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Short communication

Estrogen receptor alpha augments changes in hemostatic gene expression in HepG2 cells treated with estradiol and phytoestrogens

Lynne A. Kelly[a,*], Dana Seidlova-Wuttke[b], Wolfgang Wuttke[b], John J. O'Leary[c], Lucy A. Norris[a]

[a] Coagulation Research Laboratory, Department of Obstetrics and Gynaecology, Trinity Centre for Health Sciences, St. James Hospital, Dublin 8, Ireland
[b] Department of Endocrinology, University Medical Center Göttingen, Georg-August-University Göttingen, Germany
[c] Department of Histopathology, Trinity College Dublin, Ireland

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ABSTRACT

Phytoestrogens are popular alternatives to estrogen therapy however their effects on hemostasis in post-menopausal women are unknown. The aim of this study was to determine the effect of the phytoestrogens, genistein, daidzein and equol on the expression of key genes from the hemostatic system in human hepatocyte cell models and to determine the role of estrogen receptors in mediating any response seen. HepG2 cells and Hep89 cells (expressing estrogen receptor alpha (ERα)) were incubated for 24 h with 50 nM 17β-estradiol, genistein, daidzein or equol. Tissue plasminogen activator (tPA), plasminogen activator inhibitor-1 (PAI-1), Factor VII, fibrinogen γ, protein C and protein S mRNA expression were determined using TaqMan PCR. Genistein and equol increased tPA and PAI-1 expression in Hep89 cells with fold changes greater than those observed for estradiol. In HepG2 cells (which do not express ERα), PAI-1 and tPA expression were unchanged. Increased expression of Factor VII was observed in phytoestrogen treated Hep89 cells but not in similarly treated HepG2s. Prothrombin gene expression was increased in equol and daidzein treated HepG2 cells in the absence of the classical estrogen receptors. These data suggest that phytoestrogens can regulate the expression of coagulation and fibrinolytic genes in a human hepatocyte cell line; an effect which is augmented by ERα.

Introduction

Phytoestrogens have become increasingly popular as a 'natural' alternative to HT for treatment of menopausal symptoms and are thought to explain the lower risk of cardiovascular disease in Asia (Knight and Eden 1995). HT is associated with an increased risk of cardiovascular and thromboembolic disease particularly in older users of oral preparations. Although the pathogenesis of this increased risk is not understood, changes in the hemostatic system, originating from altered clearance and synthesis of hemostasis proteins in the liver, are thought to be involved. A growing body of evidence suggests that these effects cannot be explained solely by the classical model of steroid hormone action and that 17β-estradiol can also mediate rapid signaling events via pathways that involve transmembrane ERs, such as G-protein-coupled estrogen receptor termed GPER (Prossnitz et al. 2008).

Data on the effects of phytoestrogens on hemostasis are scarce with some small studies reporting either neutral or anti-thrombotic effects (Kris-Etherton et al. 2002). The beneficial effects of phytoestrogens have been attributed to their ability to bind ERβ (Pilsakova et al. 2010; Kuiper et al. 1998), however, we have previously shown in an animal model of the menopause that genistein can cause a dose dependent increase in the hepatic expression of many coagulation factors in the absence of ERβ (Kelly et al. 2010). This suggests that at a molecular level, phytoestrogens may not have as neutral an effect on blood coagulation as originally thought and that phytoestrogens can alter hemostatic gene expression by mechanisms other than via ERβ.

In this study, we investigated the effects of genistein, daidzein and its metabolite equol, on the expression of genes and proteins from the hemostatic system in an estrogen receptor-negative human hepatocyte cell line (HepG2) transfected to stably express ERα (Hep89). We show that phytoestrogens can increase the expression of key coagulation and fibrinolytic genes *in vitro* in a human hepatocyte cell line in the absence of ERβ.

Materials and methods

Cell culture

Hep89 were a gift from Dr D.C. Harnish (Department of Nuclear Receptors, Wyeth Ayerst Research, PA, USA) (Harnish et al. 1998).

* Corresponding author at: Molecular Pathology Research Laboratory, Department of Histopathology, Coombe Women and Infants University Hospital, Ireland. Tel.: +353 1 4085674.
E-mail address: kellyl37@tcd.ie (L.A. Kelly).

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Both HepG2 (Health Protection Agency, Wiltshire, UK) and Hep89 cells were seeded onto 6 well plates and maintained in culture with eagles minimum essential medium (EMEM), containing FBS, l-glutamine, non-essential amino acids and Pen/Strep (Sigma) at 37 °C in 5% CO_2 until they were 80% confluent. Prior to estrogen or phytoestrogen treatment, cells were switched to phenol-free medium containing charcoal stripped serum for 24 h. For stimulation experiments, cells grown to passage 5 were incubated with 50 nM of compound or vehicle control for 24 h. Cell viability was assessed using MTT assay as previously described (Kelly et al. 2010). No significant cytotoxic effects were found from genistein, equol or daidzein after a broad range of concentrations were tested.

RNA extraction and cDNA synthesis

RNA was extracted using the RNAeasy Total RNA mini kit (Qiagen) according to the manufacturers' instructions and reverse transcribed to single stranded cDNA using the High-Capacity cDNA Archive Kit (Applied Biosystems) following the manufacturers protocol.

RT-PCR

The evaluation of gene expression was performed by Taqman® RT-PCR. Commercially available TaqMan® primer and probe combinations were used to detect expression of the following genes, Factor VII (NM_000131.3), protein C (NM_000312.2), fibrinogen γ (NM_000509.4), prothrombin (NM_001992.3), protein S (NM_000313.2), plasminogen activator inhibitor-1 (PAI-1) (NM_000602.2), tissue plasminogen activator (tPA) (NM_033011.2), estrogen receptor alpha (ERα) (NM_000125.3), estrogen receptor beta (ERβ) (NM_001040275.1) and G-protein coupled receptor (GPER) (NM_001505.2). The endogenous control selected was 18s. All samples were assayed in triplicate.

Statistical analysis

Statistical analysis was performed using SPSS® (version 16.0). Unpaired *t*-tests were used to determine the significance of fold changes relative to DMSO as vehicle. $P < 0.05$ was considered significant.

Results

Hemostasis gene expression was measured initially in Hep89 cells following incubation with genistein, equol and daidzein. Genes where their expression was significantly increased were selected for further comparative studies with HepG2 cells. All effects observed were greater than those observed for estradiol for the same genes. PAI-1, tPA, prothrombin and Factor VII were selected; the effects of the phytoestrogens on the remaining genes was within the background range (data not shown).

Effect of ERα expression on the phytoestrogen induced changes in hemostasis in the HepG2 cell line (Hep89 v HepG2) (Fig. 1)

Plasminogen activator inhibitor-1 (PAI-1)

In Hep89 cells the phytoestrogens genistein ($p < 0.01$) and equol ($p < 0.05$) upregulated the expression of PAI-1 as did estradiol. This effect was significantly reduced for genistein ($p < 0.01$) and equol ($p < 0.05$) in the absence of ERα in the HepG2 cell line. Daidzein and estradiol had a similar effect on PAI-1 expression in both cell types showing an approximate 2-fold increase compared with vehicle treated cells.

Tissue plasminogen activator (tPA)

Genistein and equol treatment resulted in an up-regulation of tPA expression in Hep89 cells, an effect not observed in HepG2 cells devoid of ERα, where a down regulation was observed following equol treatment. This effect was significantly different between the two cell lines ($p < 0.001$). Daidzein increased tPA expression in Hep89 cells 3-fold whereas the effects in the HepG2 cells was similar to vehicle. Hep89 cells incubated with estradiol resulted in a 2-fold change in tPA expression, a result which was not significantly different to that observed in ER negative HepG2 cells.

Factor VII mRNA (FVII)

Equol and daidzein incubation showed an increase in Factor VII expression above background levels in Hep89 cells. Lower levels of FVII mRNA expression following estradiol ($p < 0.01$) and equol incubation ($p < 0.05$) were observed in HepG2 cells compared with those found in Hep89 cells.

Prothrombin

Genistein treatment upregulated prothrombin mRNA expression in HepG2 cells and this effect was amplified in the presence of the estrogen receptor expressed in Hep89 cells ($p < 0.05$). In contrast HepG2 cells treated with equol and daidzein upregulated prothrombin expression and showed significantly higher fold changes compared with those found in Hep89 cells on expression compared with HepG2 cells ($p < 0.05$).

Effect of phytoestrogens on estrogen receptor expression (Fig. 2)

As expected HepG2 cells did not express ERα or ERβ, however GPER was detected (data not shown). Hep89 cells expressed both GPER and ERα but not ERβ. Hep89 cells incubated with genistein, equol and daidzein showed a 2–3 fold downregulation of ERα which was significant for genistein ($p < 0.05$). A similar effect was observed with estradiol. GPER mRNA expression was upregulated by all three phytoestrogens, the effect with equol was significant ($p < 0.05$).

Discussion

Phytoestrogens are increasingly popular as a natural alternative to HT in post-menopausal women and are thought to be beneficial or at least neutral with respect to the risk of cardiovascular and thromboembolic disease; an effect largely mediated by ERβ. Contrary to these findings, we have previously shown, in a rat model of the menopause, that phytoestrogens can increase the expression of certain hemostatic genes in a concentration dependent manner (Kelly et al. 2010).

The most significant alterations in expression following phytoestrogen treatment were observed with tPA and its inhibitor PAI-1. Both have been linked to a variety of pathological conditions including thrombosis and myocardial infarction. ERα is clearly important in mediating the effects of phytoestrogens on tPA and PAI-1 expression since in the absence of this receptor, the upregulation of tPA was abolished and in the case of PAI-1, it was significantly reduced. It appears likely that this response is due to a combination of ERα mediated effects which may involve some of the following transcription factors, AP-1, NFκB or c-Jun (Nilsson et al. 2001).

Overexpression of the prothrombin gene is associated with an increased risk of thrombosis (Poort et al. 1996). Genistein induced an increase in the transcription of prothrombin in Hep89 cells which was not observed with estradiol, equol or daidzein. This suggests a ligand dependent effect. On the other hand, estradiol, equol and daidzein increased prothrombin expression in ER negative cells (HepG2 cells) suggesting that this effect is regulated by