Short Communication

Genistein Induces Bcl-2 Expression in Human Dermal Microvascular Endothelial Cells: a Short Report

(wound healing / angiogenesis / hormone replacement therapy / phytoestrogen)

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Abstract. It has been shown previously that oestradiol protects the vascular network, leading to increased skin flap viability associated with Bcl-2, VEGF and FGF-2 up-regulation. We have shown that genistein, a natural selective oestrogen receptor modulator, also increases skin flap viability in rats and induces Bcl-2 expression in human umbilical vein endothelial cells. In the present study we aimed to answer the question whether genistein increases expression of Bcl-2, a potent anti-apoptotic protein, in human dermal microvascular endothelial cells (HMVEC-d) as well. Our results showed that administration of genistein induces Bcl-2 expression in a concentration-dependent manner. Cell co-treatment with genistein and anti-ER compounds (MPP, PHTPP, ICI, G-15) diminished the observed positive effect of genistein on Bcl-2 expression. The decrease in Bcl-2 expression in HMVEC-d was most prominent after co-treatment with ICI (nuclear ER antagonist/GPR30 agonist) and PHTPP (selective ER-β antagonist). In conclusion, genistein increases Bcl-2 expression in HMVEC-d, contributing to its protective effect on the skin flap viability. However, the question whether the mechanism is ER-specific (via ER-β) has to be answered in further studies using a model of gene silencing or genetically modified cells.

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Abbreviations: Bcl-2 – B-cell lymphoma 2, FGF-2 – fibroblast growth factor 2; HMVEC-d – human dermal microvascular endothelial cells, G-15 – (3aS*,4R*,9bR*)-4-(6-bromo-1,3-benzodioxol-5-yl)-3a,4,5,9b-3H-cyclopenta[c]quinolone, ICI – ICI 182, 780, 7α,17β)-7-[9-[(4,4,5,5,5-pentafluoropentyl)sulphinyl]nonyl] oestra-1,3,5(10)-triene-3,17-diol, MPP – MPP dihydrochloride, (1,3-bis(4-hydroxyphenyl)-4-methyl-5-[4-(2-piperidinylethoxy) phenol]-1H-pyrazole dihydrochloride hydrate), PHTPP – (4-[2-phenyl-5,7-bis(trifluoromethyl)pyrazolo[1,5-a]-pyrimidine-3-yl] phenol, a.k.a. 2-phenyl-3-(4-hydroxyphenyl)-5,7-bis(trifluoromethyl)-pyrazolo[1,5-a]pyrimidine), SERM – selective oestrogen receptor modulator, VEGF – vascular endothelial growth factor.

Introduction

The beneficial effect of oestradiol on skin flap viability appeared to be attributable to the anti-apoptotic Bcl-2 protein and two key pro-angiogenic molecules, fibroblast growth factor 2 (FGF-2) and vascular endothelial growth factor (VEGF) (Toutain et al., 2009). Of note, the protective effect was not seen in ER-α knock-out mice, and thus seemed to be ER-modulated. At the *in*

vitro level, it has been shown that oestradiol enhances Bcl-2 synthesis in cultured keratinocytes (Kanda and Watanabe, 2003) and endothelial cells (Spyridopoulos et al., 1997). However, oestrogen replacement therapy (ERT) has been associated with several side-effects, for example on breast and/or uterine tissue, that were avoided in case of selective oestrogen receptor modulators (SERMs), including phytoestrogens (Smith et al., 2014; Šušaníková et al., 2019). Motivated by the oestrogen-related data we previously performed *in vivo* (using a random pattern skin flap model in ovariectomized rats) and *in vitro* (on human umbilical vein endothelial cells) investigation with genistein, a natural SERM, and found that genistein is able, similarly as oestradiol, to improve skin flap viability (Faber et al., 2018). We also found up-regulated Bcl-2 expression (evaluated by flow cytometry) in human umbilical vein endothelial cells. Assuming that the main skin flap protective mechanism is built on the reperfusion of a protective vascular network, in the present work we focused on the expression of Bcl-2 in human dermal microvascular endothelial cells (HMVEC-d) following genistein treatment that we determined by Western blot. We also co-treated cells with highly selective oestrogen receptor (ER) antagonist to answer the question whether the genistein-induced Bcl-2 expression is ER-dependent.

Material and Methods

Reagents

Highly selective ER-α antagonist MPP dihydrochloride (1,3-bis(4-hydroxyphenyl)-4-methyl-5-[4-(2-piperidinylethoxy)phenol]-1H-pyrazole dihydrochloride hydrate), selective ER-β antagonist PHTPP (4-[2-phenyl-5,7-bis(trifluoromethyl)pyrazolo[1,5-a]-pyrimidine-3-yl]phenol, a.k.a. 2-phenyl-3-(4-hydroxyphenyl)-5,7-bis(trifluoromethyl)-pyrazolo[1,5-a]pyrimidine), high-affinity and selective GPR30 (G-protein-coupled oestrogen receptor) antagonist G-15 ((3aS*,4R*,9bR*)-4-(6-bromo-1,3-benzodioxol-5-yl)-3a,4,5,9b-3H-cyclopenta[c]quinoline) and oestrogen receptor antagonist ICI 182,780 ((7α,17β)-7-[9-[(4,4,5,5,5-pentafluoropentyl)sulphinyl]nonyl]oestra-1,3,5(10)-triene-3,17-diol) and phytoestrogen genistein (all purchased from Tocris Bioscience, Bristol, UK) were diluted in DMSO according to the manufacturer's instructions.

Cell culture

Adult human dermal microvascular endothelial cells (HMVEC-d) were obtained from Lonza (Lonza Walkersville, Inc., Walkersville, MD). Cells were grown in Endopan MV (endothelial growth medium) containing epidermal growth factor (EGF), FGF-2, VEGF, ascorbic acid phosphate, R3 insulin-like growth factor 1 (R3-IGF-1), 6% foetal bovine serum (FBS), gentamicin/amphotericin (GA) and hydrocortisone (PAN-Biotech GmbH, Aidenbach, Germany) in a humidified atmosphere at 37 °C under 5% CO_2.

Immunofluorescence of oestrogen receptors

HMVEC-d were seeded on a cover glass at a density of 5,000 cells/cm^2 and cultured for 48 h in the presence or absence of genistein (1,000 nM). After that, cells were washed with PBS and fixed in 2% paraformaldehyde (pH = 7.2). Non-specific binding of secondary antibody was blocked by pre-incubation with normal swine serum (DAKO, Glostrup, Denmark) diluted in PBS for 30 min. Antibodies used in the present investigation are described in Table 1. Control of specificity was performed by replacement of the first step antibody by irrelevant antibody of the same isotype directed against antigen not occurring in the cells. The nuclei of cells were counterstained with 4′,6-diamidino-2-phenylindole (DAPI) (Sigma-Aldrich, St. Louis, MO) specifically recognizing DNA. The specimens were mounted to Vectashield (Vector Laboratories, Burlingame, CA) and inspected by an Eclipse 90i microscope (Nikon, Tokyo, Japan) equipped with NIS-Elements AR.40.00 for data storage and analysis and with a ProgRes MF CCD camera (Jenoptik Optical Systems GmBH, Jena, Germany).

MTS assay

Cell viability was evaluated by colorimetric assay using the MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulphophenyl)-2H-tetrazolium) dye (Promega, Madison, WI). HMVECs were seeded at a density of 6×10^3 cells per well in 96-well culture plates and incubated overnight. Approximately 24 h after seeding, the growth medium was substituted with fresh medium containing different concentrations of the tested ER antagonist (1,000–25,000 nM) or genistein (1–100,000 nM). After 48 h incubation period, 10 µl

Table 1. Antibodies used for immunofluorescence of HMVEC-d

Primary antibody	Dilution	Host	Produced by	Secondary antibody	Produced by	Channel
Anti-Oestrogen Receptor α	1 : 50	Rabbit polyclonal	Sigma-Aldrich St. Louis, MO	Goat anti-rabbit	Thermo Fisher Scientific, Waltham, MA	TRITC-red
Anti-Oestrogen Receptor β	1 : 100	Rabbit polyclonal	Sigma-Aldrich	Goat anti-rabbit	Thermo Fisher Scientific	TRITC-red
Anti-GPR (C-terminal)	1 : 50	Rabbit polyclonal	Sigma-Aldrich	Goat anti-rabbit	Thermo Fisher Scientific	TRITC-red

of MTS was added to each well. Absorbance at 490 nm was measured after additional 2 h using a plate reader (Infinite M200, Tecan, Männendorf, Switzerland). The measured absorbance of untreated control was 100 % and that of the tested compounds was expressed as a percentage of the control.

Western blot analysis

HMVEC-d were seeded at a density of 5×10^5 cells per tissue culture dish. Cells were pre-incubated overnight with 1,000 nM of the tested compounds (MPP dihydrochloride; PHTPP; G-15; ICI 182,780). Twenty-four hours after seeding, different concentrations of genistein (1–1,000 nM) were added. Cells were incubated in the presence of tested compounds and genistein for additional 48 h. Cells were then harvested in ice-cold PBS, centrifuged and lysed by 1× Laemmli buffer (sans 2-mercaptoethanol and bromophenol blue). Protein concentration in each sample was quantified by the Bradford assay (Bio-Rad, Hercules, CA). Equal amounts of proteins (25 µg) from each sample were resolved in 10% SDS polyacrylamide gel. The proteins were transferred onto PVDF membrane using the Trans-Blot Turbo Transfer System (Bio-Rad). The membrane was then blocked with 5% BSA in TBST buffer and incubated with Bcl-2 antibody (Cell Signalling Technology, Danvers, MA) followed by horseradish peroxidase (HRP)-conjugated secondary antibody (Invitrogen, Carlsbad, CA). Anti-β-actin HRP conjugate (Cell Signalling Technology) antibody was used as a loading control. Blots were visualized with Immobilon Crescendo Western HRP substrate (Merck Millipore, Burlington, MA). Image analysis was performed by Image Studio™ Lite (LI-COR Biosciences, Lincoln, NE).

Statistical analysis

Results from the MTS assay are expressed as mean ± SD (standard deviation). Statistical comparison of the obtained data was performed using one-way ANOVA followed by the Dunnett's test. The difference was considered to be statistically significant when $P < 0.05$.

Results

Expression of oestrogen receptors

The expression of ERs (in the presence and absence of genistein) is shown in Fig. 1. We observed only weak (+) expression of ER-α and GPR30, whereas the expression of ER-β was recorded as moderate (++). Of note, the presence of genistein did not modulate ER expression to a level observable by immunofluorescence.

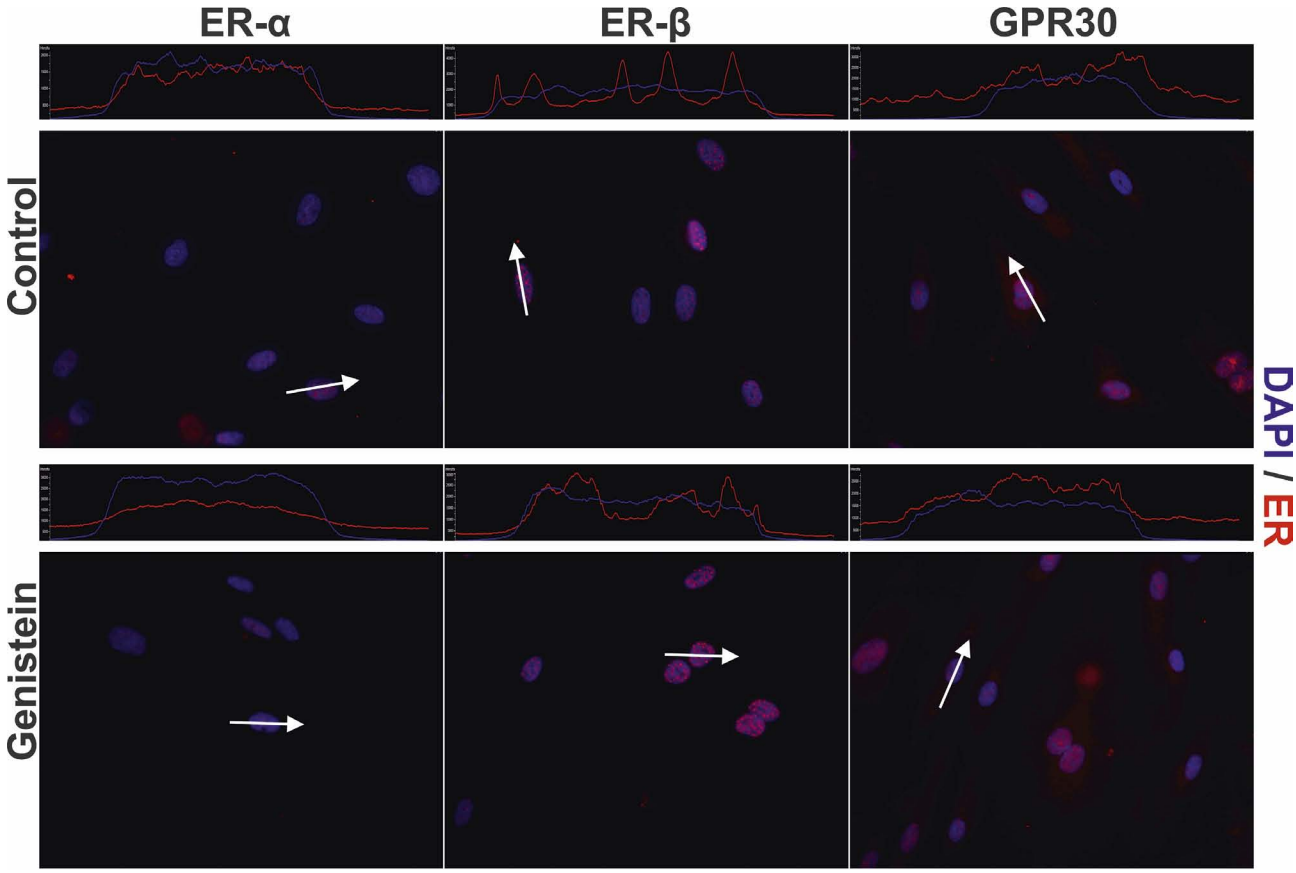

Fig. 1. Immunofluorescent analysis of oestrogen receptor (ER) expression in human dermal microvascular endothelial cells. ER-β was most highly expressed.

Cytotoxycity of ER modulators

The results from the MTS assay of HMVECs and genistein are shown in Fig. 2A. Genistein did not significantly increase cell viability at any of the tested concentrations. The highest tested concentration of genistein (100,000 nM) was found toxic. For further experiments we selected genistein in the concentration range from 1 to 1,000 nM.

The results from the MTS assay of HMVECs and ER antagonists are shown in Fig. 2B. The MTS assay was performed to find a non-cytotoxic concentration of ER antagonists that would be tested in combination with genistein to reveal whether genistein acts in an ER-specific manner. Concentrations from 1,000 to 25,000 nM of compounds PHTPP, G-15, ICI and 1,000 – 10,000 nM of MPP did not significantly modulate cell viability. The cytotoxic effect was observed only for MPP at concentration 25,000 nM. We observed a slight increase in the cell viability of cells treated with PHTPP. Therefore, for subsequent experiments with genistein, the non-cytotoxic concentration of ER antagonists at 1,000 nM was used.

Bcl-2 expression

Western blot of Bcl-2 expression in HMVEC-d is shown in Fig. 3A. Genistein increased Bcl-2 expression at concentrations from 10 nM to 1,000 nM compared to untreated control.

To study the involvement of respective ERs (ER-α, ER-β or GPR30), cells were incubated with ER antagonists, at 1,000 nM, prior to and during the treatment with genistein (1 nM – 1,000 nM). The expression of Bcl-2 protein in the treated cells was also analysed by Western blot (Fig. 3B). All anti-ER compounds (MPP, PHTPP, ICI, G-15) diminished the observed positive effect of genistein on Bcl-2 expression. The decrease in Bcl-2 expression in HMVEC-d cells was most prominent after co-treatment of genistein and ICI (nuclear ER antagonist) and genistein and PHTPP (selective ER-β antagonist). MPP (selective ER-α antagonist) and G-15 (selective GPR30 antagonist) affected the genistein-induced Bcl-2 expression only moderately in comparison with PHTPP and ICI.

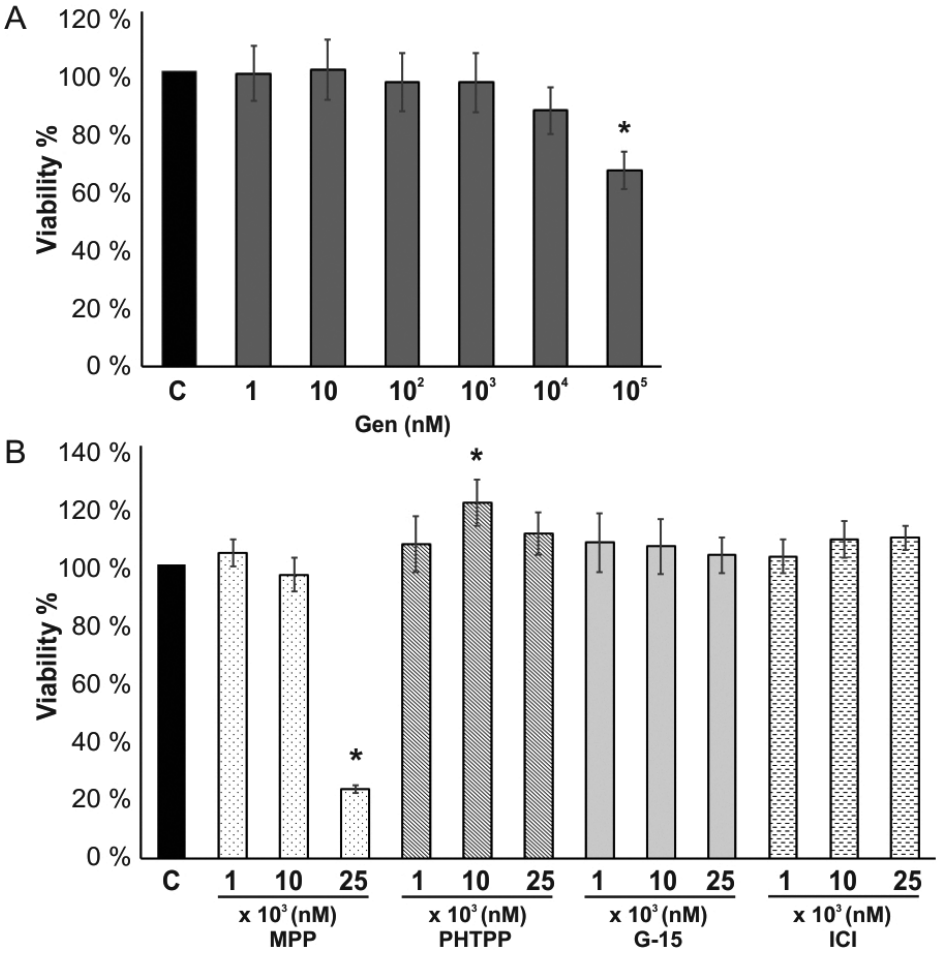

Fig. 2. MTS assay of human dermal microvascular endothelial cells following treatment with genistein (**A**) and selective oestrogen receptor modulators (**B**) (MPP – ER-α antagonist; PHTPP – ER-β antagonist; G-15 – GPR30 antagonist; ICI – fulvestrant (ER-α/β antagonist and GPR30 agonist).

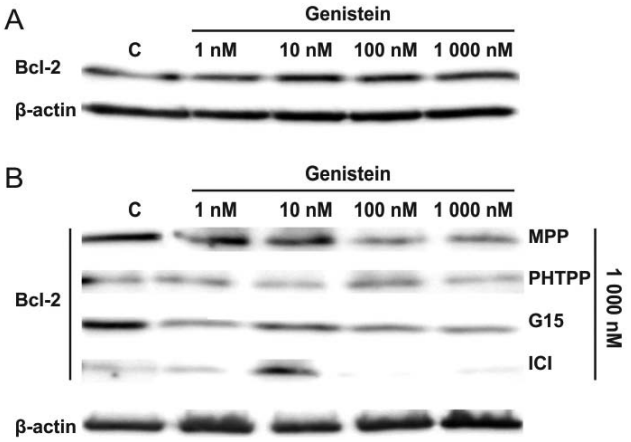

Fig. 3. Western blot of human dermal microvascular endothelial cells following treatment with genistein (**A**) and genistein in combination with selective oestrogen receptor modulators (**B**).

Discussion

Our present *in vitro* data indicate that genistein enhances anti-apoptotic Bcl-2 protein expression also in HMVEC-d cells. The most prominent inhibition was seen when the cells were co-treated either with a non-selective ER-α/β antagonist or a highly selective ER-β antagonist. Thus, we here support the assumption that positive oestrogenic effects of genistein on the skin are rather mediated via ER-β (Campbell et al., 2010), whose expression is highest among the ERs in HMVEC-d cells. Furthermore, as shown in MCF-7 cells, genistein binds to ER-β with almost the same efficiency as oestradiol, but the concentration required to induce transcription is 10^4 times higher for genistein (Morito et al., 2001).

Other protective effects of genistein may include decreased TGF-β1, -β2, and -β3 production and more importantly, genistein was also reported to modulate the BAX/Bcl-2 ratio and decrease p53 expression (Jurzak et al., 2014). Recently, genistein-3′-sodium sulphonate, a new derivative of genistein, effectively reversed LPS-induced down-regulation of Bcl-2 and activation of caspase-3 in lung endothelial cells (Yi et al., 2020), which is in accord with our evidence.

Epidemiological studies suggest that genistein, based on its oestrogenic and/or anti-oestrogenic effects, may be used as an alternative therapy for a wide range of hormonal disorders, including breast and prostate cancer, cardiovascular diseases, osteoporosis, or menopausal symptoms (Křížová et al., 2019). Intake of isoflavone reduced the serum concentration of oestradiol by feedback regulation, and genistein inhibits tyrosine kinase involved in the cell cycle. Although our previous data show that genistein stimulates migration of HMVEC-d cells (Faber et al., 2018) and up-regulates Bcl-2 expression, the concentrations of genistein required for the observed positive effects are much higher than an efficient concentration of oestradiol. This much higher concentration may also explain why consumption of soy beans may reduce the risk of cancer (Varinska et al., 2015). On the other hand, preferential expression of ER-β in skin cells and preferential binding of isoflavones to this receptor may explain the positive effect of genistein on skin repair (Haczynski et al., 2002; Thornton et al., 2003; Merlo et al., 2009).

We conclude that genistein increases Bcl-2 expression in human dermal microvascular endothelial cells. However, the question whether the inhibition via ER-β was due to its strongest expression when compared to the expression of ER-α and GPR30 has to be answered in further studies. In the context of our results, the use of a receptor antagonist instead of a gene silencing and/or knock-out cell line may therefore represent a limitation of our study.

Conflict of interest

The authors declare that they have no conflict of interest.

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