CUCURBITACIN ATTENUATES HYPERGLYCEMIA BY INCREASING GLUT2 THROUGH PI3K-PAKT IN THE HEPATOCYTES OF STREPTOZOTOCIN INDUCED DIABETIC RATS

Gani Sharmila Banu*

Department of Zoology, NKR Government Arts College for Women, Namakkal-637001, Tamilnadu, India.

ABSTRACT

The current study was undertaken to investigate the role of cucurbitacin in the regulation of hepatic expression of insulin signaling proteins and the underlying mechanism. Diabetes was induced with a single dose of streptozotocin (40 mg/kg bw/i.p). Diabetic rats were treated with cucurbitacin (5 mg/kg bw) for 45 days. Diabetic rats showed significant decrease in protein expression of PI3K, p-Akt and GLUT2, and the immunohistochemistry of IR, p-Akt and GLUT2 in liver showed abnormal changes. However, the administration of cucurbitacin in diabetic rats showed to enhance IR, p-Akt and GLUT2 expressions when compared with diabetic rats. Immunostaining showed that administration of cucurbitacin in diabetic rats demonstrated a positive GLUT2 staining in pancreas and synthesis of IR, p-Akt and GLUT2 in the hepatocytes. Based on the present findings, the antidiabetic potential of cucurbitacin could improve insulin action and glucose metabolism through activation of GLUT2 protein in liver and pancreas.

KEYWORDS: cucurbitacin, streptozotocin, GLUT2, PI3K/p-Akt, diabetes, glucose homeostasis.

Abbreviation

Akt- Protein kinase B/ a serine/threonine-specific protein kinase; ANOVA- One-way analysis of variance; b.w. – body weight; GLUT-2- glucose transporter 2; IR- Insulin receptor; IRS 1,
INTRODUCTION

Diabetes is a complex metabolic syndrome caused by various etiologies and characterized by defects in the body's ability to maintain insulin and glucose homeostasis. The International Diabetes Federation estimates that people living with diabetes will surge from 382 million to 592 million people by 2035 in developing and under-developed countries under the age of 60.[1] Insulin plays the function of regulating glucose homeostasis mainly by PI3K/Akt signaling pathway.[2] After food consumption, the pancreatic beta cells produced insulin, which passes into the blood circulation and affects insulin receptor on the surface of the liver cell membrane, making its activation of phosphorylated tyrosine located in the beta receptor subunit.[3] Activated insulin receptor makes tyrosine site of insulin receptor substrate-1/2 (IRS-1/2) after the phosphorylation, then activates PI3K; Activated PI3K can catalyze 4, 5-2 phosphatidyl inositol phosphate (PIP2) and generate PIP3, which may as the second messenger to activate Akt, and the activated Akt plays metabolism biological effects by regulating downstream molecules. In a state of insulin resistance of diabetes, phosphorylation level of Akt Ser473 decreases,[4] and insulin signal transduction subsides. Thus, any abnormality appeared along PI3K/Akt signaling pathway will affect the transduction of insulin signal, prompting the development of IR as well as diabetes. In addition, the glucose transporters (GLUT) play a crucial role in glucose homeostasis. GLUT2 is expressed in a restricted set of organs that are of critical importance for the control of glucose homeostasis.[5] Dysregulation of GLUT2 in the liver and pancreas can result of pathophysiological manifestations, which is related to diabetes in human and rodents.[6] Based on the accumulated evidence suggests that GLUT2 may serve as a potential drug target for diabetes.[7]

Though, the availability of synthetic agents currently used for the treatment of diabetes is sulfonylureas, biguanides, thiazolidinediones and α-glycosidase inhibitors, it has several undesirable side effects. Furthermore, cost of these drugs is quite expensive and alternative approaches in management of diabetes are urgently required. Significant candidates are plant triterpenoids, which are naturally occurring compounds widely distributed in vegetables, fruits and plant derived phytocompounds having minimal side effects.[8] Among natural
compounds, triterpenoids are a class of pharmacologically active and structurally rich metabolites with privileged motifs for further modifications, which are reported to act on glucose metabolism. Thus, it has become clear that triterpenoids may exert their glucose-lowering effects.

Cucurbitacins are tetracyclic triterpenes extracted from plants of the Cucurbitaceae family that have been used as an antirheumatic and purgative remedy for centuries in rural areas.[9,10] It has been reported that at least 100 species of cucurbitacins exist from about 30 genera of Cucurbitaceae families.[11] They possess a broad range of pharmacological properties, including anti-inflammatory, antipyretic, anticancer activities.[12] In particular, antiproliferative activity has been found in eight cucurbitacins, including cucurbitacin B, D, E, I, IIa, L, Q, and R.[13,14] Furthermore, the anticancer properties of several variants of cucurbitacins have been observed both in vitro and in vivo.[15-17] Cucurbitacin promotes apoptosis in colon cancer tested by tumor models of syngeneic transplanted mice[17] and induces apoptosis in pancreatic cancer cells.[18] These mechanisms indicate that inhibition of the JAK/STAT3 pathway by cucurbitacin may be effective in cancer immunotherapy. However, the role of cucurbitacins in diabetes and related metabolic complications in rodent study has not been investigated yet. Therefore, the purposes of this study were to evaluate the effects of cucurbitacin on regulation of hepatic expression of insulin signaling proteins and the underlying molecular mechanism in STZ-induced diabetic animal model.

MATERIALS AND METHODS

Chemicals

Cucurbitacin and STZ were obtained from Sigma-Aldrich. (St. Louis, MO, USA). Primary antibodies IR, PI3K, p-Akt, GLUT 2 and β-actin antibodies were purchased from Santa Cruz biotechnology, Inc., USA. Secondary antibody was purchased from Genei, Bangalore, India. All other chemicals and solvents were of analytical grade obtained from local suppliers (India).

Animals

Six-week-old adult male Wistar albino rats weighing 180–200g were procured and maintained at our department. They were maintained in environmentally controlled conditions (temperature of 22 ± 2°C, a relative humidity of 65 ± 5%, and 12/12 h light/dark cycle) for 7 days. The animal shad free access to commercial standard pellet diet before start the experiment and water was provided ad libitum throughout the period of the experiment.
The animal facilities and all experimental protocols were approved by the Institutional Animal Ethics Committee following the principles and guidelines of the committee for the purpose of control and supervision of experiments on animals (CPCSEA), India.

**Induction of experimental diabetes**

After acclimatization for 7 days, experimental rats excluding the normal control and normal treated groups were given free access to standardized high-fat diet for 2 weeks prior to single intraperitoneal injection with STZ (40mg/kg, dissolved in 0.1 M citrate buffer, pH 4.5). Citrate buffer (carrier) alone was injected to normal control and normal treated rats. STZ-injected animals were administered glucose solution (20%) to block existing drug-induced hypoglycemia. Fasting blood glucose (FBG) level was measured 5 days after the STZ induction. The rats with a FBG level above 240 mg/dl were considered diabetic and grouped further for clinical study. The treatment was started on the subsequent day and this was considered as 1st day of treatment.

**Study design**

The experimental animals were randomly divided into five groups of six animals each. Cucurbitacin were dissolved in 5% dimethyl sulfoxide and glibenclamide was diluted in water and administered orally to experimental groups using intragastric tube daily for a period of 45 days. Group I as normal control rats; Group II as normal with cucurbitacin (5 mg/kg b.w.); Group III as diabetic control rats; Group IV as diabetic with cucurbitacin (5 mg/kg b.w.); Group V as diabetic with glibenclamide (600 μg/kg b.w.). After 45 days of treatment, the animals were anesthetized using ketamine by sacrificed cervical decapitation. The fresh pancreas and liver tissues were excised immediately and used for performed immunohistochemistry and western blot.

**Immunoblotting**

Immunoblotting was performed to analyze the expression pattern of insulin signaling proteins, GLUT-4, PPAR-γ by Laemmli. The skeletal muscle tissue sample was homogenized with ice-cold RIPA lysis buffer and protein inhibitor cocktail (10μg/ml). The cells were then centrifuged at 20,000 g for 15 min at 4°C. The supernatants were used as total protein extracts. The total and nuclear protein contents were determined by the Bio-Rad protein kit with BSA as the standard. The lysate containing 20 μg of protein was subjected to sodium dodecyl sulphate-polyacrylamide gel electrophoresis. Separated proteins were transferred electrophoretically to PVDF membrane, blocked with 3% BSA solution for 1 h,
and then incubated with specific primary antibodies (diluted 1:1000) overnight at 4°C. The membranes were washed with TBST thrice for 15 min and the blots were incubated with goat anti-rabbit or goat anti-mouse IgG horseradish peroxidase-conjugated (HRP-conjugated) secondary antibody (diluted 1:1000) in blocking solution for 1 h at room temperature. Then, the membranes were washed with TBST thrice for 15 min. Each antigen-antibody complex was visualized using ECL Western Blotting Detection Reagents and detected by chemiluminescence with LAS-1000 plus (FUJIFILM, Tokyo, Japan). Band densities were determined by an image analyzer (Multi Gauge V3.1, FUJIFILM Corporation, Valhalla, NY, USA) and normalized to β-actin for total protein and nuclear protein. Bands were scanned using a scanner and quantitative by Image J, a public domain Java image processing software.

**Staining for collagen**

**Masson’s trichrome staining**
The sections in the slides were stained with Masson’s trichrome by the method provided by Prophet et al. The tissue sections (5 μm thickness) in the slides were incubated in an oven at 60°C for 30 min then brought to water via xylene and alcohol. The slides were placed into Bouin’s solution at 56°C for 1 hour. Cool to room temperature for 5 min. Wash well with running tap water. The slides were placed in weiger Iron Hematoxylin for 5 min. and then placed in running tap water for 15 min. The slides are placed in acid Fuchs in 10 min. The sections were again washed well with running tap water followed by phosphomolybdic acid for 5-15 min. Then the slides are kept in fast green for 40 min and then rinsed well in distilled water. Dehydrated and then mounted using neutral deparaffinated xylene for microscopic observation.

**Picrosirius red staining**
The sections in the slides were stained with picrosirius red by the method of Junqueira et al. The sections in the slides were incubated in an oven at 60°C for 30 min then immersed in xylene for 5 min, 4 min and subsequently in absolute ethanol for 3 min, 2 min respectively. Sections were brought into water and then stained the nuclei with an acid resistant nuclear stain. After washing well with water the slides were placed into picrosirius red solution for 90min. The sections in the slides were differentiated with 0.01M HCl for 2 min. The slides were placed in 70% ethanol for 30 sec, 90% ethanol for 30 sec and 100% ethanol for 30 sec and this sequence was repeated for 3 times and then mounted using neutral deparaffinated xylene for microscopic observation.
Immunohistochemistry

Immunohistochemistry was performed using a super sensitive polymer HRP detection system kit from BioGenex, USA. Paraffin-embedded tissue (liver and pancreas) were cut to obtain sections of about 5 μm thickness. The mounted paraffin-embedded slices are deparaffinized in xylene and rehydrated using ethanol/H₂O gradient. Heat mediated antigen retrieval step was carried out for 10 min, and then, the slides were allowed to cool for another 20 min. This was followed by peroxidase block treatment for 10–15 min and then power block treatment for another 15 min. The sections were incubated with the concerned diluted primary antibodies IR, p-AKT and GLUT2 for 2 h in 1:200 dilution followed by treatment with the super enhancer solution for 30 min and super sensitive poly-HRP solution for 30 min. In addition Chromogen substrate was added to the sections were incubated for 15 min and rinsed with distilled water. Slides were counterstained with hematoxylin for microscopic observation.

Statistical analysis

All the experimental data were expressed as the mean ± SD. Statistical analysis was performed by using SPSS 22 software. One-way analysis of variance (ANOVA) was performed. Statistically significant differences between the groups were determined by Dunnett's post-hoc test. The differences were considered significant at p<0.05.

RESULTS

The effect of cucurbitacin (5 mg/kg b.w.) on protein expressions of PI3K, p-Akt and GLUT2 in the liver of normal and diabetic treated experimental rats were depicted by immunoblotting (Figure 1). The expressions of PI3K, p-Akt and GLUT2 proteins were significantly down regulated expression (p<0.05) in diabetes as compared to the control rats. Upon treatment with cucurbitacin (5 mg/kg b.w.) and glibenclamide (600 μg/kg b.w.) to diabetic rats, the PI3K, p-Akt and GLUT2 protein levels were significantly up regulated expression (p<0.05) in the liver of diabetic rats as compared to control rats. The photomicrographs of immunohistochemistry of IR, p-Akt and GLUT2 in liver of control and experimental rats were represented in Figure 2. Streptozotocin (40 mg/kg b.w) induced diabetic rats show down regulated expression of IR and p-Akt when comparable with normal control rats. Upon treatment with cucurbitacin (5 mg/kg b.w.) and glibenclamide (600 μg/kg b.w.) showed relatively upregulated expressions when compared with diabetic control rats. The protein expression of GLUT2 significantly decreased (p<0.05) in diabetic rats when compared with
normal control rats. However, the administration of cucurbitacin (5 mg/kg b.w.) and glibenclamide (600 μg/kg b.w.) showed upregulated GLUT2 expression in the liver. Photomicrographs of immunohistochemistry of GLUT2 in the pancreas of control and experimental rats were depicted in Figure 3. The protein expression of GLUT2 down regulated in STZ induced diabetic rats when compared with normal control rats. However, the administration of cucurbitacin (5 mg/kg b.w.) and glibenclamide (600 μg/kg b.w.) showed upregulated GLUT2 expression in the pancreas. The photomicrographs of Masson trichrome and Picrosirius red staining in the liver collagen of control and experimental rats illustrated in Figure 4 and 5. Masson trichrome and Picrosirius red colour were increased based on collagen accumulation in the hepatocytes of diabetic rats. Upon administration with cucurbitacin (5 mg/kg b.w.) and glibenclamide (600 μg/kg b.w.) to diabetic rats showed the reduced collagen deposition in hepatocytes when compared to diabetic rats. Diabetic rat showed extensive area of increased collagen deposition. However, the diabetic rats treated with cucurbitacin (5 mg/kg bw) and glibenclamide (600 μg/kg b.w.) showed decreased interstitial collagen accumulation when compared to diabetic rats.

(a) immunoblot analysis

![Immunoblot Analysis](image)

**Figure 1: Effect of cucurbitacin on insulin signaling protein (PI3K, pAkt and GLUT2) expression of liver of normal and experimental rats by immunoblotting techniques.**

Lane: A- Normal control; B- Normal with cucurbitacin (5 mg/kg b.w.); C- Diabetic control; D- diabetic with cucurbitacin (5 mg/kg b.w.); E- diabetic with glibenclamide (600 μg/kg b.w.).
The histogram quantification values indicate mean ± SD of six independent experiments. One-way ANOVA is followed and statistically significant differences (p < 0.05) between the groups were determined by Dunnett's post-hoc test. aDiabetic control rats were compared with normal rats; bCucurbitacin-treated diabetic rats were compared with diabetic control rats; cGlibenclamide-treated diabetic rats were compared with diabetic control rats.

Figure 2: Effect of Cucurbitacin on IR, p-Akt and GLUT2 protein expression in the liver of control and experimental rats by immunohistochemistry.

A-Normal control; B- Normal with cucurbitacin (5 mg/kg b.w.); C- Diabetic control; D-diabetic with cucurbitacin (5 mg/kg b.w.); E- diabetic with glibenclamide (600 μg/kg b.w.).
Figure 3: Effect of Cucurbitacin on GLUT2 protein expression in the pancreas of control and experimental rats by immunohistochemistry.

A-Normal control; B- Normal with cucurbitacin (5 mg/kg b.w.); C- Diabetic control; D-diabetic with cucurbitacin (5 mg/kg b.w.); E- diabetic with glibenclamide (600 μg/kg b.w.).

Figure 4: Photomicrographs of Masson’s trichrome staining of liver tissues of control and experimental rats.

A-Normal control; B- Normal with cucurbitacin (5 mg/kg b.w.); C- Diabetic control; D-diabetic with cucurbitacin (5 mg/kg b.w.); E- diabetic with glibenclamide (600 μg/kg b.w.).
Figure 5: Photomicrographs of Picrosirius red staining of liver tissues of control and experimental rats.

A-Normal control; B- Normal with cucurbitacin (5 mg/kg b.w.); C- Diabetic control; D- diabetic with cucurbitacin (5 mg/kg b.w.); E- diabetic with glibenclamide (600 μg/kg b.w.).

DISCUSSION

Insulin induced glucose uptake is a sequential process involving many proteins which are activated by phosphorylation reaction. Insulin stimulates the activation of the insulin receptor tyrosine kinase through autophosphorylation. This leads to stimulate IRS proteins, followed by activation of PI3K/Akt and subsequent translocation of GLUT and glucose uptake by the cells.\textsuperscript{23} To explore the effect of cucurbitacin on insulin sensitivity in terms of the cascade proteins in the insulin signaling pathway in pancreas and liver was determined. The PI3K-Akt pathway act as a central part in insulin signal transduction,\textsuperscript{24} which are responsible for the mainstream of metabolic actions of insulin specifically in muscle, liver, pancreas and adipose tissue\textsuperscript{25} and is activated after IRS tyrosine phosphorylation upon stimulation by insulin. In diabetes, translocation of protein kinase B/Akt to the plasma membrane is altered due to impaired insulin.\textsuperscript{26} This mechanism proposes that decreased responsiveness to the insulin in the liver may have resulted in a fall in Akt activation. Moreover, our data revealed a significant down regulation in GLUT2 protein expression in diabetic rats. In the present study, oral administration of cucurbitacin in diabetic rats showed up-regulated the Akt protein
expression in pancreas and liver tissue. From this, we conclude that a possible link between Akt activation and GLUT2 synthesis.

GLUT2 has known to be a glucose sensor as it is mostly responsible for glucose homeostasis through its role in pancreatic β-cell and glucose uptake and disposal by the liver. In diabetic rats, reduced glucose-stimulated insulin secretion always accompanied with reduced expression of GLUT2. However, GLUT2 expression could be restored and normalized by the synthesis of insulin. Alteration in the expression of GLUT2 has been implicated in the reduction of the secretory response to glucose. Our previous study demonstrated that cucurbitacin ameliorates the hyperglycemia in diabetic rats. In the present study, there was an increase in the expression level of GLUT2 in cucurbitacin treated diabetic groups. Cucurbitacin was effective in restoring GLUT2 levels towards normal control level, which might be responsible for increased glucose uptake and release of glucose in liver and pancreas. Our present study was associated with a previous study that oleanolic acid, improves glucose metabolism in diabetic rats by upregulating GLUT2 protein, leading to increased glucose uptake in liver and pancreas. Hence, the administration of cucurbitacin may play a vital factor to show antihyperglycemic potential through PI3K-Akt-GLUT2 sequential pathways.

In the current study, we performed the immunohistochemical analysis of protein expression IR, p-Akt, and GLUT2 on liver and pancreatic tissue of normal and experimental rats. There was a significant fall in insulin immunoreactivity and the number of immunoreactive β cells in diabetic rats which is due to STZ administration. However, the administration of cucurbitacin to diabetic rats, the immunoreactivity was improved based on the observations, and these present study was positively correlated with the previous study. In diabetes, chronic hyperglycemia can cause tissue injury. Consequently, pathological response to tissue injury causes fibrosis in the organs. Previous studies have shown that glucose plays an important role in fibrosis by the involvement in accumulation of collagen in tissues through the increase in collagen synthesis at the transcriptional level. Alterations in liver functions are more common among diabetic patients, and many symptoms in the liver have been reported, including changes in the amount of collagen fibers. Diabetic rats showing extensive area of fibrosis and collagen production which is the main cause of histological changes in the STZ induced diabetic rats. Oral treatment with cucurbitacin protects the above abnormalities by suppressing the effects of STZ. Upon treatment with cucurbitacin, insulin
signaling pathway may offer information for molecular mechanism of anti-hepatofibrosis and by that it prevents further damage to the liver.

CONCLUSIONS
In conclusion, this study has shown that cucurbitacin has prominent stimulatory effects on insulin secretion in pancreas via increased protein the expression of GLUT2. These data suggest that cucurbitacin modulates glucose uptake in liver via inducing GLUT2 synthesis through $p$-Akt activation in hepatocytes of diabetic rats.

REFERENCES


