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Myrtenal ameliorates hyperglycemia by enhancing GLUT2 through Akt in the skeletal muscle and liver of diabetic rats

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ABSTRACT

Insulin signaling pathway is an important role in glucose utilization in tissues. Our Previous study has established that myrtenal has antihyperglycemic effect against diabetic rats. The aim of this study was to explore the molecular mechanism of myrtenal in Streptozotocin-induced diabetic rats. Experimental diabetes was induced by single intraperitoneal injection of Streptozotocin (STZ) (40 mg/kg bw) in Wistar albino rats. Diabetic rats were administered myrtenal (80 mg/kg bw) for a period of 28 days. Diabetic rats showed an increased the levels of plasma glucose, decreased the levels of plasma insulin, down-regulation of insulin receptor substrate 2 (IRS2), Akt and glucose transporter 2 (GLUT2) in liver and insulin receptor substrate 2 (IRS2), Akt and glucose transporter 4 (GLUT4) protein expression in skeletal muscle. However, myrtenal treated diabetic rats revealed decreased the levels of plasma glucose, improved the plasma insulin levels, up-regulation of IRS2, Akt and GLUT2 in liver and IRS2, Akt and GLUT4 protein expression in skeletal muscle. The up-regulation of glucose transporters enhances the glucose uptake in liver and skeletal muscle. The histopathology and immunohistochemical analysis of the pancreas also corroborates with the above findings. Our findings suggest that myrtenal could be a potent phytochemical in the management of diabetes.

1. Introduction

Diabetes mellitus (DM) is characterized by increased blood glucose levels and insufficiency of insulin secretion/action. Such setting causes the impairment of glucose uptake in the peripheral tissues and reduced the glucose consumption for energy purposes. Type 2 diabetes mellitus (T2DM) is due to impaired signaling resultant to the binding of insulin to its receptor. As a consequence, insulin activated signaling proteins, such as insulin receptor substrate IRS-1 and IRS-2 are attenuated in many tissues including liver, skeletal muscle and kidney [1,2].

Glucose transporter (GLUT4) is a rate limiting factor for glucose utilization in skeletal muscle, and Akt (protein kinase B) is a fundamental mediator of insulin induced GLUT4 translocation from cytosol to membrane [3]. These proteins have been involved in the pathogenesis in STZ-induced diabetic rats [4,5]. The present study was undertaken to investigate the effect of myrtenal on insulin signaling cascade proteins in skeletal muscle and liver of Streptozotocin-induced diabetic rats.

2. Material and methods

2.1. Animals

Adult male albino Wistar rats (180–200 g) were obtained from the Central Animal House, Department of Experimental Medicine, Rajah Muthiah Medical College and Hospital, Annamalai University and maintained at a constant temperature (25 ± 1 °C) on a 12 h light/12 h dark cycle with standard pellet diet (National Institute for Nutrition, Hyderabad, India) and water was provided *ad libitum*. The experimental protocol was approved by the animal ethics committee of Rajah Muthiah Medical College and Hospital (Reg. No. 160/1999/CPCSEA, Proposal number: 1000), Annamalai University.

2.2. Chemicals

Myrtenal (PubChem Substance ID: 24853054) and Streptozotocin (STZ) (PubChem Substance ID: 24899428) were purchased from Sigma-Aldrich (St. Louis, MO, USA). The Primary antibody was

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purchased from Santa-Cruz Biotechnology, Inc., USA. All other chemicals used in this study were of analytical grade obtained from Merck and Hi Media, India.

2.3. Induction of diabetes in rats

Diabetes mellitus was induced in overnight fasted experimental rats by a single intraperitoneal injection (ip) of STZ (40 mg/kg bw) dissolved in 0.1 M citrate buffer at pH 4.5. STZ injected animals were given 20% glucose solution for 24 h to prevent initial drug-induced hypoglycemia mortality. STZ injected animals exhibited hyperglycemia within a few days. Diabetic condition rats were confirmed by measuring the elevated fasting plasma glucose (by glucose oxidase method) 72 h after STZ injection. The rats with fasting blood glucose above 250 mg/dL were considered to be diabetic and used for the experiment.

2.4. Treatment schedule

The rats were divided into four groups, each comprising of six rats ($4 \times 6 = 24$ rats). Those rats with blood glucose above 250 mg/dL were considered as diabetic. Myrtenal was dissolved in 1 mL of corn oil and administered orally to the experimental rats using an intragastric tube daily for a period of 28 days [6].

Group I: normal control rats.
Group II: normal + myrtenal (myt) (80 mg/kg bw/day).
Group III: diabetic control rats.
Group IV: diabetic + myrtenal (myt) (80 mg/kg bw/day).

At the end of the experimental period, all the animals were fasted overnight, anesthetized and sacrificed by cervical dislocation. Blood samples were collected in tubes containing potassium oxalate and sodium fluoride (3:1) mixture for the estimation of plasma glucose. The liver and skeletal muscle were dissected out, washed with ice-cold saline and stored at −80 °C until used. The liver tissue was weighed and 10% tissue homogenate prepared with 0.1 M Tris–HCl buffer, pH 7.4. After centrifugation, the clear supernatant was obtained and used for various assays.

2.5. Assessment of glucose and insulin

Glucose was estimated by the method of Trinder using a commercial kit [7]. Plasma insulin content was analyzed multi-well plate reader using the rat insulin ELISA kit, Mercodia, Uppsala, Sweden, according to manufacturer instructions.

2.6. Western blot analysis

Briefly, 100 mg liver and skeletal muscle were homogenized in RIPA buffer and protease inhibitor cocktail (Sigma) using a polytron equipped homogenizer at a precise low setting on ice. The homogenate was centrifuged at 10,000 *g* for 10 min 4 °C and then the supernatant was collected. The resultant supernatant was sampled as the protein for IRS2, Akt, GLUT2 and GLUT4. Protein concentration was estimated by Lowry et al. (1951) [8] using bovine serum albumin (BSA) as a standard. Briefly, each sample (50 μg) was subjected to heat denaturation at 94 °C for 5 min with Laemmli buffer. Proteins were resolved by SDS-PAGE on 10% polyacrylamide gels and then transferred to PVDF membrane (purchased from sigma). The membrane was blocked with 5% BSA blocking buffer in TBS-T (Tris-buffered saline and Tween 20) for 2 h at room temperature. The membrane was incubated with primary antibody IRS2, Akt, GLUT2 and GLUT4 (Santa Cruz, Biotechnology) as per the instructions from the manufacturer and incubated overnight at 4 °C. The membrane was subjected to repeated wash for 3 times with TBS-T and then incubated for 1 h in horse radish peroxidase (HRP)-conjugated rabbit secondary antibody by 1:7500 dilutions in TBS-T. The membrane was again subjected to repeated wash for 3 times with TBS-T. Protein bands were visualized in WesternSure Premium Chemiluminescent Substrate (LI-COR). Densitometry was performed on C-DIGIT blot Scanner and Image J software. Later, the membranes were incubated in stripping buffer (50 ml, containing 62.5 mM Tris HCl (pH 6.7), 1 g SDS and 0.34 ml β-mercaptoethanol) at 55 °C for 40 min. Following this, the membrane was re-probed using β-actin antibody (1:5000).

2.7. Histological observation

Pancreas was instantly dissected out, excised and rinsed in ice-cold saline solution. A portion of pancreas was fixed in 10% neutral buffered formalin fixative solution. After fixation tissues were embedded in paraffin, solid sections of 4–5 μm thickness were made by using a rotary microtome. The sections were stained with hematoxylin-eosin and histological observations made under a light microscope (40×).

2.8. Immunohistochemical observation

Immunohistochemical staining performed on 5 μm thick paraffin pancreatic sections were deparaffinized with xylene and rehydrated with graded concentrations of isopropyl alcohol. Separated sections were processed. Slides were incubated overnight with anti-mouse insulin antibody (Sigma, Product No. I 2018) (1:200 dilutions). The slides were rinsed well with phosphate buffer and incubated with super enhancer reagent for 30 min. After rinsing with phosphate buffer, incubation was done with super-sensitive polymer-horseradish peroxidase immunohistochemistry detection system. Sections were washed with buffer and incubated with a 3, 3-diaminobenzidine solution for 5 min. Sections were stained with hematoxylin and observed under the light microscope.

2.9. Image analysis for insulin immunoreactivity

To assess the insulin immunoreactivity the intensity of the corresponding signals from the pancreatic tissue sections were measured. Above 10 islets in each rat group were randomly chosen

Table 1
Effect of Myrtenal on the levels of plasma glucose and insulin in normal and experimental rats.

Groups	Normal	Normal + Myrtenal (80 mg/kg)	Diabetic	Diabetic + Myrtenal (80 mg/kg)
Plasma glucose (mg/dL)	82.20 ± 2.40[a]	84.35 ± 1.96[a]	273.50 ± 7.97[b]	139.86 ± 4.80[c]
Plasma insulin (μU/mL)	16.92 ± 1.60[a]	17.45 ± 1.26[a]	5.94 ± 0.48[b]	13.86 ± 1.16[c]

Values are represented as means ± SD for 6 rats in each group. Values are not sharing a common superscript letter (a-c). Significance was accepted at $P < 0.05$ (DMRT).
[a] Normal and Normal + Myrtenal (80 mg/kg bw) significant when compared with Diabetic and Diabetic + Myrtenal (80 mg/kg bw) ($p < 0.05$).
[b] Diabetic rats significant when compared with Normal, Normal + Myrtenal (80 mg/kg bw) and Diabetic + myrtenal ($p < 0.05$).
[c] Diabetic + myrtenal (80 mg/kg bw) significant when compared with Diabetic, Normal and normal + myrtenal (80 mg/kg bw) ($p < 0.05$).