significantly different against control (p<0.05).
beta cells. This depolarizes the cell plasma membrane, inducing the opening of [Ca\(^{2+}\)] channels and the secretion of insulin (31). In this study, glibenclamide stimulated the expression of Kir6.2 without affecting insulin expression (Figure 3). However, the C. ficifolia fruit extract and DCI increased the mRNA expression of both insulin and Kir6.2; therefore, the C. ficifolia fruit extract and DCI must have a different hypoglycemia inducing mechanism than glibenclamide. This is likely the result of distinct chemical components in the C. ficifolia fruit extract other than DCI.

Previous phytochemical studies have shown the presence of active principles in the aqueous extracts of C. ficifolia fruit (32). The aqueous extract contains DCI (3.3 mg/g of extract), and it has been reported to contain flavonoids and cucurbitacins. There have been similar findings in other Cucurbitaceae species (33). Flavonoids are secondary metabolites of plants, and have been characterized as being soluble in water. Flavonoids have been principally associated with increased in insulin secretion (34-35). Flavonoids are likely to be associated with the expression of insulin mRNA, which clearly agrees with the greater effect of the aqueous extract of the C. ficifolia fruit compared to DCI alone.

The fruit of C. ficifolia has been associated with a hypoglycemic effect, and it has been shown to have additional benefits as an antioxidant and an anti-inflammatory (10,14,19). It is probable that these actions may be attributed components in the extract other than DCI, such as, flavonoids (36) or curcurbitacins (37). However, a complete characterization of the aqueous extract is necessary to correlate these effects.

C. ficifolia has been reported as a useful alternative for the treatment of T2D (10-12,14). The activity of C. ficifolia and DCI on the mRNA expression levels of proteins implicated in the production and secretion of insulin in pancreatic beta cells represents a notable finding. Other studies have focused on verifying the hypoglycemic effect of C. ficifolia in both diabetic patients and animal models (16,18-19). These studies have provided a great contribution to the study of medicinal plants. However, this study represents the first attempt to elucidate the mechanism of action of C. ficifolia: an increase in the expression of insulin and K\(^{+}\)\(_{ATP}\) channels, which is likely due to DCI.

Several nutraceuticals used in clinical practice have been shown to target the pathogenesis of diabetes mellitus (38). In our laboratory, a jelly developed from pomegranate juice with the addition of a pomegranate rind extract, has the potential to control DM (39). Therefore, in the same manner, we can develop a nutraceutical from C. ficifolia.

Conclusion

The aqueous extract of C. ficifolia fruit and DCI increased mRNA expression for insulin and Kir6.2 in RINm5F cells. These results suggest a mechanism of action involving the stimulation of insulin and Kir6.2 (a protein widely involved in the secretion of insulin) production, which represents a useful alternative for DM control. Therefore, the fruits of C. ficifolia may be considered to be a potential source of raw material for obtaining new oral hypoglycemic drugs and for the development of a new nutraceutical for DM control.

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