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Plasma Glucose-lowering Effect of Thujone and its Molecular Mechanisms of Action in Streptozotocin-induced Diabetic Rats

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ABSTRACT

Thujone, an ingredient of essential oils of many different medicinal herbs, has been demonstrated to have blood-glucose-lowering effect in various diabetic models; however, the underlying cellular hypoglycemic mechanism(s) of thujone action remains to be fully defined. This study, therefore, was designed to explore thujone's underlying hypoglycemic mechanisms using in vivo diabetic model. Male Wister rats were rendered diabetic by a single intraperitoneal injection of Streptozotocin (STZ) (55 mg/kg). Thereafter, rats were divided into four groups (non-diabetic control, diabetic control, non-diabetic with thujone and diabetic with thujone) and were orally given either thujone (60 mg/kg) or vehicle for 4 weeks. Increased plasma glucose, impaired Oral Glucose Tolerance Test (OGTT), decreased plasma insulin, impaired GLUT4 translocation and decreased AMPK and PI3K phosphorylation were recorded in diabetic control rats. After thujone administration, plasma glucose level and glucose tolerance, as estimated by OGTT were improved, whereas the plasma insulin level remained down-regulated. Moreover, thujone markedly restored the impaired GLUT4 translocation and fully ameliorated AMPK phosphorylation, while PI3-K phosphorylation remained inhibited. These results suggest that thujone has a high potentiality to induce hypoglycemia, at least in part, via the AMPK-dependent mechanism involving restoration of GLUT4 translocation.

Keywords: Hyperglycemia, Thujone, Glut4, PI3K, Akt, Skeletal muscle

INTRODUCTION

Diabetes is a kind of metabolic disorder, characterized by prolonged hyperglycemia, and occurs mainly as a result of a lack of insulin secretion and/or failure of muscle, fat or liver cells to respond properly to insulin to maintain glucose homeostasis [1]. Among the three insulin responsive tissues, skeletal muscle represents a crucial site for controlling glucose homeostasis [2] as approximately 75%-80% of the entire glucose entering circulation is removed by this tissue. As a result, controlling glucose uptake in skeletal muscle may competently resolve insulin resistance and its associated hyperglycemia.

In skeletal muscle, glucose uptake is mediated by GLUT4 translocation, a process that is highly regulated by insulin [2,3] through Phosphoinositol-3-kinase (PI3K). This enzyme, which represents one of the earliest critical events in insulin signaling pathway, facilitates the recruitment and activation of various post-receptor insulin signaling proteins involved in the recruiting of GLUT4 to the cell surface, among them is the Akt [3-6].

In addition to the insulin signal pathway, adenosine monophosphate–activated protein kinase (AMPK) is another important signaling molecule that promotes, independently from insulin, intracellular glucose uptake by a GLUT4 involved process [7,8]. Since AMPK signaling system is not disturbed in insulin-resistance states [9,10]. Therefore, one of the main strategies critically important for seeking solutions to diabetes and metabolic disease is improving the functionality of this signaling pathway.

The currently used antidiabetic agents like thiazolidinedione's are not completely effective particularly in preventing diabetic complications, together with the fact that the disease is attaining epidemic proportions justify the search for new and alternative drugs that help in treatment and preferably also in preventing the progression of this disease [11,12]. The complementary and alternative medicine approaches that include natural compounds may be promising and valuable therapeutic drugs in this regard.

It had previously been reported that thujone, an ingredient of essential oils of different herbs [13,14], exhibits a blood-glucose-lowering effect *in*

vivo that was attributed to increased hepatic glycogen synthesis via Akt/GSK-3β pathway. Therefore, the primary purpose of this study was to evaluate whether the hypoglycemic action of thujone *in vivo* could also be attributed to enhancing glucose disposal in skeletal muscle.

MATERIALS AND METHODS

Materials

All reagents were purchased from Sigma-Aldrich (Germany) unless otherwise noted. Antibodies were obtained from a variety of sources: Antiphospho-AMPK Thr 172 and anti-AMPK from Upstate (Lake Placid, NY); anti-GLUT4 from Chemicon International (Temecula, CA); Goat-anti-rabbit secondary antibodies from Chemicon International; Donkey-anti-rabbit secondary antibody from Amersham Biosciences (Oakville, Ontario, Canada).

Experimental animals

Male Wister rats (150-180 g) were housed under well hygienic conditions of 23 ± 2°C with 12 h light/12 h dark cycles. The animals consumed normal laboratory diet and water was provided ad libitum. All experimental procedures carried out in accordance with the animal experimentation ethics committee at the Hashemite University, where the research was conducted.

Induction of diabetes mellitus

Diabetes was induced in overnight fasted rats by a single Intraperitoneal (i.p) injection of 55 mg/kg STZ freshly dissolved in cold citrate buffer (0.1 M, pH 4.5) [15]. Normal control animals were injected with the citrate buffer vehicle. The STZ diabetic and control groups received an equal volume of the vehicle (1 ml physiological saline). To prevent fatal hypoglycemia that might be induced by STZ, the rats were supplied with 10% of glucose solution after 6 h of STZ administration for the next 24 h. One week after STZ injection, blood samples were collected from rat tail vein and blood glucose level was measured by the use of glucometer (ACCU-CHECK Active kit, Roche Diagnostics, Mannheim, Germany). Only those animals that exhibited a fasting blood glucose level above 200 mg/dl were used as diabetic rats for further study. The day on which hyperglycemia had been confirmed was designated as day 0.

Experimental design and tissue collection

The rats were randomly divided into four groups of 6 rats each as follows: non-diabetic group (ND), non-diabetic group administered with thujone (ND+T), diabetic group (D) and diabetic group administered with thujone (D+T). Thujone (60 mg/kg body weight) was administered orally once a day for 4 weeks. The dosage of thujone was determined based on a pilot acute oral toxicity study (data not shown).

At the end of the experiment, rats were fasted for 12 h and tail vein blood sample was collected for determination of plasma insulin and glucose levels. Thereafter, rats were sacrificed and soleus muscle was isolated gently, cleaned free of adipose and connective tissues, as well as blood, and frozen immediately for further analysis.

Measurement of blood glucose levels

Blood glucose levels were determined every week after 12 h of fasting using glucometer (ACCU-CHECK Active kit, Roche Diagnostics, Mannheim, Germany).

Measurement of blood insulin levels

Plasma insulin levels were measured as described previously [15]. Briefly, insulin was determined in duplicate using commercial ELISA kits (Crystal Chem Inc.; Downers Grove, IL, USA). Standards were run in parallel with the samples. The concentrations of insulin were calculated in reference to the corresponding standard curves.

Oral Glucose Tolerance Test (OGTT)

The OGTT was performed at the end of the experiment time (4th week). Prior to OGTT rats were fasted overnight (12 h). Thirty min following the various treatments programs, rats were orally given glucose at 2 g/kg of body weight. Subsequently, tail vein blood glucose levels were measured at 0 (prior to glucose load), 30, 60, 90 and 120 min after glucose administration.

Plasma membrane preparation

Plasma membranes were obtained from giant sarcolemmal vesicles, as described previously [16,17]. Briefly, muscle tissues were cut into thin layers (1-3 mm thick) with scalpel and then were incubated for 1 h at 34°C in 140 mM KCl-10 mM MOPS (pH 7.4), aprotinin (30 µg/ml) and collagenase (Type VII, 150 U/ml) in a shaking water bath. At the end of the incubation, the supernatant fraction was collected and the remaining muscle debris washed with KCl/MOPS and 10 mM Ethylenediaminetetraacetic Acid (EDTA), which resulted in a second supernatant fraction. The resulting supernatant fraction was pooled with the first. Percoll and aprotinin were added to the collected medium. The resulting suspension was placed at the bottom of a density gradient consisting of a 3 ml middle layer of 4% Nycodenz (wt/vol) and a 1 ml KCl-MOPS upper layer. This sample was then centrifuged at 60 g for 45 min at room temperature. Subsequently, the vesicles were then harvested from the interface of the upper and middle layers, diluted in KCl-MOPS, and re-centrifuged at 12,000 g for 5 min. The supernatant fraction was aspirated and the resulting pellet was re-suspended in KCl/MOPS. Vesicles were stored at −70°C for subsequent analysis.

Protein analysis, extraction and western blotting

he muscle was homogenized on ice in 2 ml homogenizing buffer. After homogenization, the solution was sonicated (5 sec) and set to rock end over end for 30 min at 4°C. The solution was centrifuged at 1,500 g for 15 min at 4°C and the supernatant collected. The homogenate and plasma membrane protein concentrations were analyzed for total using the bicinchonic acid assay. Proteins were separated using SDS-polyacrylamide gel electrophoresis and were detected using Western blotting, as has been reported previously [16,17].

Statistics

The All data are reported as mean ± SEM of six rats per group and statistical significance was evaluated by one-way ANOVA, followed by the post hoc test to determine statistical significance at selected points. Values were considered statistically significant when $P < 0.05$.

RESULTS

Effect of thujone on fasting blood glucose levels

During the experimental period, fasting blood glucose levels remained significantly (Figure 1; P <0.05) elevated in STZ-diabetic rats compared to non-diabetic control. However, treated STZ-diabetic rats with thujone induced a gradual and progressive reduction in blood glucose levels compared to STZ-diabetic rats (Figure 1; P <0.05). Notably, after the 3rd week of treatment, blood glucose levels in STZ-diabetic rats treated with thujone reached values similar to that in non-diabetic control rats (Figure 1; P >0.05). Thereafter, thujone treatment did not further reduce blood glucose level (Figure 1; P > 0.05). In non-diabetic rats, administration of thujone did not affect blood glucose levels (Figure 1; P >0.05) compared to the non-diabetic control that had not been treated with thujone.

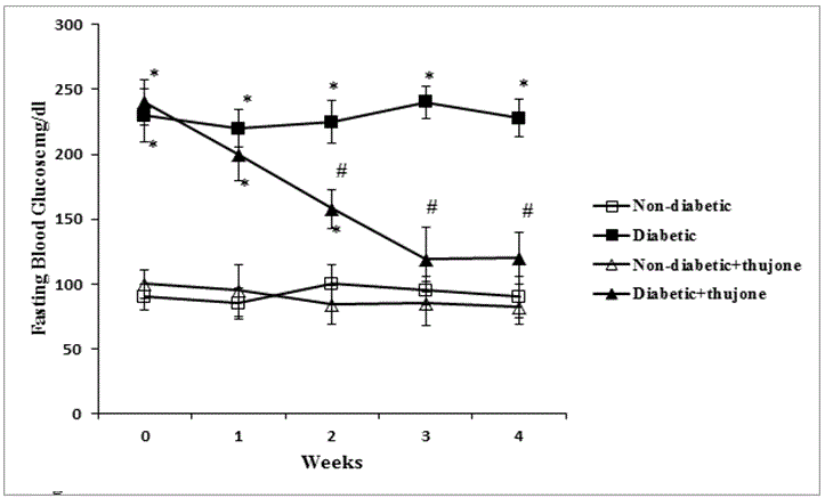

Figure 1: Effect of thujone treatment (4 weeks) on plasma glucose levels. Values are means ± SE (n=6)
*Significantly different from non-diabetic (P <0.05); #Significantly different from diabetic (P <0.05).

Effect of thujone on OGTT

Blood glucose reached its highest level at 30 min in all groups after the glucose load (2 g/kg body weight) and then gradually declined (Figure 2). Blood glucose levels in the STZ-diabetic rats were significantly (Figure 2; P <0.05) higher than those of the non-diabetic control, indicating glucose intolerance in STZ-diabetic rats. Administration of thujone displayed an intermediate improvement in the OGTT response between non-diabetic and STZ-diabetic control.

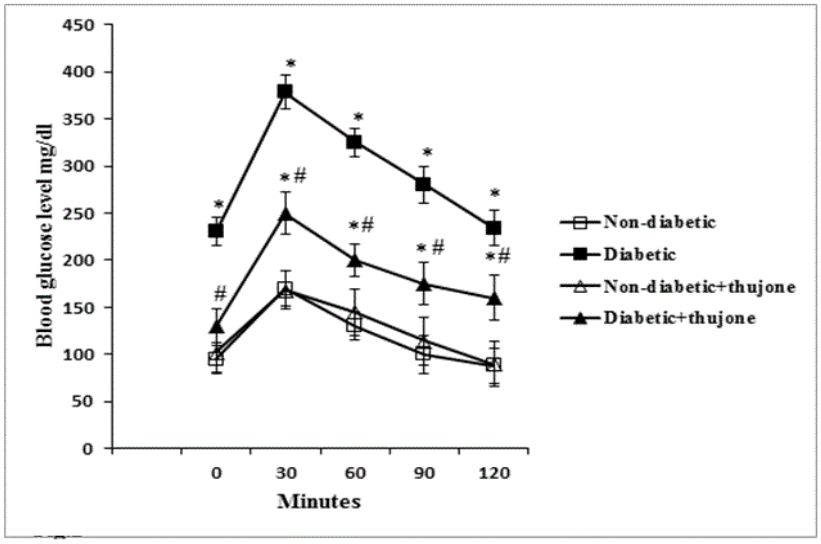

Figure 2: Effect of thujone treatment (4 weeks) on oral glucose tolerance test (OGTT). OGTT was carried out at the 4th week of the experiment period. Prior to OGTT rats were fasted overnight (12 h). Blood was taken from the tail vein at 0, 30, 60, 90 and 120 min after the oral glucose (2 g/kg body weight) administration. Data are means ± SE (n=6)
*Significantly different from non-diabetic (P <0.05); #Significantly different from diabetic (P <0.05).

Effect of thujone on blood insulin levels

Blood insulin level was decreased significantly (Figure 3; P <0.05) in STZ-diabetic rats compared to non-diabetic control. The plasma insulin levels in non-diabetic control maintained constant during the experimental time. Treatment with thujone exhibited no (Figure 3; P >0.05) changes in the blood insulin levels compared to STZ-diabetic rats.

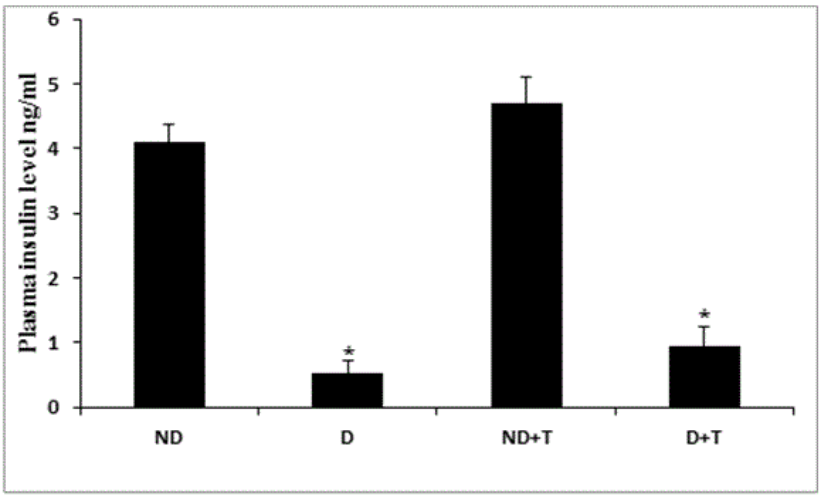

Figure 3: Effect of thujone treatment (4 weeks) on the plasma insulin levels. Values are means± SE (n=6)
*Significantly different from ND and ND+T (P <0.05); ND: Non-diabetic, D: Diabetic, ND+T: Non-diabetic+thujone, D+T: Diabetic+thujone

Effects of thujone on plasmalemmal GLUT4 translocation

The plasmalemmal Glut4, in non-diabetic control and in non-diabetic control treated with thujone, was the same (Figure 4; P > 0.05). In contrast, the plasmalemmal Glut4 in STZ-diabetic rats was decreased (Figure 4; P <0.05). The Glut4 appearance at the plasma membrane of STZ-diabetic rats treated with thujone was completely ameliorated to the levels seen in non-diabetic control (Figure 4; P >0.05). None of the treatment programs affected the expression level of Glut4 protein (Figure 4; P >0.05).

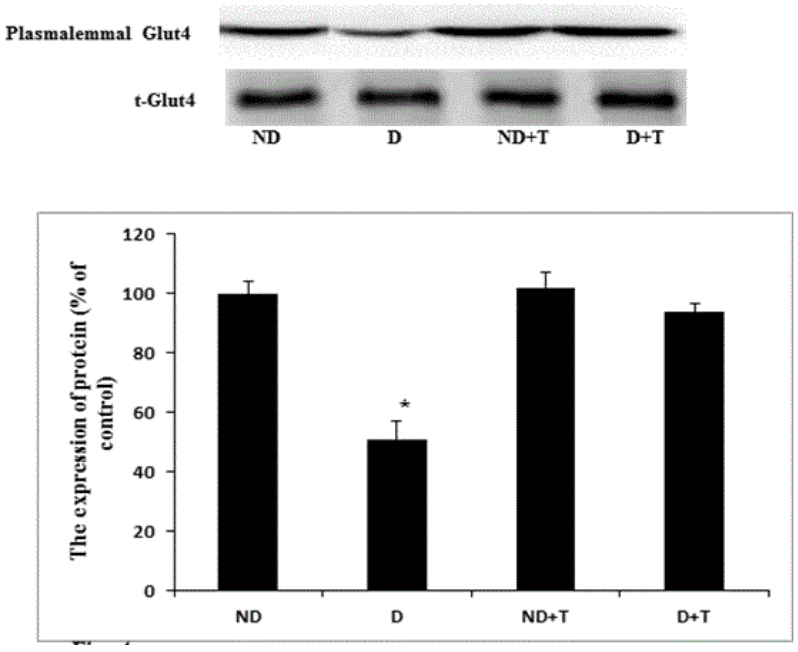

Figure 4: Effect of thujone treatment (4 weeks) on Glut4 translocation (Plasmalemmal Glut4) in skeletal muscle of rats. Values are means ± SE (n=3 independent experiments for each data point were based on pooled muscle samples from 3 animals). 30 µg of homogenate and 20 µg of plasma membrane protein were loaded
*Significantly different from other experimental treatments (P < 0.05); ND: Non-diabetic; D: Diabetic; ND+T: Non-diabetic+thujone; D+T: Diabetic+thujone

Effects of thujone on AMPK phosphorylation

The levels of AMPK phosphorylation were decreased in STZ-diabetic rats compared with non-diabetic control (Figure 5; P < 0.05). However, in STZ-diabetic rats treated with thujone, the phosphorylation levels of AMPK were fully rescued (Figure 5; P < 0.05). This increase in AMPK phosphorylation after thujone treatment was similar to that seen in non-diabetic control. Comparison to non-diabetic control, the phosphorylation of AMPK in non-diabetic rats treated with thujone was not altered (Figure 5; P > 0.05). The total expression of AMPK did not change with any of the treatment scenarios (Figure 5; P > 0.05).

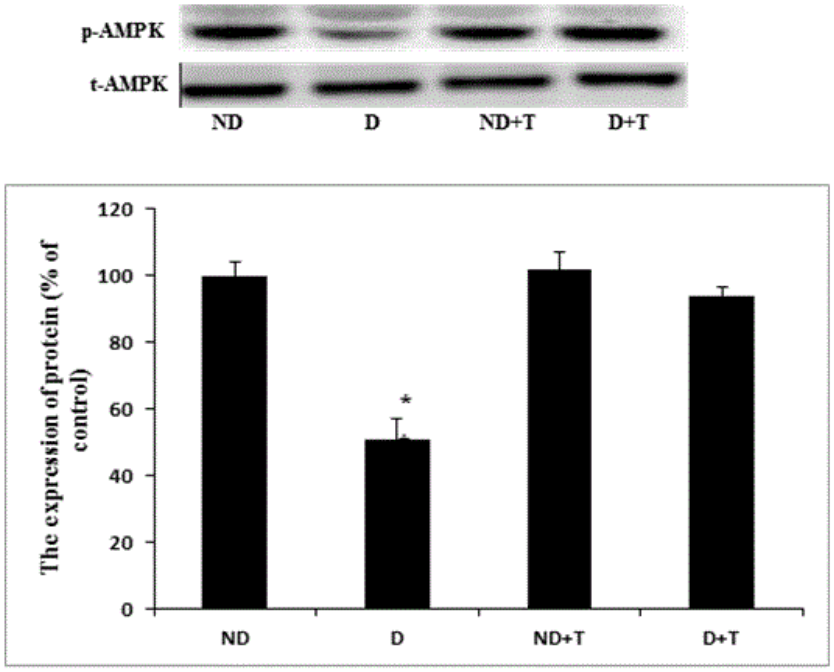

Figure 5: Effect of thujone (4 weeks) on AMPK phosphorylation (p-AMPK*α*) in skeletal muscle of rats. Values are means ± SE. Equal quantities of protein were loaded for each muscle at each time point, and loading was verified with Ponceau staining

*Significantly different from other experimental treatments (P < 0.05); ND: Non-diabetic, D: Diabetic, ND+T: Non-diabetic+thujone, D+T: Diabetic+thujone

Effects of thujone on PI3K phosphorylation

In the STZ-diabetic rats, a decrease in PI3K phosphorylation level was detected compared with non-diabetic control (Figure 6; P < 0.05). Oral administration of thujone to STZ-diabetic rats, failed to reverse the above decrease in PI3K phosphorylation (Figure 6; P < 0.05). In non-diabetic control treated with thujone, the PI3K phosphorylation level remained unchanged when compared with the non-diabetic control that had not been treated with thujone (Figure 6; P > 0.05). Of further note, the total protein expression of PI3K remained unchanged by any of the experimental treatments (Figure 6; P > 0.05).

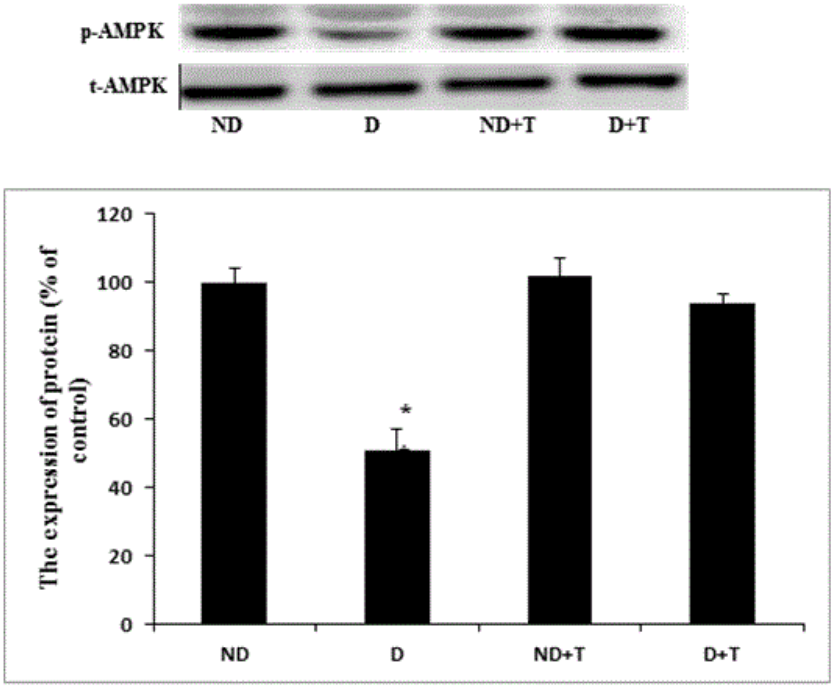

Figure 6: Effect of thujone (4 weeks) on PI3K phosphorylation (p-PI3K) in skeletal muscle of rats. Values are means ± SE. Equal quantities of protein were loaded for each muscle at each time point and loading was verified with Ponceau staining

∗Significantly different from other experimental treatments (P < 0.05); ND: Non-diabetic, D: Diabetic, ND+T: Non-diabetic+thujone, D+T: Diabetic+thujone

DISCUSSION

In this study, we adopted a small dose intraperitoneal injection of STZ (55 mg/kg) to induce an animal model of diabetes. The successful diabetic animal model showed, similar to the results of previously reported studies [18,19], hyperglycemia, impaired glucose tolerance, low levels of insulin, and reduced Glut4 content at muscle cell surface. This marked impairment in Glut4 translocation was closely associated with impairment in AMPK and PI3K phosphorylation. Therefore, STZ –induced diabetes is a suitable model to address hypoglycemic properties of thujone and its mechanistic approach.

The present study has provided novel findings on the therapeutic effects of thujone on STZ-induced diabetes and on its mechanism of action *in vivo*. More scinetifically, we found that thujone completely alleviated (i) hyperglycemia induced by STZ effectively by improving glucose tolerance, as estimated by OGTT. This marked improvement in hyperglycemia was closely associated with the complete restoration of (ii) Glut4 translocation and (iii) AMPK phosphorylation's. Our data further found that (iv) the plasma insulin level in STZ-diabetic rats remained inhibited, this finding was in line with one previous study [15], implying that STZ treatment reduced β cell mass, which followed by insulin deficiency, in turn, thus resulting in hyperglycemia. Collectively, it implies that thujone exhibited a protective effect against the diabetogenic activity of STZ mainly by an extra pancreatic mechanism.

Skeletal muscles are now widely considered to be a key, and possibly the most important tissue that accounts for whole body carbohydrate homeostasis. Indeed, skeletal muscle metabolizes ~ 85% of glucose load that enters the circulation [2]. Therefore, skeletal muscle tissues are usually used as a therapeutic target in the battle against diabetes and insulin resistance.

Our result that STZ reduced Glut4 at the plasma membrane is consistent with results from other studies [20,21]. Treatment with thujone exerted a significant increase in Glut4 translocation in skeletal muscle to a level that was comparable to non-diabetic control, suggesting that the antihyperglycemic effect of thujone could be related to increased glucose uptake by skeletal muscle. This result of thujone induced increased Glut4 appearance at the plasma membrane of skeletal muscle was in accordance with only one study [22], in which it has been demonstrated that thujone fully rescued palmitate -induced insulin resistance by partially restored Glut4 translocation in skeletal muscles.

To examine the mechanisms through which thujone mediated Glut4 translocation from an intracellular deposit site to the plasma membrane of skeletal muscle, we assessed key intracellular component involved in this process namely the AMPK [7,8] and/or PI3K [3,4]. Our STZ-induced diabetic rats exert a decrease in PI3K phosphorylation, suggesting that insulin deficiency results in this subsequent decrease in PI3K phosphorylation. Thujone treatment failed to improve the decreased PI3K phosphorylation. On the other hand, our data, consistent with previous studies [18,19], confirmed that STZ- induced diabetes is associated with impairment in AMPK phosphorylation. We also found that these impairments in AMPK phosphorylation were completely prevented by thujone treatment. To the best of our knowledge, such an effect of thujone on AMPK phosphorylation has previously been reported in only one other study [22]. This restorative effect of thujone on AMPK phosphorylation was highly correlated with the surface GLUT4. Therefore, the protective effect of thujone against the STZ-induced hyperglycemia observed herein could be due to the enhancement of AMPK phosphorylation, which in turn, contributed to the lowering of circulating glucose. However, further studies will be necessary to ascertain how the phosphorylation of AMPK underlies the action of thujone to increase sarcolemmal Glut4 contents.

CONCLUSION

In conclusion, these findings indicate that thujone possesses a potential for preventing STZ-induced hyperglycemia and ameliorating glucose tolerance through promoting GLUT4 translocation and enhancing glucose uptake by AMPK-dependent signaling pathway in skeletal muscle. Our study also suggests that thujone could be a promising alternative treatment for the prevention of hyperglycemia and diabetes mellitus.

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