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Effects of genistein on insulin pathway-related genes in mouse differentiated myoblast C2C12 cell line: evidence for two independent modes of action

Slawomir Lewicki[1], Aneta Lewicka[2], Boleslaw Kalicki[3], Agnieszka Sobolewska-Ruta[4], Bogdan Debski[5], Robert Zdanowski[1], Tomasz Syrylo[6], Malgorzata Kloc[7, 8], Jacek Z. Kubiak[1, 9]

[1]Department of Regenerative Medicine and Cell Biology, Military Institute of Hygiene and Epidemiology, Warsaw, Poland

[2]Independent Laboratory of Epidemiology, Military Institute of Hygiene and Epidemiology, Warsaw, Poland

[3]Paediatric, Nephrology and Allergology Clinic, Military Institute of Medicine, Warsaw, Poland

[4]Bioengineering Department, The Institute of Biotechnology and Antibiotics, Warsaw, Poland

[5]Division of Biochemistry, Department of Physiological Sciences, Faculty of Veterinary Medicine, Warsaw University of Life Sciences — SGGW, Warsaw, Poland

[6]Department of Urology, Military Institute of Medicine, Warsaw, Poland

[7]The Houston Methodist Hospital and the Houston Methodist Research Institute, Department of Surgery, Houston, Texas, USA

[8]University of Texas, MD Anderson Cancer Center, Houston TX, USA

[9]Univ Rennes, CNRS, IGDR — Institute of Genetics and Development of Rennes, UMR 6290, Cell Cycle Group, Faculty of Medicine, 35043 Rennes, France

Abstract

Introduction. Genistein (plant isoflavone) is a well-known anti-cancer drug with estrogenic-like properties. Genistein also regulates sugar and lipid metabolism; thus, it has anti-diabetic properties. The aim of the study was to evaluate *in vitro* effects of genistein on glucose transport, fatty acids oxidation, activation of PKB, and expression of genes related to insulin pathway in differentiated myoblast C2C12 mouse cell line.

Material and methods. Differentiated myoblast C2C12 mouse cell line was used to assess the effects of different genistein concentrations on glucose transport and fatty acids oxidation measured by radioactivity technique, activation of PKB, and expression of selected genes related to insulin signaling pathway (IR-α, IR-β, IRS-1, PKB, GLUT-4, PP2A, SH-PTP2) at the mRNA and protein levels. Cells were incubated with various concentrations of genistein under standard conditions for 0–48 hours.

Results. Genistein in low concentrations (0.1–1 μM) significantly increased glucose transport and decreased fatty acids oxidation in C2C12 cells after 48 h of incubation. High concentration of genistein (50 μM) had the opposite effect. Genistein stimulated PKB phosphorylation during the first 5–10 minutes of incubation. There

Correspondence address: Aneta Lewicka, PhD
Independent Laboratory of Epidemiology,
Military Institute of Hygiene and Epidemiology,
Kozielska 4, 01–163, Warsaw, Poland
tel: +48 261 853 101, fax: +48 261 853 133
e-mail: anet.lewicka@gmail.com

was no significant impact on the protein expression of selected genes (IR-α, IR-β, IRS-1, PKB, GLUT-4, PP2A--Cα, ER-α and ER-β) after 48 h treatment. We observed inverse correlation between genistein concentration and the expression of SH-PTP2 protein. Genistein affected the expression pattern of mRNAs for genes related to the insulin pathway, however, not the expression of the encoded proteins.

Conclusions. The results of this study showed that depending on the concentration and time of incubation genistein significantly affects glucose and lipid metabolism and at low concentration modifies expression pattern of a few genes in C2C12 cells. *(Folia Histochemica et Cytobiologica 2018, Vol. 56, No. 3, 123–132)*

Key words: genistein; C2C12 cell line; glucose transport; insulin pathway proteins; fatty acids oxidation

Introduction

Genistein (4',5,7-trihydroxyisoflavone) is a phytoestrogen found in plants from *Fabaceae* family such as soybeans. It has numerous health benefits, such as positive regulation of homeostasis, attributed to its multiple biological functions [1]. The concentration of genistein in human and animal body fluids depends on the type of diet. A human diet rich in vegetable products (including soybean and soybean-derived products) significantly increases concentration of this phytoestrogen in the body [2].

Multiple studies suggest that dietary phytoestrogens play a beneficial role in reducing obesity and protecting against diabetes, as well as in improving glucose metabolism and insulin resistance [3]. In cells, the glucose level is regulated by insulin, the major hormone controlling critical cellular energy functions. The glucose and lipid metabolism is regulated by activation and regulation of the insulin cellular pathway-associated genes including receptors and related proteins, *i.e.* insulin receptors (IRs), insulin receptor substrate 1 (IRS-1), phosphoinositide 3-kinase (PI-3K) and protein kinase B (PKB) [4]. Perturbations of this pathway lead to development of type 2 diabetes that manifests by increased chronic insulin resistance and the loss of function of pancreatic β-cells. Because of the growing incidence of type 2 diabetes and complications related to this widespread disease, the identification of effective drugs protecting β-cells functions is extremely important [5]. Genistein is one of the drugs with such a potential [6].

Many studies have demonstrated that genistein has a direct anti-diabetic effect after administration to animals and humans [7]. It increases β-cell proliferation and regeneration [8], regulates glucose-stimulated insulin secretion (GSIS) [9] and protects cells against apoptosis [10]. These effects may be independent of genistein functions as an estrogen receptor agonist, antioxidant, or tyrosine kinase inhibitor [11, 12]. Genistein regulates a plethora of molecular pathways critical for cell homeostasis and survival. It affected *i.e.* activation of NF-κB pathway, PI3K/Akt/mTOR signaling pathway or MAPK/ERK pathway [13]. In low concertation (up to 10 μM) genistein induces cell proliferation, studied mainly in cancer cell lines (*i.e.* human breast cancer cell line MCF-7) [14] or human uterine leiomyoma [15]). Higher concertation of genistein (above 10 μM) significantly reduced proliferation of many cell types (adipocytes 3T3 L1 [16], muscle — RASMCs [17], endothelial cells — HCAEC [18] or epithelial cell line RWPE-1 [19]) and induced apoptosis [20].

The aim of the present study was to investigate the effects of genistein on energy metabolism, gene transcription profile and the expression of proteins related to the insulin pathway. Experiments were carried out on mouse myoblast cell line C2C12, because these cells exhibit strong reactivity to the modification of glucose metabolism.

Materials and methods

The work was approved by institutional review boards and the State Committee for Scientific Research (KBN, Poland).

Cell culture. Experiments were performed *in vitro* on mouse C2C12 myogenic cell line obtained from the European Collection of Animal Cell Cultures (Porton Down, UK). Cells were grown in Dulbecco Modified Eagle's Medium (DMEM) supplemented with 20% fetal bovine serum (FBS), antibiotics: penicillin-streptomycin (50 IU/mL) and fungizone (2.5 μg/mL) (all purchased from Life Technologies, Warsaw, Poland) in an atmosphere of 5% CO_2/95% humidified air at 37°C. Confluent culture (about 90% of confluence) was differentiated in DMEM containing 2% of horse serum (Life Technologies) for three days. On the 3rd day, medium was removed and cells were washed twice with phosphate buffered saline (PBS, Life Technologies, Warsaw, Poland). DMEM without serum was added for the next 24 h. Then the cells were washed twice with PBS and the experiments were commenced.

Genistein. Genistein was purchased from SigmaAldrich (Poznan, Poland). The stock solution was dissolved in ethanol (99.8%, SigmaAldrich). The working solutions were stored at -80°C. Used concentrations of genistein (0.1; 1 and

50 μM) were based on the results obtained in preliminary study (data not shown).

Measurement of glucose transport. The experiment was performed on cells cultured in 24-well plates. Genistein (0-control, 0.1; 1 or 50 μM) was added to the medium (DMEM without FBS) for 1, 3, 6 and 48 h. Each time-point experiment was repeated 3 times. At each time-point medium was removed, cells were rinsed twice with PBS, and incubated with 2-deoxy-D-[1,2-^{3}H]-glucose (radioactivity 277.5 Bq, Amersham, UK) in PBS. After 15 min cells were washed twice with PBS. Then 200 μl of 2 M NaOH was added. Following incubation for 24 h, 50 μl of cells lysates were taken for protein assay [21] and the radioactivity of the remaining sample was analyzed in a liquid scintillation counter (Packard TRI-CARB, Packard, Ramsey, MN, USA). Results are presented as % of control value (mean ± SD) from 4 independent experiments.

Measurement of fatty acids oxidation. Level of β-oxidation was analyzed according to Manning *et al.* [22]. The experiment was performed on cells cultured in 24-well plate. Genistein (0-control, 0.1; 1 or 50 μM) was added to the medium (DMEM without FBS) for 1, 3, 6 and 48 hours. After given time of incubation medium was removed, cells were rinsed twice with PBS and incubated with tritium labeled [9,10-^{3}H] palmitic acid (radioactivity 280.1 Bq, Amersham, UK) in PBS. After 60 min of incubation (37°C, 5% CO_2, 95% humidity) cells were washed twice with PBS. Then 200 μl of 2 M NaOH was added. Following incubation for 24 h cells were lysed. A fifty μl of the lysates were used for protein assay [21] and the remaining samples were transferred to AG-1-X8 column (Bio-Rad, Munich, Germany). The column filtrate was analyzed in a liquid scintillation counter (Packard TRI-CARB). The activity of β-oxidation in C2C12 cells was determined by the amount of tritiated water formed from the oxidation of ([9,10-^{3}H] palmitic acid. Results are presented as percentage of control value (mean ± SD) from 4 independent experiments.

Western blot. The experiment was performed on cells cultured in 6-well plates. After genistein treatment (0, 0.1, 1 or 50 μM) total proteins were extracted from cells using RIPA buffer (Sigma Aldrich) and centrifuged at 2000 g for 20 min. Protein extracts were separated in 4–12% denaturing polyacrylamide gel. Proteins were blotted onto nitrocellulose membrane (Life Technologies, Warsaw, Poland) and blocked with 5% fat-free dry milk in Tris-buffered saline Tween (TBST, Sigma Aldrich) at 4°C. After washing with TBST, proteins were incubated overnight at 4°C with primary antibodies (Santa Cruz Biotechnology, Heidelberg, Germany): rabbit polyclonal IgG anti-p(ser437)Akt-1/2/3, rabbit polyclonal IgG anti-Akt 1/2/3, rabbit polyclonal IgG anti-IRα and β, rabbit polyclonal IgG SH-PTP2, rabbit polyclonal IgG

anti-PP2A-Cα, rabbit polyclonal IgG anti-GAPDH, rabbit polyclonal IgG anti-IRS1, rabbit polyclonal IgG anti-ERα and chicken polyclonal IgG anti-ERβ503 received from Prof. Jan-Åke Gustafsson and Dr. Margaret Warner from the Department of Biosciences and Nutrition, Karolinska Institutet, Stockholm, Sweden. The bound antibodies were detected by second peroxidase-conjugated antibodies: donkey polyclonal IgG anti-rabbit HRP and rabbit polyclonal IgG anti-chicken HRP (Santa Cruz Biotechnology) and chemiluminescence (ECL detection reagent; Amersham). For densitometry analysis X-Ray films were scanned, and integrated optical density (IOD) was measured using Kodak 1D 3.5 software (New Haven, CT, USA). The densitometric values of the investigated proteins were normalized to GAPDH (glyceraldehyde-3-phosphate dehydrogenase). All western blots analyzes were performed at least in triplicate. Results are presented as % of control value (mean ± SD).

Microarray analysis. Microarray analysis was performed using SuperArray kit (Bio-Rad, Munich, Germany). Genistein (1 μM) was added for 4 h (short stimulation) or 24 h (long stimulation) to cells cultured as described above. RNA extraction, cRNA and cDNA synthesis and hybridization were performed according to the manufacture's protocols. Hybridization results were visualized on X-ray plates, scanned (CanoScan Lide 60, Ōta, Tokio, Japan), and analyzed in GEArray Expression Analysis Suite (SuperArray software, available on the manufacturer's website SABiosciences Corp., Frederick, MD, USA). The densitometry values of the investigated genes were normalized to GAPDH, rps27a (ribosomal protein S27a) and b2m (β-2 microglobulin), hsp90ab1 (heat shock protein 90 kDa α — cytosolic, class B member 1) and ppia (peptidylprolyl isomerase A). Based on this study we compiled the list of genes which expression was changed at least 100% comparing to the control values.

Statistical analysis. The significance of the difference was assessed by parametric t-test for a normal distribution of values and Kruskal-Wallis test for a non-parametric distribution of values (GraphPad Prism 4 software, La Jolla, CA, USA). P values < 0.05 were considered significant.

Results

Glucose transport in differentiated myoblast C2C12 cell line

We examined the effects of genistein (Gen) on the intracellular glucose transport. Genistein at 50 μM concentration, but not at lower concentrations, caused dramatic (~85%) reduction of glucose transport (p < 0.001) regardless of the incubation time (Fig. 1). Incubation of cells for 6 h with 1 μM genistein stimulated slightly glucose transport (108.4%, p < 0.05), while the
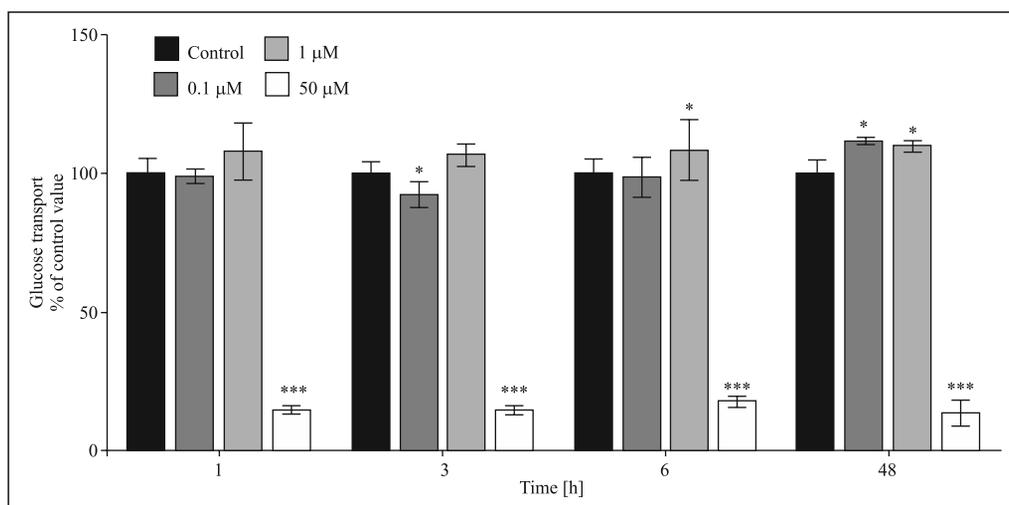
Figure 1. Genistein effect on the glucose uptake by differentiated myoblast C2C12 cell line. Genistein (0.1, 1 and 50 μM) was added on the 4th day of cell differentiation for 1, 3, 6 or 48 hours. Glucose transport was measured as described in Methods. The results are shown as the mean values ± SD. Statistical differences were calculated relative to the values obtained for control. *P < 0.05; ***P < 0.001.

values obtained for the 0.1 μM genistein fluctuated near the control values with a slight decrease (92.3%, p < 0.05) after incubation for 3 h (Fig. 1). After 48 h of incubation these two concentrations caused a slight 10% (Gen 0.1 μM, p < 0.05; Gen 1 μM, p < 0.001) increase in glucose uptake (Fig. 1).

Fatty-acids β-oxidation in differentiated myoblast C2C12 cell line

We tested the effect of genistein on fatty acids oxidation. One-hour incubation of differentiated myoblast C2C12 cell line with 50 μM genistein increased β-oxidation by 34.4%, p < 0.001). This effect dissipated with time of incubation: 115.2% after 3 hours and 83.8% after 6 hours (Fig. 2). After 48 h of incubation with genistein there was a dose-dependent reduction in the β-oxidation activity: 0.1 μM — 73.9% (p < < 0.001), 1 μM — 92.1% (p < 0.05) and 50 μM — 97.8% (p > 0.05) relative to the control value (Fig. 2).

Effects of genistein on insulin signaling pathways proteins

Protein kinase B (PKB) is one of the major proteins of the insulin pathway, which regulates cell response to the glucose level. One μM genistein caused a very rapid and significant increase (by 40%, p < 0.01) in the PKB kinase phosphorylation level during the first 15 min of incubation. This effect disappeared after 30 min of incubation (Fig. 3).

There were no significant changes in the expression level of the other studied insulin pathway proteins, *i.e.*

insulin receptor IR-beta, IRS-1 or PKB kinase when cells were incubated with 0.1, 1 and 50 μM genistein (Suppl. Fig. 1A, C, D). There was also no effect of genistein (0.1, 1 and 50 μM) on the expression of IR receptors (Suppl. Fig. 1A, B), GLUT-4 (Suppl. Fig. 1E) and estrogen receptors (ER-α and ER-β, Suppl. Fig. 1F, G) in differentiated myoblast C2C12 cell line.

Results from western blot analysis revealed that genistein caused no change in the expression of the phosphatase PP2A-Cα. Only at 50 μM concentration, genistein increased PP2A-Cα expression by 20% after 48 h incubation (Suppl. Fig. 1H). In contrast, there was a positive correlation between the genistein concentration and expression of SH2 protein tyrosine phosphatase 2 (SH-PTP2) (Suppl. Fig. 1I).

Effects of genistein on gene expression pattern

Microarray analysis was focused on 119 genes related to insulin signaling pathway. The analysis of genes' expression in the control conditions after 4 and 24 h incubation of the differentiated myoblast C2C12 cell line with the genistein revealed only slight effects (Table 1). Addition of 1 μM genistein for 4 h caused increase in the expression of 21 genes, among which the 11 genes were activated by genistein (not detected in control condition, Table 2). Only one gene (*nck1*, Table 2) showed reduced expression. Genistein-affected genes belonged to the following families and pathways: insulin receptor family, PI-3 kinase pathway, MAPK kinase pathway, insulin pathway, carbohydrate metabolism, lipid metabolism, transcrip-
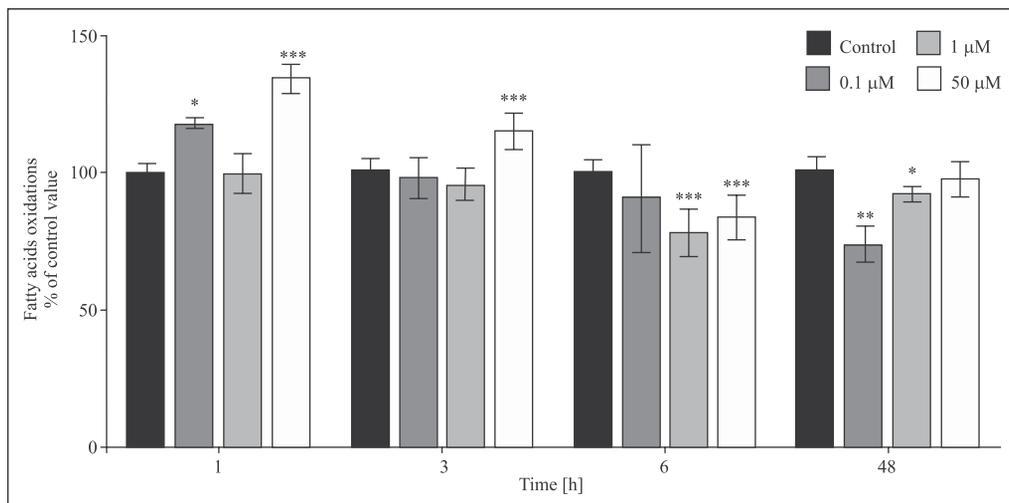
Figure 2. Genistein effect on β-oxidation of fatty acids in differentiated myoblast C2C12 cell line. Genistein (0.1, 1 and 50 μM) was added on the 4th day of differentiation and cells were incubated for 1, 3, 6 or 48 hours. β-oxidation of fatty acids was measured as described in Methods. The results are presented as the mean values ± SD. Statistical differences were calculated relative to the control values. *P < 0.05; **P < 0.01; ***P < 0.001.
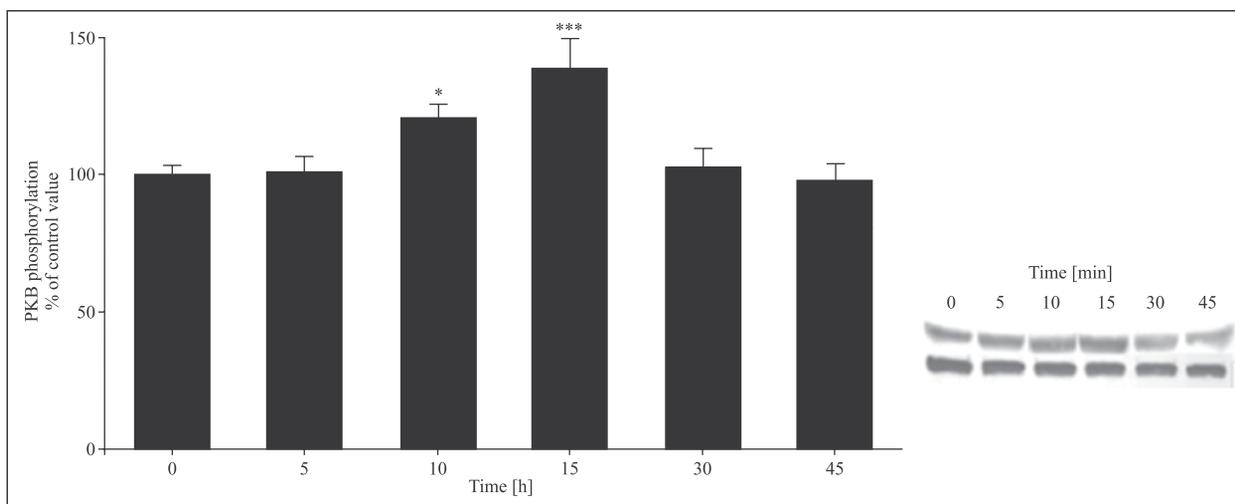

Figure 3. Western blot analysis of the effects of 1 μM genistein and time of incubation (0–45 minutes) on the p(ser437)Akt-1/2/3 kinase level in differentiated myoblast C2C12 cell line. Results are presented as mean ± SD. Statistical differences were calculated relative to the values obtained for control. *P < 0.05; ***P < 0.001. The densitometry values were normalized to glyceraldehyde 3-phosphate dehydrogenase (GAPDH) expression levels. PKB — protein kinase B.

tion factors and regulatory genes of the cell cycle or differentiation. Incubation of the cells for 24 h in the presence of 1 μM genistein resulted in significantly less-pronounced effects on the insulin pathway genes expression. There was reduction in the expression of three genes: *cbl, gck, sorbs1*, and the increased expression of *akt1* gene. Results of microarray analysis are shown in Table 2.

Disscusion

Comparison between populations of Asian and Western developed countries showed that a diet rich in phytoestrogens reduces the incidence of cardiovascular diseases and disorders of the carbohydrate and lipid metabolism [23]. Similar health effects were observed for a diet rich in soy-based foods; it caused

Table 1. Differences in insulin pathway gene expression in differentiated myoblast C2C12 cell line after 4 and 24 h incubation in control condition. Underlined are genes with switched on expression after 24 h; " ↑ " — genes with increased expression; " ↓ " — genes with decreased expression. Information of gene functions is quoted according to the UniProtKB database

Gene expression in control condition (after 4 and 24 hour incubation)

Gene symbol	Gene name	Function	Expression level
bcl2l1	Acetyl-Coenzyme A carboxylase 2	Catalyzes the ATP-dependent carboxylation of acetyl-CoA to malonyl-CoA; inhibition of fatty acid and glucose oxidation; regulation of mitochondrial fatty acid oxidation through malo-nyl-CoA-dependent inhibition of carnitine palmitoyltransferase 1	↑2.58
Cbl	Casitas B-lineage lymphoma	Proto-oncogene	↑2.76
dok4	Docking protein 4	Transmembrane receptor protein tyrosine kinase signaling pathway (MAPK)	↑2.16
g6pdx	Glucose-6-phosphate dehy-drogenase X-linked	Catalase oxidative pentose-phosphate pathway	↑2.33
map2k2	Mitogen activated protein kinase kinase 2	Protein serine/threonine kinase activity; MAPK signaling pathway	↑2.72
Rras	Harvey rat sarcoma oncogene, subgroup R	Regulates the organization of the actin cytoskeleton with OSPBL3; modulates integrin beta-1 (ITGB1) activity	↑2.41
slc27a4	Solute carrier family 27 (fatty acid transporter), member 4	Involved in translocation of long-chain fatty acids (LFCA) across the plasma membrane; the principal fatty acid transporter in small intestinal enterocytes; formation of the epidermal barrier; required for fat absorption in early embryogenesis	↑2.22
sorbs1	Sorbin and SH3 domain containing 1	Tyrosine phosphorylation of CBL by linking CBL to the insulin receptor; required for insulin-stimulated glucose transport. Involved in formation of actin stress fibers and focal adhesions	↑2.38

Abbreviations: CBL (Casitas B-lineage Lymphoma) — E3 ubiquitin-protein ligase involved in cell signaling and protein ubiquitination; LFCA — long-chain fatty acids

a decrease in total cholesterol, low-density lipopro-tein (LDL) and triglycerides without modification of high-density lipoprotein (HDL) concentration in serum of postmenopausal women [24]. Studies on male CD-1 mice also confirmed positive effects of soy products on animal health. Soy-containing diet caused reduction in serum insulin level, pancreat-ic insulin content, AMP-activated protein kinase (AMPK) phosphorylation, activated expression of genes responsible for the fatty acids oxidation (in the adipose tissue) and increased glucose transport activity in muscle cells [25]. It has been shown in mice that soy-enhanced diet prevented obesity and related disorders [26].

Genistein is one of the most active soy isoflavones. It is involved in the regulation of lipid and glucose metabolism, but the exact role, the mechanism of action and the degree of its positive health impact are poorly understood. Genistein is structurally similar to 17β-estradiol, which allows its binding to the estro-gen receptors and the activation of protein kinase A (PKA) [27]. Our previous studies on differentiated myoblast C2C12 cell line showed that 10 nmol of 17 β-estradiol significantly affected the expression of genes related to the insulin pathway [28]. This study suggested that genistein might affect sugar and lipid metabolism through interactions with estrogen receptors. There are also reports indicating a non-es-trogenic effect of genistein. The studies of Liu *et al.* [12] showed that genistein regulated the secretion of insulin in two insulin-secreting cell lines INS-1 and MIN6, through the signal cascade closely related to the cAMP activation. Genistein increased intracellu-lar cAMP level and activated PKA by a mechanism unrelated to estrogen receptor or protein tyrosine kinase (PTK) but associated with the stimulation of adenylate cyclase activity.

It has been also suggested that genistein may mod-ulate the activity of glucose transport at the cellular level. Our results obtained in the present studies are consistent with the results of Shuo-Bin *et al.* [29] stud-ies on C2C12 cells. We showed that 50 μM genistein caused inhibition (approximately by 85%) of glucose uptake. We also showed that low doses of genistein had only slight but statistically significant effect on glucose transport in C2C12 cells (± 10% change) both after short (1–6 h) and long (48 h) incubation (Fig. 1). Similar results were obtained by Nomura *et al.* [30]. In their studies the 1 h incubation with low concertation (1–5 μM) of genistein did not affect

Table 2. Effect of genistein on the insulin pathway gene expression in differentiated myoblast C2C12 cell line after 4 h and 24 h incubation. Underlined are genes with switched on expression compare to the appropriate control condition (4 h or 24 h); " ↑ " — genes with increased expression; " ↓ " — genes with decreased expression. Information on gene functions is quoted according to the UniProtKB database

Gene symbol	Gene name	Function	Expression level
List of gene expressed after 4 hours of cell incubation with 1 μM genistein			
acacb	Acetyl-Coenzyme A carboxylase 2	Catalyzes ATP-dependent carboxylation of acetyl-CoA to malonyl-CoA; inhibition of fatty acid and glucose oxidation; regulation of mitochondrial fatty acid oxidation through malonyl-CoA-dependent inhibition of carnitine palmitoyltransferase 1	↑2.61
acox1	Acyl-Coenzyme A oxidase 1, palmitoyl	Catalyzes the desaturation of acyl-CoAs to 2-trans-enoyl-CoAs	↑2.26
<u>*aebp1*</u>	AE binding protein 1	Regulates MAP-kinase activity in adipocytes; regulates NF-κB activity in macrophages; transcriptional repressor	↑3.98
<u>*akt2*</u>	Thymoma viral proto-oncogene 2	Related to serine/threonine-protein kinase; regulates metabolism, proliferation, cell survival, growth and angiogenesis	↑3.34
<u>*araf*</u>	Serine/threonine-protein kinase A-Raf	Transduction of mitogenic signals from the cell membrane to the nucleus; regulates the TOR signaling cascade	↑2.77
cap1	CAP, adenylate cyclase--associated protein 1	Regulates filament dynamics; implicated in complex developmental processes, including mRNA localization and the establishment of cell polarity	↑2.04
<u>*cbl*</u>	Casitas B-lineage lymphoma	Proto-oncogene	↑4.06
<u>*cebpb*</u>	CCAAT/enhancer binding protein (C/EBP), beta	DNA binding; regulation of transcription	↑2.55
<u>*cebpd*</u>	CCAAT/enhancer binding protein (C/EBP), delta	Transcription factor regulating the expression of genes involved in immune and inflammatory responses	↑2.27
<u>*dok4*</u>	Docking protein 4	Transmembrane receptor protein tyrosine kinase signaling pathway (MAPK)	↑4.34
g6pdx	Glucose-6-phosphate dehydrogenase X-linked	Catalase oxidative pentose-phosphate pathway	↑5.28
<u>*hk2*</u>	Hexokinase 2	Involved in the metabolism of hexoses	↑2.71
<u>*klf10*</u>	Kruppel-like factor 10	Regulates the circadian expression of genes involved in lipogenesis, gluconeogenesis, and glycolysis in the liver	↑2.01
map2k1	Mitogen activated protein kinase kinase 1	Protein serine/threonine kinase activity, MAPK signaling pathway	↑3.35
<u>*map2k2*</u>	Mitogen activated protein kinase kinase 2	Protein serine/threonine kinase activity, MAPK signaling pathway	↑4.19
<u>*nck1*</u>	Non-catalytic region of tyrosine kinase adaptor protein 1	Adapter protein which associates with tyrosine-phosphorylated growth factor receptors	↓0.46
Ptprf	Protein tyrosine phosph-atase, receptor type, F	Transmembrane receptor protein tyrosine phosphatase signaling pathway; role in cell adhesion	↑2.54
raf1	V-raf-leukemia viral oncogene 1	Protein serine/threonine kinase activity; Cell growth and/or maintenance; Regulation of cell cycle	↑2.41
rras	Harvey rat sarcoma oncogene, subgroup R	Regulates the organization of the actin cytoskeleton with OSP-BL3; modulates integrin β-1 (ITGB1) activity	↑2.02
rras2	Related RAS Viral (R-Ras) Oncogene Homolog 2	A plasma membrane-associated GTP-binding protein with GTPase activity; transduces growth inhibitory signals across the cell membrane exerting its effect *via* an effector shared with the Ras proteins but in an antagonistic fashion	↑3.09
slc27a4	Solute carrier family 27 (fatty acid transporter), member 4	Involved in translocation of long-chain fatty acids (LFCA) across the plasma membrane; the principal fatty acid transporter in small intestinal enterocytes; formation of the epidermal barrier; required for fat absorption during early embryogenesis	↑2.07
sorbs1	Sorbin and SH3 domain containing 1	Involved in tyrosine phosphorylation of by linking CBL to the insulin receptor; required for insulin-stimulated glucose transport; involved in formation of actin stress fibers and focal adhesions	↑3.20

→

Table 2 (cont.). Effect of genistein on the insulin pathway gene expression in differentiated myoblast C2C12 cell line after 4 h and 24 h incubation. Underlined are genes with switched on expression compare to the appropriate control condition (4 h or 24 h); "↑" — genes with increased expression; "↓" — genes with decreased expression. Information on gene functions is quoted according to the UniProtKB database

Gene symbol	Gene name	Function	Expression level
List of genes expressed after 24 hours of cell incubation with 1 μM genistein			
akt1	serine/threonine-protein kinase1	Regulates many processes including metabolism, proliferation, cell survival, growth and angiogenesis	↑ 2.30
<u>*cbl*</u>	Casitas B-lineage lymphoma	Proto-oncogene that encodes a RING finger E3 ubiquitin ligase. The encoded protein is one of the enzymes required for targeting substrates for degradation by the proteasome	↓ 0.37
gck	Glucokinase	Catalyzes the initial step in utilization of glucose by the beta-cell and liver at physiological glucose concentration	↓ 0.45
sorbs1	Sorbin and SH3 domain containing 1	Involved in tyrosine phosphorylation of CBL by linking CBL to the insulin receptor; required for insulin-stimulated glucose transport; involved in formation of actin stress fibers and focal adhesions	↓ 0.39

Abbreviations as in the description of Table 1.

glucose uptake in mouse MC3T3-G2/PA6 cells differentiated into mature adipocytes. Higher concertation of genistein (10 μM) significantly decreased glucose uptake and this inhibitory effect was not reversed by addition of insulin. We also observed similar correlation; genistein at low concentration did not affect insulin-stimulated glucose uptake; however, at high concentration (50 μM) it inhibited insulin-stimulated glucose transport (our unpublished data). Nomura *et al.* [30] also showed that genistein (10 μM) had no effect on insulin-stimulated phosphorylation of PKB. Also Bazuine *et al.* [31] showed in 3T3-L1 adipocytes that the higher genistein concentration (above 20 μM) caused inhibition of glucose uptake, which was independent of protein kinase PKB inhibition. These authors concluded that genistein affected function of proteins such as the GLUT4 transporter and that, in 3T3-L1 adipocytes, genistein is a potent and direct inhibitor of GLUT-4 insulin-stimulated glucose uptake [31]. Another study showed that the effect of genistein in 3T3-L1 adipocytes was unrelated to insulin-stimulated GLUT4 translocation from the low-density microsomes to the plasma membrane [32]. Authors suggested that observed effect might be related to the decrease in the intrinsic activity of the glucose uptake by conformational change of GLUT-4 transporter. Interestingly, we found that high concentration of genistein (50 μM) adversely affected energy metabolism in C2C12 cells. It also enhanced fatty acids metabolism during a short-term period (1–3 hours) of incubation. This observation may explain a positive therapeutic effect of genistein on human obesity and diabetes [6]. We think that the inhibitory

effect of 50 μM genistein on the glucose uptake can be related to the increased level of membrane phosphatase SH-PTP2 responsible for muting the signal from induced insulin receptor. This phosphatase may play a role in the inhibition of the glucose transport by genistein, because its cell content increased parallely to increased genistein concentration.

In addition, some *in vitro* studies have demonstrated positive effects of genistein on glucose transport in the C2C12 cells (stimulation of about 100%, at concentrations from 0.01 to 100 μM) that was associated with the increased phosphorylation level of the insulin receptor substrate IRS-1 [29]. Genistein also increased the Akt phosphorylation level in aged rats, which may be related to the increase in the upstream interaction of ERβ with p85α protein [6]. We observed that the C2C12 cells incubated with 0.1 μM genistein showed about 10% increase in glucose transport and 50% increase in the amount of GLUT4 protein at the cell membrane (data not showed). In contrast, the high concentration of genistein (50 μM) resulted in 85% decrease in C2C12 cells growth rate and reduction in the amount of GLUT4 protein at the cell membrane after insulin stimulation (15 min, 100 nM concentration). Also Wang *et al.* [1] showed that in adipoyctes (3T3-L1 cells) genistein had antiglycemic properties associated with the activation of AMPK and induction of GLUT4 translocation to the cell membrane.

Our results demonstrated that 1 μM genistein rises PKB kinase activity by about 40% as compared to the control, but only during the first 10 to 15 min of incubation. The observed effect was not associated

with the increase in glucose transport. The studies on mouse cell line MC3T3-G2/PA6 showed that 1 h incubation with 10 μM genistein has no effect on the inhibition of insulin-stimulated proteins phosphorylation: beta-IR, IRS-1 and Akt [30]. This suggests that the inhibition of glucose transport caused by genistein concentrations higher than 1 μM does not depend on the phosphorylation of insulin pathway kinases.

Our microarray studies showed no significant changes in the level of mRNAs of the above-described proteins associated with insulin signaling pathway. We observed that genistein treatment resulted in different gene expression activation profile than estradiol treatment and modulated the expression level of insulin pathway genes to the lower extent than estradiol [28]. This may suggest that genistein regulates the insulin pathway protein levels by different mode of action than estradiol. It is also of interest that the 24 hours incubation with 1 μM genistein reduced the mRNAs levels of glucokinase (GCK) and Sorbs1, and increased mRNA level of kinase Akt1. The Sorbs1 protein is related to the regulation of cell response to insulin and its mutation causes insulin resistance [33]. In turn, glucokinase regulates carbohydrate metabolism in response to rising or falling levels of glucose [34]. Moreover, it has been shown that genistein is a potent alpha-glucosidase inhibitor [35].

In conclusion, genistein, depending on concentration, causes different biological effects in C2C12 cells. High concentrations strongly decreased glucose transport and cell proliferation as showed by us previously but did not affect β-oxidation of fatty acids. Results obtained in the present study indicate that genistein (at a low concertation of 1 μM) may modulate glucose and lipid metabolism by two independent modes of action. The first would be a direct stimulation of different cell receptors (*i.e.* Insulin-like Growth Factor receptor [36] or estrogen receptors [37, 38]) — after short stimulation there is a time-dependent increase in PKB phosphorylation and the stimulation of fatty acids metabolism. The second mode, a long-term stimulatory effect, would be related to the modulation at the transcriptional level expression of genes associated with insulin signaling pathway. Further detailed studies on these two modes of regulation may help to develop genistein-based anti-obese and anti-diabetic therapies.

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Disclosure statement

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References

1. Wang M, Gao XJ, Zhao WW, et al. Opposite effects of genistein on the regulation of insulin-mediated glucose homeostasis in adipose tissue. Br J Pharmacol. 2013; 170(2): 328–340, doi: 10.1111/bph.12276, indexed in Pubmed: 23763311.
2. He FJ, Chen JQ. Consumption of soybean, soy foods, soy isoflavones and breast cancer incidence: Differences between Chinese women and women in Western countries and possible mechanisms. Food Science and Human Wellness. 2013; 2(3-4): 146–161, doi: 10.1016/j.fshw.2013.08.002.
3. Talaei M, Pan An. Role of phytoestrogens in prevention and management of type 2 diabetes. World J Diabetes. 2015; 6(2): 271–283, doi: 10.4239/wjd.v6.i2.271, indexed in Pubmed: 25789108.
4. Fu Z, Gilbert ER, Liu D. Regulation of insulin synthesis and secretion and pancreatic Beta-cell dysfunction in diabetes. Curr Diabetes Rev. 2013; 9(1): 25–53, indexed in Pubmed: 22974359.
5. Miller BR, Nguyen H, Hu CJH, et al. New and emerging drugs and targets for type 2 diabetes: reviewing the evidence. Am Health Drug Benefits. 2014; 7(8): 452–463, indexed in Pubmed: 25558307.
6. Behloul N, Wu G. Genistein: a promising therapeutic agent for obesity and diabetes treatment. Eur J Pharmacol. 2013; 698(1-3): 31–38, doi: 10.1016/j.ejphar.2012.11.013, indexed in Pubmed: 23178528.
7. Gilbert ER, Liu D. Anti-diabetic functions of soy isoflavone genistein: mechanisms underlying its effects on pancreatic β-cell function. Food Funct. 2013; 4(2): 200–212, doi: 10.1039/c2fo30199g, indexed in Pubmed: 23160185.
8. Fu Z, Zhang W, Zhen W, et al. Genistein induces pancreatic beta-cell proliferation through activation of multiple signaling pathways and prevents insulin-deficient diabetes in mice. Endocrinology. 2010; 151(7): 3026–3037, doi: 10.1210/en.2009-1294, indexed in Pubmed: 20484465.
9. Fu Z, Liu D. Long-term exposure to genistein improves insulin secretory function of pancreatic beta-cells. Eur J Pharmacol. 2009; 616(1-3): 321–327, doi: 10.1016/j.ejphar.2009.06.005, indexed in Pubmed: 19540219.
10. You F, Li Q, Jin G, et al. Genistein protects against Aβ induced apoptosis of PC12 cells through JNK signaling and modulation of Bcl-2 family messengers. BMC Neurosci. 2017; 18(1): 12, doi: 10.1186/s12868-016-0329-9, indexed in Pubmed: 28081713.
11. Lee SJ, Kim HE, Choi SE, et al. Involvement of Ca2+/calmodulin kinase II (CaMK II) in genistein-induced potentiation of leucine/glutamine-stimulated insulin secretion. Mol Cells. 2009; 28(3): 167–174, doi: 10.1007/s10059-009-0119-7, indexed in Pubmed: 19756396.
12. Liu D, Zhen W, Yang Z, et al. Genistein acutely stimulates insulin secretion in pancreatic beta-cells through a cAMP-dependent protein kinase pathway. Diabetes. 2006; 55(4): 1043––1050, indexed in Pubmed: 16567527.
13. Uifălean A, Schneider S, Ionescu C, et al. Soy Isoflavones and Breast Cancer Cell Lines: Molecular Mechanisms and Future Perspectives. Molecules. 2015; 21(1): E13, doi: 10.3390/molecules21010013, indexed in Pubmed: 26703550.

14. Murata M, Midorikawa K, Koh M, et al. Genistein and daidzein induce cell proliferation and their metabolites cause oxidative DNA damage in relation to isoflavone-induced cancer of estrogen-sensitive organs. Biochemistry. 2004; 43(9): 2569–2577, doi: 10.1021/bi035613d, indexed in Pubmed: 14992594.

15. Moore AB, Castro L, Yu L, et al. Stimulatory and inhibitory effects of genistein on human uterine leiomyoma cell proliferation are influenced by the concentration. Hum Reprod. 2007; 22(10): 2623–2631, doi: 10.1093/humrep/dem185, indexed in Pubmed: 17725991.

16. Choi EJ, Jung JY, Kim GH. Genistein inhibits the proliferation and differentiation of MCF-7 and 3T3-L1 cells via the regulation of ERα expression and induction of apoptosis. Exp Ther Med. 2014; 8(2): 454–458, doi: 10.3892/etm.2014.1771, indexed in Pubmed: 25009600.

17. Yu JY, Lee JJ, Lim Y, et al. Genistein inhibits rat aortic smooth muscle cell proliferation through the induction of p27kip1. J Pharmacol Sci. 2008; 107(1): 90–98, indexed in Pubmed: 18490856.

18. Kayisli UA, Guzeloglu-Kayisli O, Guzel E, et al. Genistein inhibits cell proliferation and stimulates apoptosis in human coronary artery endothelial cells. Gynecol Obstet Invest. 2013; 75(4): 235–242, doi: 10.1159/000345936, indexed in Pubmed: 23594697.

19. Wang X, Clubbs EA, Bomser JA. Genistein modulates prostate epithelial cell proliferation via estrogen- and extracellular signal-regulated kinase-dependent pathways. J Nutr Biochem. 2006; 17(3): 204–210, doi: 10.1016/j.jnutbio.2005.07.005, indexed in Pubmed: 16198100.

20. Shafiee G, Saidijam M, Tavilani H, et al. Genistein Induces Apoptosis and Inhibits Proliferation of HT29 Colon Cancer Cells. Int J Mol Cell Med. 2016; 5(3): 178–191, indexed in Pubmed: 27942504.

21. LOWRY OH, ROSEBROUGH NJ, FARR AL, et al. Protein measurement with the Folin phenol reagent. J Biol Chem. 1951; 193(1): 265–275, indexed in Pubmed: 14907713.

22. Manning NJ, Olpin SE, Pollitt RJ, et al. A comparison of [9,10-3H]palmitic and [9,10-3H]myristic acids for the detection of defects of fatty acid oxidation in intact cultured fibroblasts. J Inherit Metab Dis. 1990; 13(1): 58–68, doi: 10.1007/bf01799333, indexed in Pubmed: 2109149.

23. Gil-Izquierdo A, Penalvo JL, Gil JI, et al. Soy isoflavones and cardiovascular disease epidemiological, clinical and -omics perspectives. Curr Pharm Biotechnol. 2012; 13(5): 624–631, indexed in Pubmed: 22122477.

24. Ramdath DD, Padhi EMT, Sarfaraz S, et al. Beyond the Cholesterol-Lowering Effect of Soy Protein: A Review of the Effects of Dietary Soy and Its Constituents on Risk Factors for Cardiovascular Disease. Nutrients. 2017; 9(4), doi: 10.3390/nu9040324, indexed in Pubmed: 28338639.

25. Cederroth CR, Vinciguerra M, Gjinovci A, et al. Dietary phytoestrogens activate AMP-activated protein kinase with improvement in lipid and glucose metabolism. Diabetes. 2008; 57(5): 1176–1185, doi: 10.2337/db07-0630, indexed in Pubmed: 18420492.

26. Tanigawa T, Shibata R, Kondo K, et al. Soybean β-Conglycinin Prevents Age-Related Hearing Impairment. PLOS ONE. 2015; 10(9): e0137493, doi: 10.1371/journal.pone.0137493.

27. Ng WWH, Keung W, Xu YC, et al. Genistein potentiates protein kinase A activity in porcine coronary artery. Mol Cell Biochem. 2008; 311(1-2): 37–44, doi: 10.1007/s11010-007-9691-3, indexed in Pubmed: 18165926.

28. Lewicki S, Lewicka A, Kalicki B, et al. Chromium III ions imitate the estradiol influence on expression of insulin pathway related genes and proteins in mouse myocytes cell line C2C12. Trace Elements and Electrolytes. 2016; 33(01): 28–34, doi: 10.5414/tex01405.

29. Jou SB, Huang CC, Liu IM, et al. Activation of alpha1A-adrenoceptors by genistein at concentrations lower than that to inhibit tyrosine kinase in cultured C2C12 cells. Planta Med. 2004; 70(7): 610–614, doi: 10.1055/s-2004-827182, indexed in Pubmed: 15254853.

30. Nomura M, Takahashi T, Nagata N, et al. Inhibitory Mechanisms of Flavonoids on Insulin-Stimulated Glucose Uptake in MC3T3-G2/PA6 Adipose Cells. Biological & Pharmaceutical Bulletin. 2008; 31(7): 1403–1409, doi: 10.1248/bpb.31.1403.

31. Bazuine M, van den Broek PJA, Maassen JA. Genistein directly inhibits GLUT4-mediated glucose uptake in 3T3-L1 adipocytes. Biochem Biophys Res Commun. 2005; 326(2): 511–514, doi: 10.1016/j.bbrc.2004.11.055, indexed in Pubmed: 15582607.

32. Smith RM, Tiesinga JJ, Shah N, et al. Genistein inhibits insulin-stimulated glucose transport and decreases immunocytochemical labeling of GLUT4 carboxyl-terminus without affecting translocation of GLUT4 in isolated rat adipocytes: additional evidence of GLUT4 activation by insulin. Arch Biochem Biophys. 1993; 300(1): 238–246, doi: 10.1006/abbi.1993.1033, indexed in Pubmed: 8424658.

33. Germain M, Pezzolesi MG, Sandholm N, et al. SORBS1 gene, a new candidate for diabetic nephropathy: results from a multi-stage genome-wide association study in patients with type 1 diabetes. Diabetologia. 2015; 58(3): 543–548, doi: 10.1007/s00125-014-3459-6, indexed in Pubmed: 25476525.

34. Rui L. Energy metabolism in the liver. Compr Physiol. 2014; 4(1): 177–197, doi: 10.1002/cphy.c130024, indexed in Pubmed: 24692138.

35. Lee DS, Lee SH. Genistein, a soy isoflavone, is a potent alpha-glucosidase inhibitor. FEBS Lett. 2001; 501(1): 84–86, indexed in Pubmed: 11457461.

36. Chen WF, Wong MS. Genistein enhances insulin-like growth factor signaling pathway in human breast cancer (MCF-7) cells. J Clin Endocrinol Metab. 2004; 89(5): 2351–2359, doi: 10.1210/jc.2003-032065, indexed in Pubmed: 15126563.

37. Ponnusamy S, Tran QT, Harvey I, et al. Pharmacologic activation of estrogen receptor β increases mitochondrial function, energy expenditure, and brown adipose tissue. FASEB J. 2017; 31(1): 266–281, doi: 10.1096/fj.201600787RR, indexed in Pubmed: 27733447.

38. Hamilton D, Minze L, Kumar T, et al. Estrogen receptor alpha activation enhances mitochondrial function and systemic metabolism in high-fat-fed ovariectomized mice. Physiol Rep. 2016; 4(17): e12913, doi: 10.14814/phy2.12913, indexed in Pubmed: 27582063.