

Anti-diabetic and Pharmacological Activities, of Phytoestrogens

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Abstract

Isoflavones class of phytoestrogens including, genestein, daidzein and formononetin found in human dietary and show wide range of biological effect. In this study the impact of these phytoestrogens on glucose uptake was investigated in Hepatocellular carcinoma (HepG2 cells), using, 2-(N-(7-nitrobenz-2-oxa-1, 3- diazol-4-yl) amino)-2-deoxyglucose and Omega FluoStar plate reader. Incubation of cells (10^4 /ml, in 24 well plate at 37 °C in 5% CO₂ / air) with three phytoestrogens at concentration of 10^{-4} M to 10^{-9} M in two studies mood, short term treatment (one hour) and long term treatment (24 hours) revealed, daidzein stimulates uptake of glucose, with a greater effect after a short treatment of one hour compared with treatment 24 hours. The effect was broadly higher at the lower concentrations tried, 1 μM to 1nM, at which glucose uptake was increased four to five fold after a one hour treatment. Interestingly the higher concentrations tried, 10 and 100 μM, showed less effect. Longer treatments resulted in a lesser stimulation of glucose uptake, and a peak effect was found at 0.1 μM. genestein exerted slightly inhibitory effect after one hour treatment compared with control, with the exception of treatment at 1 μM, which stimulated uptake about three-fold compared with control. Longer treatments with 10^{-4} M to 10^{-6} M genistein resulted in gradual increase in glucose uptake to 2.4 times more than control, and thereafter a decline. Overall there was significant difference in glucose uptake between short and long term treatments, as indicated by two-way ANOVA, but the differences depend on the concentration of genistein, with higher concentrations of 10 and 100 μM inducing greater uptake after 24 hours treatment but at lower concentrations, uptake was stimulated more after a one hour treatment. A short treatment with formononetin inhibited glucose uptake, while longer treatments had variable effects, with an approximately two fold stimulation across a range of concentrations. HepG2 cells showed a significant increase in glucose uptake after treatment with phytoestrogens compared to the control.

Keywords: Phytoestrogens, Hypoglycaemia, In vitro study



Introduction

Diabetes is an endocrine disease characterised by a chronic increase in blood sugar levels caused by a deficiency of insulin production, which leads to type 1 diabetes, or by a loss of tissue response to insulin, which leads to type 2 diabetes. The disease leads to disruption of metabolism, vascular damage and damage to the nervous system, as well as damage to other organs and systems. Diabetes mellitus (DM) is an important public health concern that affects more than 170 million individuals worldwide. It is expected that the number of people suffering from diabetes in the UK will reach approximately 5 million, or almost 10% of the population, by 2025 and it is one of the leading causes of death worldwide (Abeeleh *et al.* 2009). Diabetes is a multi-organ disease independent of age, race and gender. Efforts have accordingly been increased towards developing and refining treatments as well as to addressing the underlying causes of the disease. Many parts of the world have a documented history of the use of phytochemicals to treat diabetes, and these can be an attractive and alternative to expensive pharmaceutical medicines. Accordingly there is an increasing interest in identifying new phytochemicals with proven pharmacological effects on diabetes. Dietary phytoestrogens have been shown to play a beneficial role in obesity and diabetes, so this study investigated the effect of phytoestrogens on glucose uptake. Three phytoestrogens (daidzein, formononetin and genestin, which are naturally occurring isoflavones) were chosen.

Material and methods

HepG2 cells were obtained from King's College Medical School Liver Unit. DMEM (Dulbecco's Modified Eagle Medium) glucose free and with glucose, methanol, dimethyl sulfoxide (DMSO), Costar 24 well plates, 2-(N-(7-nitrobenz-2-oxa-1, 3-diazol-4-yl) amino)-2-deoxyglucose (2-NBDG), daidzein, formononetin and genestin were obtained from Sigma Aldrich (Poole, Dorset, UK).

Cell Culture

The human hepatoma HepG2 cell line was used in this work, and was grown in DMEM with 11 mM glucose (Gibco) and 10% foetal calf serum. Glucose was added to 11 mM for most experiments unless otherwise stated. HepG2 cell cultures were started from frozen cells, using standard protocols (Hanaa *et al.* 2011). A vial of cells was collected from liquid nitrogen storage and the lower half submerged in a water bath at 37 °C. After allowing to thaw until a small amount of ice remained in the vial (usually 1-2 minutes), the vial was transferred to a sterile safety cabinet. The vial was wiped with a tissue moistened with 70% alcohol, the cap removed and the cells were removed by slow pipetting to pre-warmed growth medium. The cells were centrifuged at 1000 rpm at room temperature for 5 minutes to remove the DMSO in the cryopreservation medium. The medium was carefully removed and replaced with new medium and the cells slowly pipetted to a flask. Cells were grown at 37 °C in 5% CO₂/ air.

The HepG2 cell line is anchorage dependent and must be cultured while attached



to a solid or semi-solid substrate (adherent or monolayer culture). The fibroblastic morphology of the cells was examined by microscopy at each sub-culture

Glucose uptake in HepG2 cells

For *in vitro* glucose uptake assays using 2-NBDG, it was important to optimize the conditions of uptake of 2-NBDG by HepG2. 2-NBDG is a fluorescent derivative of glucose modified with a 2-[N-(7-nitrobenz-2-oxa-1, 3-

diazol-4-yl) amino group at the C-2 position (Molecular Weight = 342.26) (Yoshioka 1996). The probe shows intense fluorescence at 542 nm when excited at 467 nm. 5mg 2-NBDG was dissolved in 1.46 mL of methanol in order to make a 10mM as a stock solution. 5 mM, 1 mM, 0.5 mM and 0.1mM solutions were prepared in methanol, as shown in Table 2.1. Methanol only was used as a vehicle control.

Table 2.1- Dilutions of 2NBDG used for optimising the uptake assay.

Final concentration of 2-NBDG (mM)	Stock/Vol.	Vol MeOH
5	10mM/100 μ L	100 μ L
1	5mM/40 μ L	160 μ L
0.5	1mM/100 μ L	100 μ L
0.1	0.5Mm/40 μ L	160 μ L

HepG2 cells were sub-cultured using DMEM medium with glucose, 10mL of medium was added and cells were transferred into flasks, cells were seeded at a density 5.0×10^4 cells per mL and incubated with 10 mL of medium. Cells were counted using a haemocytometer and diluted to 0.5×10^5 cells/mL. Then the cells were aliquotted at 0.5 mL per well in a 24-well plate and incubated for 24 hours. 5 μ L of 0, 0.1, 0.5, 1, 5, or 10 mM 2-NBDG, was added to columns one to six respectively (from right to left), so that the 2-NBDG was diluted 100 times. Cells were incubated at 37 °C for 15 minutes then washed with PBS and fluorescent uptake was measured using an Omega FluoStar (BMG LabTech, Ayelsbury, Bucks, UK) plate reader

with $\lambda_{ex} = 485\text{nm}$, $\lambda_{em} = 530\text{nm}$, using the bottom optic and a gain of 1000.

Various modifications of the procedure were tried. One involved replacing normal medium with glucose-free medium after 24 hours. Another concern was the optimal period of incubation with 2-NBDG, and uptake was accordingly monitored from 15 minutes to 2 hours. The glucose uptake increase was checked by fluorescence microscopy.

Glucose uptake measurement after treating confluent HepG2 cells with three phytoestrogens.

This part of the project was started by making serial dilutions of three phytoestrogens genestein, daidzein and formononetin (Sigma



Aldrich, UK). Stock solutions of 10 mM, 1 mM, 100 μ M, 10 μ M, 1 μ M, and 0.1 μ M were prepared in DMSO. HepG2 cells were cultured and treated as described in section 2.4.

Results

Glucose uptake measurement in HepG2 cells treated with daidzein genestein and formononetin.

HepG2 cells were cultured and seeded in a 24 well plate at a concentration of 0.5×10^5 cells/mL, and the next day the cells were treated with 5 μ L daidzein, genestein and formononetin, with six different concentrations, started from 10^{-4} M to 10^{-9} M. For short-term treatments the cells were incubated only for one hour after treatment, while for long term treatment the cell were treated for 24 hours. After the treatment period the medium was replaced with glucose free medium and 2-NBDG added. The measurement was performed after 15 minutes of incubation with the fluorescent analogue.

Discussion and Conclusion

Analysing the hypoglycaemic effect of phytoestrogens was purpose of this work where we applied the optimised method for usage of 2-NBDG to assess glucose uptake effect of the phytoestrogens. HepG2 cells were treated with Isoflavones phytoestrogens

including daidzein, genestein and formononetin and each treatment was separately performed and analysed. The daidzein data, Fig.1.1 revealed that in both modes of study the uptake were stimulated and all the concentrations exerted increased effect on the uptake. However a better effect came from short term treatment, and concentration of 10^{-9} M is the most potent treatment. With genistein (Fig. 1.2) the situation is different, as in both modes of study, the change in glucose uptake fluctuated. In the one hour test the only concentration of genistein that showed an increase effect on the uptake was 10^{-6} M and it was about 2 times more than control. The rest of the concentrations did not have a positive effect on glucose consumption in HepG2 cells. In long treatments only two of the genistein concentrations appeared to have a positive effective on modulating the uptake, 10^{-6} M and 10^{-5} M.

The information from formononetin assay and its effect on glucose uptake (Fig 1.3), shows that only the long term method of treatment can stimulate the consumption of glucose, where the lowest concentration 10^{-9} M is the most potent compared with the rest of concentrations, and increased the uptake around 2.5 times more than control.



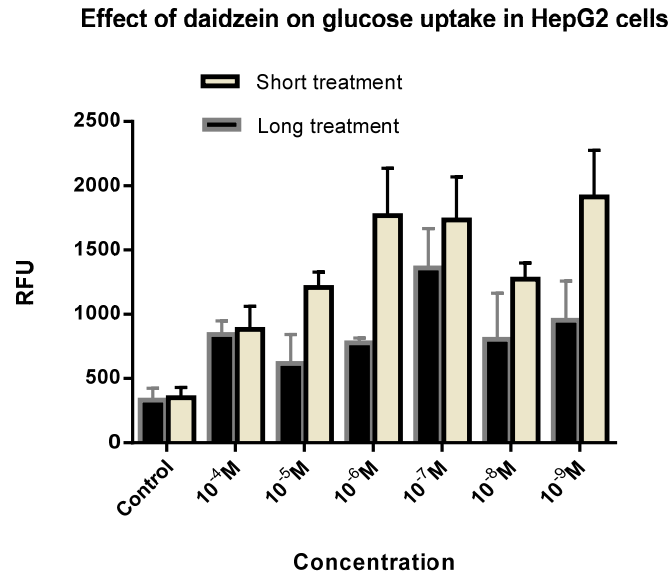


Figure 1.1- Effect of daidzein on glucose consumption in HepG2 cells. Uptake of the fluorescent glucose analogue 2-NBDG was measured in HepG2 cells treated with daidzein for short and long term periods at the indicated concentrations and compared to control untreated cells. The data are mean+S.D. of three independent experiments in triplicate. $P^{****} < 0.0001$ comparing duration of treatment, $P^{****} < 0.0001$ comparing daidzein with control, as determined by two-way ANOVA.

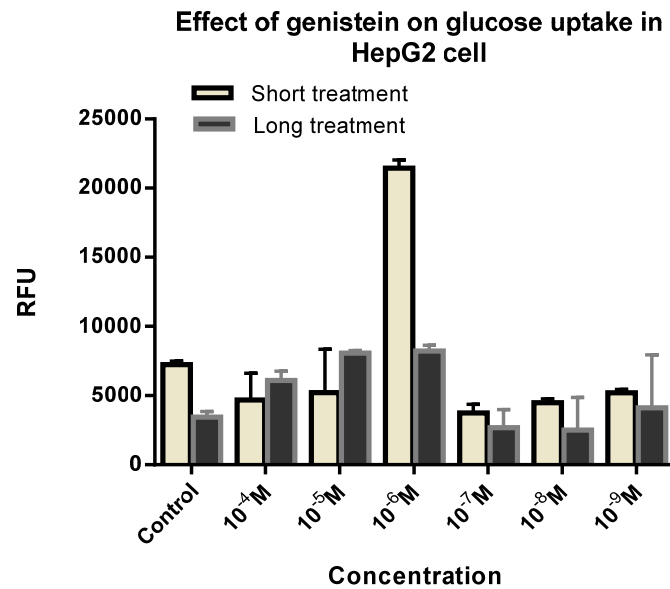


Figure 1.2- Evaluation of genistein on glucose uptake in HepG2 cells. Glucose consumption test was performed in HepG2 cells treated with range of genistein concentrations from 10⁻⁴ M to 10⁻⁹ M, for one hour and 24 hour compared with untreated control. The data are mean+S.D. of three independent experiments in triplicate. $P^{****} < 0.0001$ comparing duration of treatment, $P^{****} < 0.0001$ comparing genistein with controls determined by two-way ANOVA.



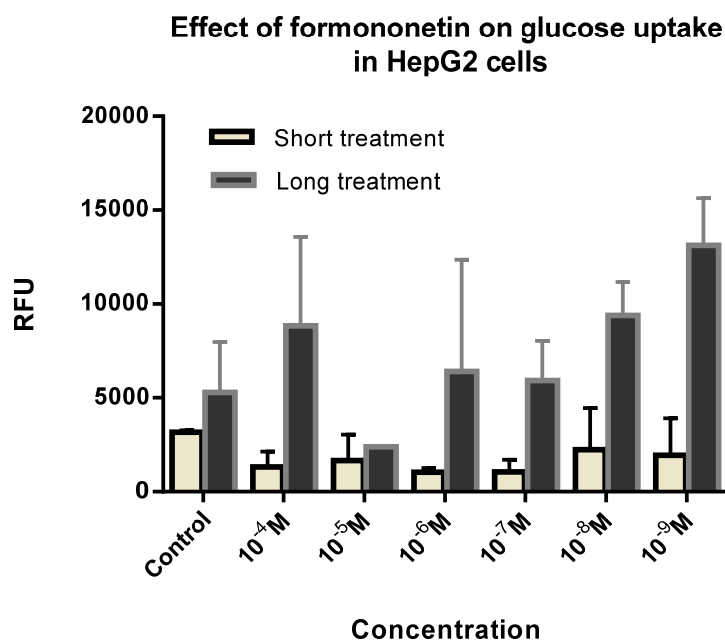


Figure 1.3- Effect of formononetin effect on glucose uptake in HepG2 cells. Glucose uptake measured in HepG2 cells after short and long treatments with six different concentrations of formononetin compared with untreated control. The data are mean+S.D. of three independent experiments in triplicate. P**** 0.0001 comparing duration of treatment, P***<0.0001 as determined by two-way ANOVA.

The effect of phytoestrogens, mainly in type 2 diabetes, have been extensively studied previously (Jayagopal *et al.* 2002; Azadbakht *et al.* 2008; Hermansen *et al.* 2001; Liu *et al.* 2010; Clerici *et al.* 2011; Anderson *et al.* 1998; Teixeira *et al.* 2004; Gobert *et al.* 2010).

In this work we assessed the effect of daidzein, genistein and formononetin on glucose uptake in an *in vitro* study. The finding in this section in terms of the isoflavones' hypoglycaemic effect is supported by previous studies.

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