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Interaction of phytochemicals with hypoglycemic drugs on glucose uptake in L6 myotubes

Pranav Kumar Prabhakar, Mukesh Doble*

Department of Biotechnology, Indian Institute of Technology Madras, Chennai 600 036, India

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ABSTRACT

The present study analyses the effect of eugenol, arecoline and vanillic acid alone and in combination with two oral hypoglycemic drugs (OHD), namely, metformin and 2,4-thiazolodinedione (THZ), on 2-deoxyglucose (2DG) uptake in L6 myotubes. 2DG uptake in L6 myotubes was determined using an enzymatic assay developed by Yamamoto et al. (2006). Lipid content inside the cells has been estimated with oil red O assay. The absorption, distribution, metabolism, and excretion (ADME) and drug likeness properties of these phytochemicals are estimated using software QikProp®. All the three phytochemicals enhance 2DG uptake both in time- and dose-dependent manner. Eugenol and arecoline enhances 2DG uptake synergistically with both the OHD; whereas vanillic acid showing partly synergy with THZ and antagonistic activity with metformin on 2DG uptake. Eugenol and arecoline significantly increase the expressions of the glucose transporter type 4 (GLUT4) and phosphoinositide 3-kinase (PI3K) genes, but not the peroxisome proliferator-activated receptor (PPAR) gamma. Whereas vanillic acid does not has any significant effect on the expressions of these genes, the ADME results indicate that these phytochemicals are satisfying all the conditions to have a good oral bioavailability. These findings suggest that these phytochemicals can replace the commercial drugs in part, which could lead to a reduction in toxicity and side effects caused by the later as well as reduce the secondary complications.

Introduction

Diabetes is recognized as a group of heterogeneous disorders with the common elements of hyperglycemia and glucose intolerance, due to insulin deficiency, impaired effectiveness of insulin action, or both (Harris and Zimmet 1997). According to WHO, with all the complications, diabetes mellitus (DM) ranks as the third leading cause of death. The diabetic population in the world is estimated to double from 110 million in 1994 to 220 million by 2010 (Wild et al. 2004). There is a need for new hypoglycemic agents which will have therapeutic efficacy as well as less side effects. There are reports to manage diabetes with medicinal plants (Prabhakar and Doble 2008a,b) which is having less side effects, better effectiveness, multiple target sites and are of relatively low cost. Also, if the therapeutic concentration of the oral hypoglycemic drugs (OHD) could be reduced by replacing it with a phytochemicals, the side effects caused by the OHD could be reduced to a large extent (Prabhakar and Doble 2009). For such therapeutic strategy one has to consider the interaction between the OHD and phytochemicals and on the metabolism as well as the bioavailability of the phytochemicals.

Eugenol is, a phenylpropanoid, extracted from certain essential oils including clove oil (*Eugenia aromaticum* or *Eugenia caryophyllata*), nutmeg, cinnamon, and bay leaf. Tradionally it has been used in dentistry, for abdominal pain, and as an acaricidal, local antiseptic and anesthetic. It is rapidly absorbed and metabolized after oral administration and it is almost completely excreted in the urine within 24 h (Fischer and Dengler 1990). Eugenol is also able to enhance the activities of some of the detergents such as lysozyme, Triton X-100 and SDS in damaging the bacterial cell membrane. The hydrophilic antibiotics such as vancomycin and β-lactam antibiotics which have a marginal activity on gram negative bacteria exhibit an enhanced antibacterial activity when pretreated with eugenol (Hemaiswarya and Doble 2009).

Arecoline is an alkaloid type of natural product found in the fruit of the areca palm (*Areca catechu* L.). It is known to be a partial agonist of muscarinic acetylcholine M_1, M_2 and M_3 receptors. Arecoline is extensively metabolized to multiple products; none appear to be due to cytochrome P450. The metabolism involves mainly oxidation reactions exclusively, *N*-oxidations. *N*-Methylnipecotic acid is a novel metabolite arising from carbon–carbon double-bond reduction (Giri et al. 2006).

Vanillic acid, a benzoic acid derivative present in the root of *Angelica sinensis* [Oliv.] Diels. is used in traditional Chinese

* Corresponding author. Tel.: +91 4422574107; fax: +91 4422574102.
E-mail addresses: prabhakar.iitm@hotmail.com (P.K. Prabhakar), mukeshd@iitm.ac.in (M. Doble).

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medicine. There are no reports about the metabolism of vanillic acid in human but there are 23 strains of Zygomycetes which produce extracellular phenoloxidases which are involved in the reduction of vanillic acid to vanillyl alcohol (Guiraud et al. 1992).

This paper describes the effect of the three phytochemicals in combination with two commercial OHD, namely, metformin and thiazolodinedione (THZ), on 2-deoxyglucose (2DG) uptake in L6 myotubes. A nonradioisotope, enzymatic assay is used to estimate the uptake of 2DG (Yamamoto et al. 2006).

Materials and methods

Materials

L6 myoblasts, derived from skeletal muscles of rat, were purchased from NCCS, Pune, India. The culture media DMEM (Dulbecco modified Eagle's medium), FBS (fetal bovine serum), trypsin, antibiotics (penicillin, streptomycin and amphotericin B) were purchased from PAN BIOTECH GmbH, Germany. 2DG, hexokinase, G6PDH (glucose-6-phosphate dehydrogenase), diaphorase, rezazurine, ATP, $NADP^+$, and all the primers were obtained from Sigma–Aldrich, Bangalore, India. THZ and BSA were purchased from Himedia Laboratories Pvt. Ltd., Mumbai, India and metformin and dimethyl sulfoxide (DMSO) were ordered from Merck, Mumbai, India. All other chemicals were purchased from SRL Mumbai, India. All the plastic wares were obtained from Tarson, Kolkata, India. Medox easy spin column total RNA Miniprep super kit was purchases from Medox Biotech, Chennai, India, and used for total RNA extraction and RobusT I RT-PCR kit from Finzymes, Finland, was used for RT-PCR.

Determination of 2DG

2DG was estimated in a 24-well plate by an enzymatic diaphorase-NADPH amplifying system assay as reported by Yamamoto et al. (2006). 100 μl of 2DG solution of different concentrations (0.25, 0.5, 1, 2, 5 and 10 μM) was dispensed into each well in a 24-well plate. After the addition of 300 μl of a reaction cocktail, consisting of 50 mM TEA (pH 8.1), 0.02% BSA, 50 mM KCl, 0.5 mM $MgCl_2$, 670 μM ATP, 0.12 μM $NADP^+$, 25 μM resazurin sodium salt, 5.5 units/ml hexokinase, 16 units/ml G6PDH, and 1 unit/ml diaphorase, the mixture was incubated for 90 min. This cocktail was prepared just before the assay from the refrigerated stock solutions. After incubation, the fluorescence at 590 nm with excitation at 530 nm was measured with FP-6500 research grade fluorescence spectrometer (M/s. Jasco International Co. Ltd., Japan), to detect the conversion of resazurin to resorufin. If the reaction goes to completion the amount of resorufin formed, should be stoichiometrically equivalent to the amount of 2DG uptake. A standard curve was initially prepared between fluorescence readings with different concentrations of 2DG in a well of the culture plate without the cells. 2DG is more convenient than glucose itself because it is phosphorylated to a stable and impermeable derivative, 2-DG-6-phosphate, by hexokinase or glucokinase.

Cell culture of L6 myotubes

L6 myoblasts (3.5×10^5 cells/well) were grown in each well of a 24-well plate in DMEM supplemented with 10% fetal bovine serum and antibiotics (100 units/ml penicillin, 100 μg/ml streptomycin, and 5 μg/ml amphotericin B) at 37 °C in a humidified atmosphere composed of 5% CO_2 and 95% air. The medium was changed every third day. For differentiation, the L6 myoblasts were transferred to DMEM having 2% FBS for 4–6 days post-confluency. The extent of differentiation was established by observing the formation of elongated and multinucleate myotubes. These differentiated cells were used for further studies.

MTT assay

The cell viability was measured using MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay as described in the literature (Mosmann 1983). This assay is based on the reduction of MTT into purple formazan pigment by the mitochondrial succinate-tetrazolium reductase system (Gomez et al. 1997). Changes in MTT reductase activity is detected even before the membrane lysis, making this assay ideal for detecting cellular viability (Tollefson et al. 1996). The cells are seeded at 5×10^4 cells/ml density and incubated with both the phytochemicals and OHDs at various concentrations (0, 10, 20, 50 and 100 μM) for 24- and 48-h. After the specified time the medium is replaced with MTT solution (0.5 mg/ml in PBS) for 4-h at 37 °C. The formazan formed inside the cells is dissolved in 0.04 N HCl taken in isopropanol and the absorbance is measured spectrophotometrically at 595 nm with a Spectramax Plus384® Spectrophotometer (Molecular Devices, CA, USA). The viable cell number is directly proportional to the production of formazan.

Triglyceride assay by Oil red O method

Accumulation of triglyceride inside the cells is determined by the Oil red O (ORO) staining of neutral lipids in a 24-well plate (Laughton 1986). After the appropriate incubation with test compounds myotube cultures were fixed with perchloric acid. Then cells were washed with distilled water and submerged with ORO dissolved in propylene glycol (2 mg/ml). The wells were washed with PBS three times after overnight incubation at room temperature. ORO was extracted using isopropanol for 10 min at room temperature. The absorbance was measured at 490 nm with a V-670 research grade UV–vis spectrometer (M/s. Jasco International Co. Ltd., Japan) and blanked to cell-free well.

2-Deoxyglucose uptake assay

The differentiated myotubes were starved with serum for 4-h in DMEM and they were rinsed twice in KRPH (Krebs–Ringer-phosphate-Hepes) buffer (pH 7.4, 20 mM HEPES, 5 mM KH_2PO_4, 1 mM $MgSO_4$, 1 mM $CaCl_2$, 136 mM NaCl, 4.7 mM KCl). Then they were incubated with 350 μl/well of DMEM and 2% FBS in the presence of the test compounds (both phytochemicals and OHDs) for specified time. Cells were washed twice with KRPH buffer containing 0.1% BSA and were incubated with the same buffer containing 1.5 mM 2DG and 0.1% BSA for 30 min at 37 °C in 5% CO_2 atmosphere. After incubation they were rinsed with the same buffer containing 0.1% BSA and 50 μl of NaOH (0.1 N). 50 μl of HCl (0.1 N) were added to neutralize the alkalinity of the wells and followed by the addition of 50 μl of 150 mM of TEA buffer (pH 8.1). The fluorescence is measured by the enzymatic method described above, and the obtained values correspond to the 2DG concentration inside the cell.

In this study five different concentrations of OHD (2.5, 5, 10, 15, and 20 μM) and various concentrations of phytochemicals were tested. All the experiments were performed in triplicates and the average with the SD were reported here.

Synergistic interaction of phytochemical with commercial OHD is explained here on the basis of a parameter termed as combination index (CI) (Zhao et al. 2005). It is estimated as

$$\mathrm{CI} = \frac{C_a}{IC_a} + \frac{C_b}{IC_b}$$

where C_a and C_b are the concentrations of compound A and compound B used in combination to achieve a fixed effect (in this case