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Upregulation of Glut-4 and PPARγ by an isoflavone from *Pterocarpus marsupium* on L6 myotubes: a possible mechanism of action

R. Anandharajan[a], K. Pathmanathan[a], N.P. Shankernarayanan[c], Ram A. Vishwakarma[b,1], Arun Balakrishnan[a,*]

[a] *Centre For Biotechnology, Anna University, Chennai 600025, Tamil Nadu, India*
[b] *Bio-organic Chemistry Laboratory, National Institute of Immunology, JNU complex, New Delhi 110 067, India*
[c] *V.H.S Leprosy project, Shakthi Nagar, Erode 638315, Tamil Nadu, India*

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Abstract

The purpose of the present study is to analyse the influence of *Pterocarpus marsupium* methanolic extract and isolated *Pterocarpus marsupium* isoflavone on a battery of cellular targets Glut-4, PPARγ and PI3 kinase. *Pterocarpus marsupium* is an anti-diabetic plant indigenous to South India. Sequential extraction performed with different solvents were analysed for glucose uptake activity at each step. Fraction-9 showing maximum glucose activity on glucose uptake was purified by column chromatography and the structure was elucidated as 7-O-α-L-rhamnopyranosyl oxy-4′-methoxy-5-hydroxy isoflavone using NMR and mass spectroscopy. The significant glucose uptake showed by *Pterocarpus marsupium* crude and pure was comparable with insulin and rosiglitazone. Elevation of Glut-4 and PPARγ gene expression in parallel with glucose uptake supported the in vitro glucose uptake activity of *Pterocarpus marsupium* methanolic extract and *Pterocarpus marsupium* isoflavone. The inhibitory effect of cycloheximide on *Pterocarpus marsupium* methanolic extract and *Pterocarpus marsupium* isoflavone-mediated glucose uptake suggested that new protein synthesis is required for elevated Glut-4 protein expression. PI3 kinase plays an important role in glucose transport and activated by *Pterocarpus marsupium* methanolic extract but not the isolated pure isoflavone. Therefore, we postulate that the isoflavone from *Pterocarpus marsupium* may activate glucose transport by a PI3 kinase independent pathway, which require further analysis.

Keywords: *Pterocarpus marsupium*; Glucose uptake; L6 myotubes; PPARγ; PI3 kinase

1. Introduction

Natural products from medicinal plants continue to form a common platform for the discovery of new chemical entities in the modern drug discovery programmes. A wide array of plant derived active principles for possible use in the treatment of type 2 diabetes mellitus has been reported (Bailey and Day, 1989). Heartwood of *Pterocarpus marsupium* (family: leguminoceae) has been used in ayurvedic medicine for centuries for its anti-hyperglycemic activity. Earlier studies reported that the phenolic constituents of *Pterocarpus marsupium* significantly lowered the blood glucose level in diabetic rats (Manickam et al., 1997).

Glucose transport is the rate-limiting step in glucose utilisation in insulin targeted skeletal muscle. This transport is mediated by the major glucose transporter proteins, Glut-4 and 1 in skeletal muscle of streptozotocin induced diabetic rats (Ziel et al., 1988). Glut-1 and 4 were also shown to be expressed in human skeletal muscle by cloning and

Abbreviations: NIDDM, non insulin dependent diabetes mellitus; GLUT, glucose transporter; PI3 K, phosphatidylinositol 3′ kinase; PPARγ, peroxisome proliferator activator receptor gamma; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; DMEM, Dulbecco's modified eagles medium; FCS, fetal calf serum; HEPES, *N*-2-hydroxy ethyl piperazine-*N*′-2-ethane sulphonic acid; TLC, thin layer chromatography; RT-PCR, reverse transcriptase polymerase chain reaction; SDS, sodium dodecyl sulphate; AMLV, avian Moloney leukemic virus

* Corresponding author. Tel.: +91 44 22350772; fax: +91 44 22350299.
E-mail addresses: ram@nii.res.in (R.A. Vishwakarma), arunb@annauniv.edu (A. Balakrishnan).
[1] Tel.: +91 11 26703614; fax: +91 11 26162125.

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characterization of insulin responsive glucose transporters (Fukumoto et al., 1989**)**. Insulin resistance in type 2 diabetes is manifested by decreased insulin stimulated glucose transport and metabolism in adipocytes and skeletal muscle resulting in down-regulation of the major insulin-responsive glucose transporter, Glut-4 (Kellerer and Lammers, 1999).

Impaired Glut-4 translocation and reduced expression of PI3 kinase and PPARγ have been studied in detail under diabetic condition. Studies explaining the role of PI3 kinase and PPARγ in insulin signalling enumerate the upregulation of insulin-dependent glucose transport and Glut-4 translocation in cultured L6 myotubes (Tsakiridis et al., 1995; Ciaraldi et al., 1995).

PI3 kinase, a key molecular switch in insulin signalling cascade mediates the metabolic effects of insulin, glucose transport and Glut-4 translocation (Okada et al., 1994). PPARγ, a transcription factor belonging to the nuclear receptor superfamily (Desvergne and Wahli, 1999) is essential for adipocyte differentiation (Ntambi and Young-Cheul, 2000). Activation of PPARγ on its binding with agonists directly enhances insulin signalling and glucose uptake in muscle cells (Ciaraldi et al., 1995).

The current investigation was aimed to elucidate the mechanism of action of *Pterocarpus marsupium* at cellular level. The effect of *Pterocarpus marsupium* on glucose transport, PI3 kinase and PPARγ expression were analysed using L6 myotubes, which express similar glucose transport kinetic profile to that of adult muscle (Klip et al., 1982). Chromatographic separation techniques guided with in vitro based glucose uptake assay at each step lead to the isolation of *Pterocarpus marsupium* active molecule, the structure of which was elucidated as 7-O-α-L-rhamnopyranosyl oxy-4′-methoxy-5-hydroxy isoflavone.

2. Materials and methods

2.1. Chemicals and reagents

All cell culture solutions and supplements were purchased from Life Technologies Inc. All fine chemicals were obtained from Sigma–Aldrich, St. Louis. 2-Deoxy-D-[1-^{3}H] glucose and hybond C membrane were obtained from Amersham Pharmacia Biotech, UK. Trizol reagent and AMLV reverse transcriptase, dNTP, Taq polymerase was obtained from GIBCO BRL, USA and New England Biolabs, UK, respectively. GLUT-4 antibody was procured from Santa Cruz Biotechnology, USA. Insulin, cycloheximide and cytochalasin B were obtained from Sigma–Aldrich. Medicinal plants procured from a reliable source were authenticated by a taxonomist. TLC plates (60 F_{254} grade) were from Merck, Germany. Rosiglitazone was a kind gift from Dr. Reddy's Laboratories, Hyderabad. All other chemicals and organic solvents used were of the highest analytical grade. Primers were synthesised from GIBCO BRL.

2.2. Plant extraction

The dried plant powder (100 g) of *Pterocarpus marsupium* was extracted sequentially from non-polar to polar solvents namely hexane, dichloromethane, ethylacetate and methanol at room temperature. All of these extracts were rotaevaporated under reduced pressure and concentrated and final yield was approximately 1–2 g of each respective extract. One milligram of dried extracts were reconstituted to 1 ml of the respective parent solvent and serially diluted to make 10, 1, 100, 20 and 1 ng/ml as the final concentrations, used for the glucose uptake studies.

2.3. Cell culture of L6 myoblasts and myotubes

L6 cells, a differentiating monolayer myoblast culture (obtained from ATCC—CRL-1458) was maintained in DMEM with 10% FCS and supplemented with penicillin (120 units ml^{-1}), streptomycin (75 μg ml^{-1}), gentamycin (160 μg ml^{-1}) and amphotericin B (3 μg ml^{-1}) in 5% CO_2 environment. For differentiation of L6 cells they were transferred to DMEM with 2% FCS for 4–6 days post-confluence. The extent of differentiation was established by observing multinucleation of cells. In the present experiment, ∼85–90% of the myoblasts were fused into myotubes.

2.4. Measurement of 2-deoxy-D-[1-^{3}H] glucose

L6 myoblast cells grown in 12-well plate (Corning, NY) were subjected to glucose uptake as reported (Yonemitsu et al., 2001). In brief, differentiated myotubes were serum starved for 5 h and were incubated with the plant extracts for the required time duration as indicated. After experimental incubation, cells were rinsed once with HEPES-buffered Krebs Ringer phosphate solution (118 mM NaCl, 5 mM KCl, 1.3 mM $CaCl_2$, 1.2 mM $MgSO_4$, 1.2 mM KH_2PO_4 and 30 mM HEPES—pH 7.4) and were subsequently incubated for 15 min in HEPES-buffered solution containing 0.5 μCi/ml 2-deoxy-D-[1-^{3}H] glucose. The uptake was terminated by aspiration of media. Cells were washed thrice with ice cold HEPES buffer solution and lysed in 0.1% SDS. An aliquot was used to measure the cell-associated radioactivity by liquid scintillation counting. Glucose uptake values were corrected for non-specific uptake in the presence of 10 μM cytochalasin B, (5–10% of total uptake). Glucose transport was also performed in the presence of 1 μg/ml of cycloheximide and all the assays were performed in triplicate.

2.5. Thin layer chromatography of Pterocarpus marsupium

TLC analysis was performed to optimise the best solvent system for chromatographic separation. Following solvent systems were used: (a) 40% ethyl acetate in hexane and (b) 5% methanol in dichloromethane for better resolution of compounds. Resolved components were visualised under UV