

C2C12 Cell Line is a Good Model to Explore the Effects of Herbal Extracts on GLUT4 Expression and Translocation

Fereshteh Ahmadipour¹, Tahereh Vakili², Abdolrahim Absalan³, Javad Mohiti-Ardakani¹, Hosein Hadinedoushan⁴, Mohammadali Khalili⁵, Fatemeh Pourrajab^{1*}

1- Department of Biochemistry and Molecular Biology, Faculty of Medicine, Shahid Sadoughi University of Medical Sciences, Yazd, Iran

2- Department of Clinical Biochemistry, Urmia University of Medical Sciences, Urmia, Iran

3- Department of Clinical Biochemistry, Faculty of Medical Sciences, Tarbiat Modares University, Tehran, Iran

4- Department of Immunology, Faculty of Medicine, Shahid Sadoughi University of Medical Sciences, Yazd, Iran

5- Research and Clinical Center for Infertility, Shahid Sadoughi University of Medical Sciences, Yazd, Iran

* Correspondence:

Fatemeh Pourrajab, Research and Clinical Center for Infertility, Shahid Sadoughi University of Medical Sciences, Yazd, Iran.

Tel: (98) 351 820 3412

Email: mina_poorrajab@yahoo.com

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Abstract

Objective: GLUT4 is a type of glucose transporter and plays a central role in whole-body metabolism of carbohydrates. The muscle is the major site of GLUT4 and cell line models, to explore GLUT4 behaviors under new therapeutic approach, such as herbal components, should be evaluated. Here, C2C12 cell line is evaluated for GLUT4 translocation from intracellular compartment into the cell membrane.

Materials and Methods: C2C12 cell lines were cultured and differentiated into myotubes. Cinnamon/Turmeric-water soluble extract (CWE and TWE) were prepared and differentiated myotubes were exposed to the 100, 1000 µg/ml CWE, 100 µg/ml TWE or dimethylsulfoxide for 3 hours. Intracellular/cytoplasmic membrane compartments were separated using ultracentrifugation. GLUT4 percentages quantities were measured using western blotting. Data analysis of 3 sample sizes was done by comparing mean±SD of GLUT4 quantities and independent samples t-test.

Results: CWE enhances GLUT4 translocation from intracellular compartment into the cytoplasmic membrane; its effect is also dose-dependent meaning that 1000 µg/ml concentration has a more potent effect than 100 µg/ml ($p < 0.05$). However, 100 µg/ml TWE had a reverse effect ($p < 0.05$).

Conclusion: Here we have shown that C2C12 is a good model for exploring GLUT4 changes under the effect of herbal extract. Induction or blockade of GLUT4 maybe under control of different signals transduction pathways. Furthermore, although turmeric ingredients are declared to have somewhat anti-diabetic effects, here we have shown that such effect is not applied via TWE effects on GLUT4 intracellular compartments movement into the cell membrane.

Keywords: Cinnamon, Turmeric, GLUT4, Diabetes, Herbal ingredient

Introduction

Cytoplasmic cell membrane lipid bilayers are nonpolar structures which make an impermeable barrier to polar molecules such as glucose and hexoses. Glucose transporters (GLUTs), a type of transmembrane glycoproteins, do transport of

glucose and hexoses via facilitative transmembrane mechanism (1-4). Different isoforms of GLUTs have been discovered in different tissues (5). Insulin resistant, the main cause of diabetes type 2 is associated with disturbed GLUTs metabolism (6,7). As the main regulator of glucose metabolism, i.e. insulin, acts via GLUT4, the main insulin-regulated glucose transporter (2,3,6,8-10), researchers explore a cell line model to investigate anti-diabetic agents such as herbs and spices to describe unknown aspects of cellular, molecular and biochemical mechanisms of their action (11-13).

Adipose and muscular tissues have the most potency for glucose expenditure. Also, these tissues are the main targets of insulin where it regulates GLUT4 metabolism, gene induction, cell trafficking and movement (1-3,5,6,9,10,14-26). Generally, cell culture models for GLUT4 variations under different interventional or lab trial studies have been limited mostly to the 3T3-L1, L6 and C2C12-derived cell lines from mouse (15,23,25,27-29). However, in human body, more than 75% of glucose is metabolized by muscular tissue (29) and adipocytes have a little role in this context (1,10,18). Most of the molecular studies on GLUT4 metabolism have been done using L6 (10,17,29-34) and C2C12 (5,10,19,22,24,25,35-40) cell lines. As there are a few studies on cellular and molecular aspects of herbs and spices, in the present study we have studied if C2C12 cell line maybe a candidate for exploring GLUT4 changes in cell compartments under cinnamon and turmeric water-soluble extracts, as two selected spices.

Materials and Methods

C2C12 cell line (code No. ATCC :CRL-1772) was purchased from National Cell Bank of Iran, Pasteur Institute, Tehran, Iran. Cinnamon and turmeric powder were purchased from herbal markets routinely importing them into Iran from china and India, respectively. Acetic acid, absolute ethanol, Tris, Sodium Dodecyl Sulfate (SDS), NaCl and Na₂EDTA all were

from (Merck Inc. Frankfurter Straße Darmstadt, Germany). Dimethylsulfoxide (DMSO), Dulbecco's modified Eagle's medium (DMEM) cell culture media, phenylmethylsulfonyl fluoride (PMSF) and sucrose were from Sigma-Aldrich Manufacturing, LLC (US, Saint Louis). Foetal bovine serum (FBS) and heat inactivated horse serum (H.S) were from Gibco. (Life Technologies Corporation, US). Bradford reagent and NaHCO₃ were from Sigma-Aldrich Manufacturing, LLC (US, Saint Louis). Penicillin G and streptomycin were, HEPES, Nonidet P-40, sodium deoxycholate were from AppliChem, LLC (Darmstadt, Germany). Nitrocellulose membrane was from Millipore (UK, Croyley Green Business Park Watford). ECL Advance Western immunoblotting kit was from Amersham Inc. (UK, Stanley Hill, Buckinghamshire, Amersham). Mouse anti-GLUT4 antibody 1F8 and goat anti-mouse IgG-HRP were from Santa Cruz Biotech (US, California, Santa Cruz). Mammography films were from Kodak (US, New-York, Eastman Kodak Company, Rochester).

Cinnamon Water-soluble Extract (CWE)

It was prepared according to the method described by Anderson et al (41) with modifications; 200 grams of cinnamon powder dissolved in 500 ml of 0.1N acetic acid, boiled for 20 minutes and centrifuged. One part of the 2000rpm supernatant mixed with 4 parts of absolute ethanol and kept in 4°C overnight; then the mixture was filtered using Whatman filter paper and dried in a 60°C oven; resulted powder dissolved in dimethylsulfoxide (DMSO) with a concentration of 100 mg/ml as stock solution.

Turmeric Water-soluble Extract (TWE)

To compare the effect of turmeric and cinnamon on GLUT4 contents in cell compartments we prepared a water-soluble extract of turmeric as same as CWE. We prepared a 1000 µg stock solution in DMSO of curcumin as described for CWE.

C2C12 primary culture and differentiation

About 8×10^4 C2C12 myoblasts were seeded in DMEM containing 4 mM/l glutamine, 0.025 mol/l glucose, 1mmol/l sodium pyruvate, 0.018 mol/l NaHCO₃, 100 U/ml Penicillin G, 100 µg/ml streptomycin and 10% foetal bovine serum in 250 ml tissue culture flasks. Cell culture medium was exchanged each day until reaching near confluent conditions. In all steps, cell culture flasks were kept in a humidified incubator with 5% CO₂ and 37°C. Myoblasts differentiation to myotubes induced by addition of DMEM supplemented with 2% heat inactivated horse serum (H.S) and polynucleated myotubes formation monitored microscopically until day 5 of differentiation induction. Differentiation medium was replenished each 48 hours.

Affecting CWE and TWE on myotubes and subcellular fractionation

For CWE, two different concentrations of 100mg/ml and 1000mg/ml were prepared in DMEM+2% H.S. Also a 100µg concentration of TWE in DMEM+2% H.S was prepared to assess if CWE and TWE have any effect on GLUT4 content of cytoplasmic membrane (CM) and nuclear/endoplasmic reticulum (N/ER). Also, a control group was included without any additive except 1% DMSO in DMEM+2% H.S (DDH). Ultimately CWEs, TWE and DDH were added to culture flasks containing differentiated myotubes on day 6 post-differentiation. For each category 3 flasks were considered. As Cao et al have shown the best results for GLUT4 enhancement in 3T3-L1 under CWE treatment on time exposure of 3 hours (15), we also kept myotubes in such exposure time status. After 3 hours exposure to CWEs, TWE or DMSO, myotubes were washed 3 times with ice cold phosphate buffer (pH=7.4) and then detached from flasks with scraper and homogenized with tissue homogenizer for 3 minutes in HES buffer, pH 7.4; 225 mM sucrose; 4 mM Na₂EDTA, 20 mM HEPES; 1 mM PSMF and 1 tablet/dl anti-protease cocktail. Cell homogenate was centrifuged according to the instruction

presented in Tortorella et al published paper (23); the first spin performed at 19000g for 20 minutes; To crude plasma membrane or N/ER of myotubes resulted pellet was homogenized in HES buffer and layered on a high sucrose buffer (HSB) composed of 1.12 M sucrose in 20 mM HEPES and 1 mM Na₂EDTA buffer and centrifuged further at 100000g for 1 hour. Interphase portion of this step was contained plasma membrane particles that were floated on HSB and resulted pellet included of N/ER particles. Interphase particles were aspirated and centrifuged at 40000g for 20 minutes to be pelleted; the pellet was re-suspended in PBS, pH 7.4, containing protease inhibitors. N/ER particles were re-suspended in a buffer containing 50 mM NaCl, 2% Nonidet P-40, 0.5% sodium deoxycholate, 0.2% SDS, 20 mM Tris (pH 7.4) and protease inhibitor cocktail. All centrifugation steps were done at 4°C with Beckman coulter ultracentrifuge and Type 90 Ti rotor. Also g force and round per minute (RPM) values for each step of centrifugation were calculated by the Beckman coulter ultracentrifuge calculating software which is available online. (www.beckmancoulter.com).

Sodium Dodecyl Sulphate Polyacrylamid Gel Electrophoresis (SDS-PAGE) and Western blotting

Total protein concentrations were analyzed using Bradford reagent and proteins were separated by a 10% SDS-PAGE according to the Laemmli instruction (24). Briefly 100 µg protein was loaded to SDS-PAGE wells and separated with 30 mA for 3 hours. Separated proteins were transferred to the nitrocellulose membrane at 4°C for 400 mA overnight. Blotted membranes were blocked using 2% blocking agent included in immunoblotting kit and was prepared in Tweenated phosphate buffer saline containing 1% Polysorbaat-20 (TBS), pH 7.5, for 3 hours with gently shaking. After 3×15 min washing in TBS, blotted paper immersed in 2% blocking agent containing mouse anti-GLUT4 antibody 1F8 with a dilution of 1:200 for 4

hours. After repeating wash steps, goat anti-mouse IgG-HRP was added with a concentration of 1:1000 in 2% blocking agent for 5 hours with gently shaking. After rewashing the membrane, chemiluminescent detection reagents were added according to the kit instruction and in a dark room. GLUT4 bands were visualized using mammography films and its related reagents.

Quantifications of GLUT4 bands and data analysis

Resulted bands from western blotting were quantified by scanning and comparing with together by Photo EP software. Data were a percent that divided between intervention group and its equal vehicle. Comparative histograms plotted in Excel and data were analyzed by SPSS software ver. 11.5 with two independent samples t-test.

Results

To study the effects of water-soluble extracts and also to examine whether each of those concentrations affects the GLUT4 expression,

we investigated the effects of two concentrations (100 and 1000 mg/ml) of CWE and TWE on CM and N/ER, separately, using chemiluminescent technique.

Chemiluminescent detection reagents were chosen for this study to determine whether the inducible or inhibitory effects of herbal extracts are dependent to concentration or not. Quantification and paired comparison of immunoblotting bands are presented in Table 1 and Figures 1 and 2. As it is stated in interpretation column of Table 1, GLUT4 contents were higher in N/ER fractions of Quantification and paired comparison of immunoblotting bands are presented in Table 1 and Figures 1 and 2. As it is stated in interpretation column of Table 1, GLUT4 contents were higher in N/ER fractions of control categories than CWE-exposed myotubes; also, 100µg/ml CWE-exposed myotubes had higher GLUT4 contents than 1000µg/ml CWE-exposed. However, CM fractions of control category had lower GLUT4 contents in comparison to the CWE-exposed myotubes; moreover, 100µg/ml

Table 1. GLUT4 contents of intervention and control categories determined using immunoblotting method

Fraction	Category	GLUT4 (Mean±SE)	p-value*	Interpretation
N/ER ¹	Vehicle	58.60±1.1	0.001	GLUT4 contents were reduced in N/ER but increased in CM
	100 µg/ml CWE	41.40±1.4		
CM ²	Vehicle	45.59±0.45	0.000	
	100 µg/ml CWE	54.41±0.48		
N/ER	Vehicle	55.54±1.01	0.001	GLUT4 contents were reduced in N/ER but increased in CM
	1000 µg/ml CWE	44.46±1.01		
CM	Vehicle	36.08±1.9	0.000	
	1000 µg/ml CWE	62.92±3.25		
N/ER	100 µg/ml CWE	59.46±2.56	0.006	GLUT4 contents were lower in N/ER but higher in CM of category exposed to 1000 µg/ml CWE in comparison to 100 µg/ml CWE
	1000 µg/ml CWE	40.54±2.56		
CM	100 µg/ml CWE	46.13	0.000	
	1000 µg/ml CWE	53.87		
N/ER	Vehicle	43.83±0.8	0.000	GLUT4 contents were reduced in CM but increased in N/ER
	100 µg/ml TWE	56.17±0.8		
CM	Vehicle	62.8	0.000	
	100 µg/ml TWE	37.2		

* Obtained p-values are calculated for independent samples t-tests with $\alpha=0.05$.

¹ Nuclear/Endoplasmic Reticulum

² Cytoplasmic membrane

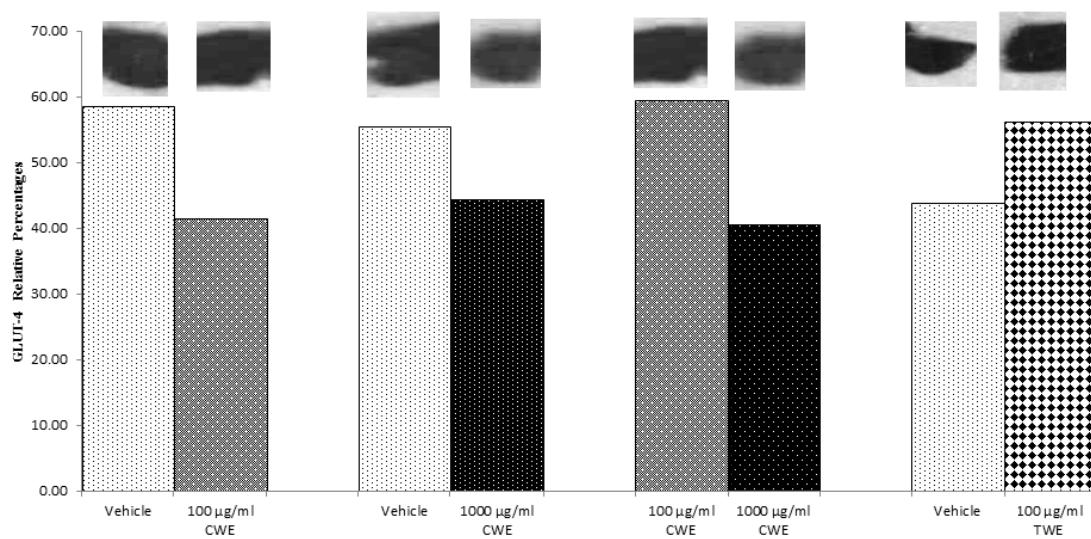


Figure 1. Quantification and paired comparisons of GLUT4 relative percentages obtained from Nuclear/Endoplasmic Reticulum (N/ER) fractions of myotubes between categories 100 and 1000µg/ml Water-soluble Cinnamon Extract (CWE), 100µg/ml Turmeric water-soluble extract (TWE) treated and their concurrent vehicle (1% DMSO) myotubes. Data were gathered from western-blot band scans. In all cases, Mean±SD were compared using independent samples t-test.

CWE-exposed myotubes had lower GLUT4 contents than 1000µg/ml CWE-exposed. But results for 100 µg/ml TWE-treated were in contrast to the results obtained for CWE. In all cases, all comparisons were significant at 95% confidence interval as showed in Table 1.

Discussion

Glucose is transported into the adipose and muscular tissues mainly via a GLUT4-dependent facilitated transport (10,18). In the other hand, about 75% of total glucose metabolism of the body is done by muscle after insulin secretion. However, adipose tissue has a slight role in glucose expenditure (31,29,26). So the most suitable tissue for exploring glucose metabolism, especially for determination of molecular mechanisms, is a muscle-derived *ex vivo*-based systems. Lauritzen et al. have stated that it is difficult to clarify GLUT4 measurement or relocation regulatory mechanisms due to the muscular tissue complexities and t-tubulin structures. Also they have declared that currently there is not a suitable cell line model to explore GLUT4 relocation in muscular tissue. Also

Michael and coworkers have certified that almost all cell culture models of muscular tissues do not have the machineries for synthesis of GLUT4 or such machineries maybe in low quantities (13-15,27-29). Most studies on GLUT4 metabolism in cell line models under the effect of natural or pharmaceutical agents have been focused mainly on 3T3-L1, an adipose tissue-derived cell line. But this cell line is not a good model to survey of glucose metabolism regulating agents such as GLUT4 mainly because of two important aspects: firstly, the most glucose utilizing tissue in the human body is muscular tissue; then, 3T3-L1 could not be a good candidate for the mentioned purpose; and secondly, GLUT1 which its synthesis and metabolism regulation is not regulated by positive effectors such as insulin, are enhanced, despite GLUT4 down-regulation, under long-term exposure to insulin (10,35-40).

This was particularly that the whole-body GLUT4 itself may be less informative due to up-regulation of compensatory mechanisms that may promote survival of the animal. Thus,

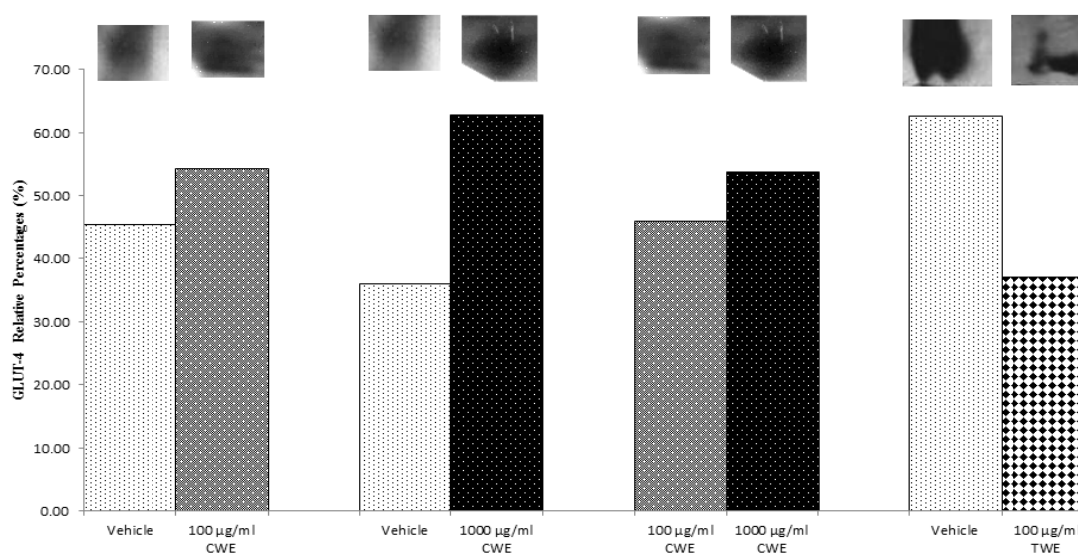


Figure 2. Quantification and paired comparisons of GLUT4 relative percentages obtained from cytoplasmic membrane (CM) fractions of myotubes between categories 100 and 1000µg/ml Cinnamon Water-Soluble Extract (CWE), 100 µg/ml Turmeric water-soluble extract (TWE) treated and their concurrent vehicle (1% DMSO) myotubes. Data were gathered from western-blot band scans. In all cases, Mean±SD were compared using independent samples t-test.

GLUT4 in muscle and adipose tissue is indispensable for normal global glucose homeostasis, while insulin receptor in these tissues appears much less critical. However, adipose tissue accounts for only a small fraction of total body glucose disposal and also lack of the lipogenic and antilipolytic actions of insulin in adipocytes, which make muscle cells more favorable in vitro model for glucose uptake studies. Interestingly, decrement and overexpression of GLUT4 protein in muscle tissue show the expected insulin resistance and propensity toward diabetes or normalizes insulin sensitivity and glucose tolerance, respectively that is consistent with a major role of GLUT4 in glucose disposal. So, researchers are looking for a cell line that could be a good representative for muscular tissue with the ability to synthesize GLUT4 and its relocating machinery (27-34).

In other side, other works have demonstrated that chemicals such as 5-aminoimidazole-4-carboxamide-1-β-D-ribofuranoside (AICAR) that is an AMPK activator, Indinavir that is a protease inhibitor of human immunodeficiency virus (HIV), and Wortmannin, a

phosphatidylinositol-3 kinase (PI3K) inhibitor, affect glucose uptake and some AMPK pathway components (39-41).

The current study showed differentiated C2C12 cells are a good model to explore GLUT4 changes under herbal extracts exposure. Cinnamon hydro-alcoholic extract-treated cells had more GLUT4 contents in CM compartments and lower contents in intracellular membranes, and N/ER, in comparison to their concurrent DMSO-treated controls. These results propose that GLUT4 is translocated from intracellular compartments in to the cell surface or cytoplasmic membranes. Also, comparison of two different concentrations of cinnamon hydro-alcoholic extracts shows that myotubes treated by higher concentrations of CWE had more GLUT4 contents in cytoplasmic membrane and lower contents in their intracellular compartments. This means that GLUT4 containing vesicles which transfer GLUT4 molecules from intracellular environments to the cell surface of C2C12-driven myotubes in response to herbal extracts in a dose-dependent manner. In the other hand, TWE-exposed myotubes did

not transfer GLUT4-containing vesicles from intracellular into the cell surface compartments in comparison to their concurrent DMSO-treated control myotubes. Importantly, DMSO-treated myotubes had more contents of GLUT4 in their cytoplasmic membrane compartments. This observation may be due to the effect of turmeric ingredients, especially curcumin, which may induce invagination and reverse movement of GLUT4 vesicles strictly in contrast to the effects seen for cinnamon. Acute hyperglycemia provides an insulin-independent inducer for GLUT4 translocation in C2C12 myotubes and rat skeletal muscle. In addition, our study showed that in concurrent control categories of both cinnamon-treated myotubes, GLUT4 contents were mostly present in nuclear or endoplasmic reticulum membranes in contrast to the cinnamon-treated cells.

Our results are in agreement with other studies. For example; Ralston and Ploug have shown that GLUT4 is mostly localized in the core of the unstimulated C2 myotubes (20). Electron micrographs had been revealed that GLUT4 containing vesicles are mainly

localized in tubulovesicular structures of unstimulated C2 myotubes (35).

Considering results for extracts of two different herbs both of which are candidates for diabetes treatment, it could be discussed that reverse and dose-dependent effects, due to herbal ingredients were seen for both extracts on GLUT4 intracellular or cytoplasmic membrane contents of C2C12-derived myotubes. So, differentiated C2C12 cells could be a good model for *ex vivo* investigation of GLUT4 variations and metabolism under the effect of herbal extracts. In other words, C2C12-derived myotubes had enough sensitivity for such investigations.

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