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Polyphenols activate energy sensing network in insulin resistant models

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ABSTRACT

Unhealthy diet deficient in fruits and vegetables but rich in calories is considered to be one factor responsible for the increased prevalence of insulin resistance and type 2 diabetes (T2D). The consumption of fast foods and soft drinks increases fructose consumption *per se* and this is of major concern since prolonged fructose intake induces insulin resistance and thereby T2D. The energy homeostasis is regulated by a network consisting of "fuel gauze" called AMP-activated protein kinase (AMPK), the NAD^+ dependent type III deacetylase (SIRT1) and peroxisome proliferator-activated receptor-γ coactivator-1α (PGC-1α) which is disrupted in T2D. The present study was aimed to investigate the action of naringenin and quercetin on energy sensing molecules in insulin resistant models. L6 myotubes and albino Wistar rats were rendered insulin resistant with palmitate and fructose respectively. Naringenin, quercetin or metformin were used for treatment. Fructose and palmitate treatment resulted in insulin resistance as evidenced by decreased glucose transporter 4 (GLUT4) translocation. The translocation of GLUT4, phosphorylation of AMPK and the expression of SIRT1 and PGC-1α which were reduced in insulin resistant cells, were increased upon treatment with polyphenols. Further, naringenin and quercetin showed binding affinity with energy sensing molecules. We conclude that drugs from natural resources that target energy sensing molecules might be helpful to prevent insulin resistance.

1. Introduction

AMP-activated protein kinase (AMPK), a serine/theronine kinase plays an important part in determining whole body energy metabolism. AMPK is an energy sensor in cells that is activated by acute increases in the cellular AMP/ATP ratio. AMPK regulates the expression of genes involved in energy metabolism in coordination with nicotinamide adenine dinucleotide $(NAD)^+$-dependent type III deacetylase called silence information regulator T1 (SIRT1) [1]. SIRT1 serves as a link between metabolism and gene regulation by deacetylating a wide range of transcriptional factors including peroxisome proliferation activated receptor, liver X receptor, forkhead box O, p53 and PPAR coactivator 1 alpha (PGC-1α) which are important for physiological and cellular processes including cellular energy metabolism, fatty acid oxidation and mitochondrial biogenesis [2].

PGC-1α is expressed in tissues with high energy oxidative capacity, like heart, skeletal muscle, liver, brown adipose tissue and brain, and is robustly induced in conditions of energy requirement [3]. PGC-1α coordinately increases mitochondrial biogenesis, respiration rates, and uptake and utilization of substrates for energy production by coactivating several nuclear receptors and non-nuclear receptor transcription factors [4,5]. PGC-1α can be activated both by direct phosphorylation by AMPK on two critical residues, threonine-177 and serine-538 and by deacetylation by SIRT1 [6]. Thus the actions of AMPK, SIRT1 and PGC-1α are interlinked and these molecules form the energy sensing network.

Polyphenols modulate the expression/action of a wide range of enzymatic systems such as tyrosine and serine-theronine protein kinase [7], activate the signaling pathways such as phosphatidylinositide-3-kinase (PI3K) and extracellular signal-regulated protein kinase (ERK) [8] and regulate the transcription factors such as nuclear factor κ B (NF-κB) [9] and are potentially useful for treating many diseases. Dietary phytochemicals, naringenin and quercetin

ABBREVIATIONS: AMPK, AMP-activated protein kinase; SIRT1, silence information regulator T1; PGC-1α, peroxisome proliferator-activated receptor-γ coactivator-1α; GLUT4, glucose transporter 4; PDB, protein data bank; α-MEM, α-modified minimal essential media; FBS, fetal bovine serum; MTT, 3-(4-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide; HFD, high fructose diet.

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are the major flavonoids present in most common plants and vegetables. The insulin sensitizing effect of these phytochemicals has been already reported [10–12]. During insulin resistance conditions, there is a disrupted energy homeostasis resulting from the reduced levels of the sensors [13]. The present study was therefore carried out to explore the effect of administration of these polyphenols on energy sensing molecules under insulin resistant conditions.

2. Materials and methods

2.1. Retrieval of biological data for docking analysis

The three dimensional crystal structure of AMPK (PDB: 2UV5), SIRT1 (PDB: 4I5I) and PGC-1α (PDB: 1XB7) were obtained from protein data bank (www.rcsb.org).

2.2. Preparation of ligand structure

The ligands were drawn using Chemsketch software from the Advanced Chemistry Development, Inc. (ACD/Labs). The LigPrep module of Schrodinger was used to prepare ligand structures, generate variation and optimize the structures by minimizing energy levels. Schrodingers Optimization Potential for Ligand Simulation (OPLS 2005) force field is a molecular mechanics force field that includes Lennard-Jones terms and Coulombic interactions was used for LigPrep application.

2.3. Protein structure preparation

The PDB structures of the enzymes were prepared by the Protein Preparation Wizard of Schrodinger Suite. The crystal structure was pre-processed and assigned by including bond orders and missing hydrogen atoms and the bond length and angles were also corrected. The water molecules within 5 A° of the protein were removed during the pre-processing and redocked during the docking protocol. The pre-processed protein was then subjected to constrained IMPACT Protein Refinement (IMPREF) minimization and to the Root Mean Squared Deviation (RMSD) of 0.3 A°.

2.4. Receptor grid generation

The receptor grid was generated by excluding co-crystallized ligand from the receptor which determines the position and the size of the active site for docking. Grid generation of receptor requires a structure with all atoms and appropriate bond orders and charges. The crystal structure and docking information of proteins previously reported in the literature and Q-Site finder software (www.bioinformatics.leeds.ac.uk/qsitefinder) were used for generating the grid. This grid was made of a box that has default dimensions around the binding site. No constraints were set while building the grid.

2.5. Molecular docking studies

Molecular docking attempts to predict the intermolecular complex between two molecules, namely the target protein and ligand. The protein-ligand docking was performed in Glide XP extra precision mode [14]. During the docking procedure, the ligand was flexible and the protein was rigid. The best docked pose was saved and the results of the best poses for proteins with its ligand were analyzed using the G score, Dock score and number of H-bond. The ligand binding energy was calculated using

$$\text{Ligand Binding Energy}(\Delta E) = E_{complex} - E_{ligand} - E_{protein}.$$

The negative binding energy score indicates higher affinity of the ligand to the protein. The more negative the score the better the binding of the ligand to the protein.

2.6. Biochemicals, kits, primers, antibody, reagents and solvents

Anti-GLUT4, anti-AMPK and anti-pAMPK were purchased from Cell Signaling Technology, Danvers, MA, USA. Anti-SIRT1 and anti-Na^+K^+ ATPase were purchased from Santa Cruz Biotechnology, CA, USA. TriZol reagent was purchased from GeNei, Bangalore, India. Primers for PGC-1α was purchased from Sigma Aldrich, MO, USA. Immunohistochemistry kit was purchased from Biogenex Pvt. Ltd., CA, USA. SYBR GREEN was purchased from Fermentas, Waltham, USA. Chemiluminescence assay kit was purchased from Thermo Scientific, Rockford, IL, USA. L6 skeletal muscle cells were purchased from National Centre for Cell Science, Pune, India. The rest of the chemicals and solvents of analytical grade and chemicals for cell culture were purchased from Himedia Laboratories Pvt. Ltd., Mumbai, India.

2.7. Maintenance of L6 skeletal muscle cell and its differentiation into myotubes

Rat L6 skeletal muscle cell line was maintained in α-modified minimal essential media (α-MEM) containing (10% fetal bovine serum (FBS) and 1% antibiotic-antimycotic solution) in 75-cm^2 flasks in an atmosphere of 5% CO_2 at 37 °C. L6 cells were cultured to greater than 80% confluency prior to the initiation of differentiation. L6 myotubes were rendered quiescent in α-MEM containing 2% FBS for 4 days to promote fusion into myotubes. Two hours before performing the experiments, myotubes were placed in serum free-α-MEM containing 2% BSA.

2.8. Cell viability assay

Percentage cell viability of L6 myotubes after 16 h incubation with different concentrations of naringenin, quercetin (1 μM, 10 μM, 25 μM, 50 μM, 75 μM and 100 μM) and metformin (0.5 mM, 1 mM, 2 mM, 5 mM and 10 mM) was determined by 3-(4-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) assay. After differentiation and treatment with the compounds, L6 myotubes (2×10^4 cells/ml) were washed and MTT (0.5 g/l), dissolved in α-MEM, was added to each well for the estimation of mitochondrial dehydrogenase activity as described previously by Mosmann (1983) [15]. After incubation for 4 h at 37 °C in a CO_2 incubator, the media was carefully removed and 150 μl of 0.5% Dimethyl Sulfoxide (DMSO) was added to each well to dissolve the purple formazon crystals. The absorbance of the solubilized formazan product was measured at 490 nm using a microplate reader. The optimum concentrations of naringenin, quercetin and metformin required for further studies were fixed at 75 μM, 50 μM and 2 mM respectively.

2.9. Induction of insulin resistance using palmitate

Insulin resistance was induced by incubating the cells for 16 h with palmitate (750 μM) dissolved in ethanol and diluted 1:100 in α-MEM [16]. Palmitate was dissolved in 50% ethanol and conjugated with MEM containing 2% free fatty acid (FFA) free bovine serum albumin (BSA) and stirred well before use. After the requisite time, the cells were stimulated with insulin (100 nM for 15 min).

2.10. Treatment of L6 myotubes

L6 myotubes (2×10^4 cells/ml) were left untreated (CON) or