EFFECT OF GENISTEIN ON SIGNALING PATHWAYS INVOLVED IN THE REGULATION OF PHOSPHOENOLPYRUVATE CARBOXYKINASE (CYTOSOLIC) IN HepG2 CELLS

ABSTRACT

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Type 2 diabetes mellitus (T2DM) is a common and increasingly prevalent disease, which has become a major public health concern worldwide and is also currently one of the most costly and burdensome chronic diseases. One of the hallmarks of diabetes is the inability of insulin to inhibit hepatic glucose production resulting in aberrant activation of gluconeogenesis. It is now well recognized that regulation of blood glucose levels in diabetic patients should be the primary concern upon which the treatment is based. In this context, the cytosolic form of phosphoenolpyruvate carboxykinase (PEPCK-C), a rate-limiting enzyme of hepatic gluconeogenesis, has been found to be dysregulated in T2DM. This observation emphasizes the role that PEPCK-C plays in the complications of, and in some cases, perhaps the etiology of diabetes thus making this enzyme an important target for combatting this disease. Despite the availability of several drugs for the treatment of diabetes, adverse effects and drug resistance are of great concern. Therefore, there is an urgent need to continue working on the prevention and control of this pathology, and as a promising alternative, researchers are seeking natural products to prevent or treat diabetes because of their potential beneficial effects on health and their safety. Recently, soy isoflavones, have attracted increasing attention due to their beneficial effects on human health, such as lowering incidences of some diseases, including prostate cancer, cardiovascular disease, osteoporosis, obesity, and T2DM. Nutritional intervention studies performed in animals and humans suggest that the ingestion of soy protein associated with isoflavones reduces serum insulin and improves glucose control and insulin resistance. In studies of human subjects with or without diabetes,
soy protein also appears to moderate hyperglycemia and reduce body weight, hyperlipidemia, and hyperinsulinemia, supporting its beneficial effects on obesity and diabetes. Genistein, predominantly found in soybean and soybean derived products, is a major source of phytoestrogens in human diets. Genistein is the most studied isoflavone and has been previously investigated for its potential beneficial effects on cancer treatment, cognitive function, and cardiovascular and skeletal health, with a primary focus on exploring its potential hypolipidemic, anti-oxidative, and estrogenic effects. While studies on whether genistein has an effect on diabetes are quite limited, available data shows that administration of genistein moderately lowers plasma glucose in diabetic patients and animal models. The anti-diabetic effects of genistein have been correlated with its insulin sensitizing effects, stimulation of β- cell proliferation, and activation of peroxisome proliferator-activated receptors (PPAR) and AMP-activated protein kinase (AMPK) in animal models. However, the effect of genistein on the expression of the gluconeoncic enzyme, PEPCK-C, has not been thoroughly investigated. Available reports suggest that genistein alters PEPCK-C activity in diabetic animals and also modulates PEPCK-C expression in cultured cells. However, the underlying mechanisms involved in the regulation of PEPCK-C expression by genistein still remain elusive.
Therefore, the objectives of the present investigation include the following aspects:

- to study the effect of genistein on signalling pathways, particularly Akt/PKB and MAPK, responsible for PEPCK-C (NM_002591) expression in HepG2 cells, and
- to evaluate cross talk, if any, between the pathways, which is responsible for PEPCK-C expression.

In order to accomplish the objectives in this study, the toxicity of genistein over a range of concentration (1-50 µM) was first determined in HepG2 cells by MTT assay. The lowest and most realistic range of genistein doses (1-30 µM) was then used to investigate the expression of PEPCK-C in HepG2 cells. To achieve this, the cells were treated with different concentration of genistein for 24 h and PEPCK-C gene expression was studied by real-time PCR and western blot. The effect of genistein on PEPCK-C activity, promoter activity, and glucose production was also studied. For real-time PCR, gene specific primers for human PEPCK-C and GAPDH were designed. Total RNA was isolated from the treated HepG2 cells followed by first strand cDNA synthesis. The effect of genistein on the abundance of PEPCK-C transcripts in the treated cells was monitored by real-time PCR using GAPDH as the endogenous control. The effect of genistein on the expression of PEPCK-C protein levels was also studied by western blot. For determining the effect of genistein on PEPCK-C promoter activity, gene-specific primers were similarly designed using the human PEPCK-C promoter sequence (range -1500 to +298 bp) obtained from
Eukaryotic Promoter Database (EPD, http://epd.vital-it.ch/). DNA was isolated from the treated cells and the promoter region (-686/+83 bp) was amplified by PCR using the designed primers. The amplified promoter region was purified and cloned into pGL3 basic vector (Promega) and the resulting PEPCK-C promoter insert was used to transfect HepG2 cells with pRL-SV40 vector as the internal control. The effect of genistein on PEPCK-C promoter activity was assayed by luciferase assay and basal promoter activity was determined in the absence of genistein. To investigate the possible pathways modulated by genistein, the expression of PKB/Akt, AMPK and MEK/ERK pathway proteins and the nuclear levels of phosphorylated Foxo1 and ERK½ was studied by western blot. In addition, the effect of genistein on the recruitment of Foxo1 to PEPCK-C promoter was also investigated by Chromatin immunoprecipitation (ChIP) assay. Molecular docking was also performed to ascertain the role of genistein as a possible AMPK modulator using Discovery Studio 4.1 software (Accelrys-Biovia) (DS 4.1). For studying the pathways followed by genistein in regulating PEPCK-C expression, the cells were pre-treated with various pathway inhibitors such as compound C, LY29004 and PD98059 prior to genistein treatment, and PEPCK-C expression was studied by real-time PCR and western blot. The biological relevance of the \textit{in vitro} findings in HepG2 cells was also tested \textit{in vivo} using alloxan-induced diabetic mice. The optimal dose of genistein was first determined by glucose tolerance test (GTT) in normal mice. The optimised dose (50 mg/kg body weight) was then used to treat alloxan-induced diabetic animals on alternate days for 2 weeks. The effect of genistein on fasting blood glucose levels was monitored after 1 and 2 weeks of treatment and the hepatic expression of
PEPCK-C AMPK, ERK½ and Foxo1 was studied by western blot at the end of the study.

The results obtained in this study are:

1. Genistein reduced PEPCK-C expression and activity in HepG2 cells in a dose and time-dependent manner. Out of all the doses tested 30 µM was found to be the most effective in reducing PEPCK-C mRNA levels (~0.8-fold) and protein expression (~0.6-fold). Consistent with these findings, genistein also decreased PEPCK-C promoter activity (~0.3-fold) and glucose production (~50%).

2. The PKB/Akt pathway played no significant role in mediating the effects of genistein on PEPCK-C expression in HepG2 cells. Further, the nuclear localization of Foxo1 and its recruitment to the PEPCK-C promoter was also unaltered by genistein as demonstrated by ChIP analysis.

3. The phosphorylation status of AMPK was increased by genistein treatment in a dose-dependent manner (up to ~3.2-fold). Genistein and AICAR reduced PEPCK-C protein levels by about 0.5-fold and 0.4-fold, respectively; whereas, compound C increased PEPCK-C levels by ~2.5-fold. Similar findings were also demonstrated by real-time PCR, thereby, indicating the involvement of AMPK in regulating PEPCK-C expression.

4. Docking study suggested that like other AMPK activators, such as AICAR and A-769662, genistein also showed similar interactions with AMPK. However, genistein closely resembled A-769662 in terms of its interactions with AMPK.
5. Genistein activated the MEK/ERK pathway by increasing the phosphorylation status of MEK½ (~1.4-fold) and ERK½ (~6.8-fold). In agreement with these findings, the level of p-ERK½ was also increased by genistein in the nuclear (~3.5-fold) and cytoplasmic extracts (~2.5-fold). Further, pre-treatment of cells with PD98059 greatly diminished genistein effects on PEPCK-C expression and glucose production.

6. Pre-treatment of cells with PD98059 and LY29004 suggested that there was no cross talk between the PKB/Akt and MEK/ERK pathways.

7. Pre-treatment of cells with the AMPK inhibitor compound C had no effect on genistein-induced activation of MEK/ERK pathway suggesting that the latter acted independent of AMPK. The two pathways, however, appeared to have additive effects on PEPCK-C expression and glucose production.

8. Genistein significantly increased the phosphorylation status of CRTC2 (~1.4-fold), an effect which was greatly reduced in the presence of compound C. Hence AMPK appeared to regulate PEPCK-C expression by targeting its downstream target, CRTC2.

9. GTT showed that genistein (50 mg/kg body weight) was more effective than the lower dose (10 mg/kg body weight) in lowering blood glucose levels in normal mice.

10. Genistein treatment slightly prevented body weight loss in diabetic mice as compared to the diabetic control group. There was no significant change in body weight between the genistein-treated normal mice and normal control group. Genistein also reduced water consumption without affecting food intake in
diabetic mice as compared to the diabetic control group. No significant change was also observed in the liver weight of all the groups after 2 weeks of genistein treatment.

11. Treatment of alloxan-induced diabetic mice with genistein (50 mg/kg body weight) for 2 weeks significantly lowered blood glucose levels (189.00 mg/dl ±6.56) compared to diabetic control (372.33 mg/dl±6.02). There was, however, no significant difference in the blood glucose levels between the genistein-treated normal mice (116.00 mg/dl ±12.77) and the normal control group (127.00 mg/dl ±7.57) after 2 weeks of treatment.

12. No significant difference was observed in the liver glycogen content and ALT and AST activities of diabetic mice (both control and genistein treated) and normal mice (both control and genistein treated)

13. Genistein increased the phosphorylation states of AMPK (~2-fold) and ERK½ (1.4-fold), and lowered PEPCK-C (~0.4-fold) protein expression in alloxan-induced diabetic mice.

Taking the results together, we conclude that genistein is an effective candidate for preventing metabolic disorders such as T2DM and that it acts via the AMPK-CRTC2 and MEK/ERK signalling pathways. Thus, AMPK and MEK/ERK signalling cascades might contribute toward improving human health. The thesis submitted herewith by me contains eighteen (18) figures and eight (8) tables, and four hundred fifty two (452) references are cited.