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Effects of some natural 5-hydroxy-isoflavones on cultured human endothelial cells in presence and absence of hydrogen peroxide

Alexandra Paulo and Helder Mota-Filipe

Abstract

Due to their biological activity, it has been suggested that consumption of isoflavone-rich diets may prevent prostate and breast cancers, osteoporosis and cardiovascular diseases. Preventive effects of isoflavones on cancer and cardiovascular diseases have been associated with their oestrogenic and antioxidant properties. However, concerns still exist about the potential dangers of consuming high levels of these compounds, since it is known that some of them have cytostatic or cytotoxic properties, depending on the concentration. To evaluate the potential cytotoxic risk and antioxidant benefit of natural 5-hydroxy-isoflavones (5-OH-isoflavones) for human vascular endothelium, the effect of some natural 5-OH-isoflavones was evaluated on cultured human endothelial cells, in the presence and absence of H_2O_2 (3 mM for 4 h). None of the isoflavones tested were able to prevent oxidative damage to endothelial cells at maximal extracellular concentrations of 1 mM. The low antioxidant capacity of these compounds was also shown by the DPPH (1,1-diphenyl-2-picrylhydrazyl radical) method. On the other hand, genistein and biochanin A, having a free 7-OH group, were toxic to the human endothelial cells in a dose-dependent manner, at concentrations $\geq$300 μM and $\geq$100 μM, respectively. These results indicate that the non-specific cytotoxic effect of 5-OH-isoflavones is associated with the free 7-OH group. In conclusion, we were not able to show that 5-OH-isoflavones are beneficial to human endothelial cells when the cells were exposed to oxidative stress caused by 3 mM of H_2O_2, but it can be concluded that consumption of 5-OH-isoflavones is of no direct cytotoxic risk to the human vascular endothelium since toxic concentrations are believed to be unreachable in-vivo.

Introduction

Isoflavones are a class of phenolic plant metabolites found almost exclusively in the subfamily Papilionoideae of the Leguminosae. The simplest 5-hydroxy-isoflavone is genistein, a soybean constituent extensively studied and recently reviewed (Dixon & Ferreira 2002). Isoflavones are selective oestrogen receptor modulators (Cos et al 2003), tyrosine kinase and DNA topoisomerase inhibitors (Akiyama et al 1987; Okura et al 1988), antioxidants (Pietta 2000), and are cytostatic or cytotoxic, depending on concentration (Constantinou et al 1998). Preventive effects of isoflavones on cancer and cardiovascular diseases have been associated not only with their oestrogenic modulation properties but also with their antioxidant activity (Wei et al 1995; Anthony et al 1998; Djuric et al 2001; Sarkar & Li 2003). Due to its pharmacological properties, genistein is considered to be of chemotherapeutic value in the treatment of B-cell precursor leukaemia (Uckun et al 1995). The antioxidant activity of isoflavones is still not well studied and results are spread in the literature. The antioxidant activity of genistein has been demonstrated through its inhibition of lipid peroxidation in several in-vitro systems (Kerry & Abbey 1998; Vedavanam et al 1999; Lin et al 2002). The genistein derivatives genistin and prunetin have been shown, in an inorganic medium, to have radical scavenging activity similar to Trolox (Pietta 2000) and to be able to inhibit superoxide radical generation by xanthine/xanthine oxidase (Wei et al 1995), respectively. Genistein was reported to be cytotoxic to cancer cells at concentrations of 100–300 μM (Constantinou et al 1998). Cos et al (2001) reported that genistein (160 μM) inhibited 50% growth of fibroblasts and, due to this cytotoxicity, a low antioxidant selectivity index was established for this compound by the use of two different biological systems.

CECF, Faculty of Pharmacy, University of Lisbon, Av. Prof. Gama Pinto, 1649 003 Lisboa, Portugal

Alexandra Paulo

Unit of Pharmacology and Pharmacotoxicology, Faculty of Pharmacy, University of Lisbon, Av. Prof. Gama Pinto, 1649 003 Lisboa, Portugal

Helder Mota-Filipe

Correspondence: A. Paulo, Faculty of Pharmacy, University of Lisbon, Av. Prof. Gama Pinto, 1649-003 Lisbon, Portugal.
E-mail: mapaulo@ff.ul.pt

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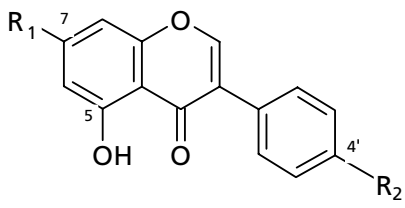
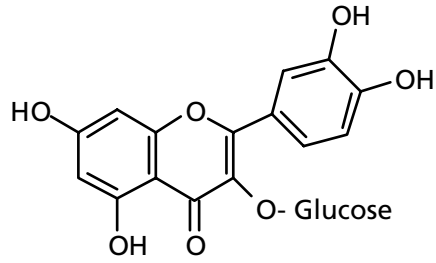
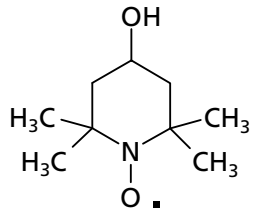


Genistin	R_1 = O-glucose, R_2 = OH
Genistein	R_1 = OH, R_2 = OH
Biochanin A	R_1 = OH, R_2 = OMe
Sissotrin	R_1 = O-glucose, R_2 = OMe
Prunetin	R_1 = OMe, R_2 = OH



Isoquercitrin



Tempol

Figure 1 Structures of 5-hydroxy-isoflavones tested (genistin, genistein, biochanin A, sissotrin, prunetin) and reference compounds (isoquercitrin and tempol).

To evaluate the cytotoxic risk and the antioxidant benefit of some common natural 5-hydroxy-isoflavones (5-OH-isoflavones) in the same cellular system and, at the same time, in a cellular environment of crucial importance in healthy and pathological situations, the effect of different concentrations of natural structurally-related isoflavones (Figure 1) on cultured human endothelial cells was evaluated in the presence and absence of H_2O_2, a source of hydroxyl radicals.

Materials and Methods

Genistein, genistin and biochanin A were purchased from Fluka and isoquercitrin and tempol from Sigma. Prunetin and sissotrin were isolated from *Pterospartum tridentatum* (L.) Wk. & Lge. (Leguminoseae) as described elsewhere (Vítor et al 2004) and the purity of the isolated compounds was determined by HPLC to be ≥95%.

Radical DPPH scavenging capacity assay

For each compound, 8, 4, 2, 1 and 0.5 mM methanolic solutions were prepared. Then 50 μL of each solution was added to 5 mL of a 0.004% methanolic solution of 1,1-diphenyl-2-picrylhydrazyl radical (DPPH). The decrease in absorbance of DPPH at 517 nm was measured at room temperature after 30 min. Methanol was used as blank solution and DPPH solution served as control. BHT (di-*tert*-butylhydroxytoluene) and isoquercitrin were used as reference antioxidants. All tests were performed in triplicate. Radical scavenging capacity was expressed as percentage inhibition of DPPH solution absorbance.

Measurement of endothelial injury in the presence and absence of H_2O_2

Human umbilical vein endothelial cells (HUVEC), purchased from Promocell (Spain) (passages 4–8), were seeded onto 96-well plates and cultured to confluence in endothelial cell growth medium (C-22010; Promocell) at 37° C and 5% CO_2. Preliminary experiments were performed to evaluate the degree of cell injury induced by H_2O_2. In these experiments, the cells were exposed to various concentrations of H_2O_2 (0.03, 0.1, 0.3, 1, 3 and 10 mM, n = 4, data not shown). In the subsequent intervention studies, the cells were challenged with a submaximal concentration of H_2O_2 (3 mM) for 4 h. Samples were dissolved in 10% dimethyl sulfoxide (DMSO) to a concentration of 10 mM.

For antioxidant assays, the cells were pre-incubated (10 min before H_2O_2) in medium in the absence or presence of increasing concentrations of isoflavones (Figure 1; genistein, genistin, prunetin, biochanin A and sissotrin) and reference compounds isoquercitrin and tempol (1 μM to 1 mM). For cytotoxic assays, cells without exposure to H_2O_2 were incubated with all samples at all concentrations (n = 4). Cell injury (i.e., reduction in mitochondrial respiration) was assessed by measuring the mitochondrial-dependent reduction of MTT (3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyltetrazolium bromide) to formazan. Cells in 96-well plates were incubated at 37° C with MTT (0.2 mg mL^{-1}) for 1 h. Culture medium was removed by aspiration and the cells were solubilized in DMSO (100 μL). The extent of reduction of MTT to formazan within cells was quantified by the measurement of absorbance at 550 nm.

Statistics

In all studies the values were expressed as means ± s.e.m. of n observations. The results on HUVEC were analysed by one-way analysis of variance followed by a Bonferroni post-hoc test for multiple comparisons. $P < 0.05$ was considered significant.

Results and Discussion

Due to the reported pharmacological activity of isoflavones, consumption of isoflavone-rich diets, such as soybeans and herbal drugs based on soybean or supplemented with isoflavones, have been encouraged worldwide. However, concerns

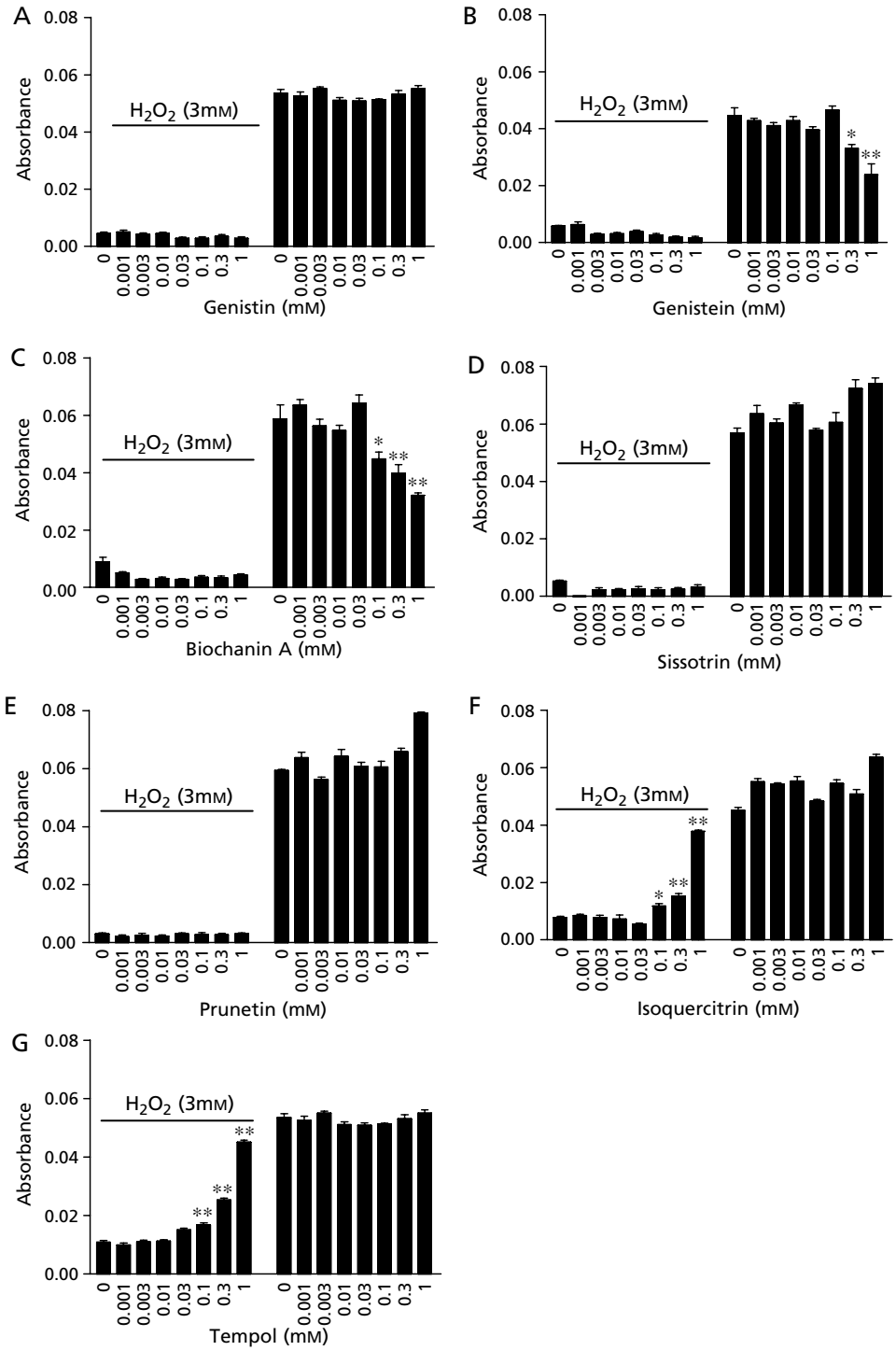


Figure 2 Effect of genistin (A), genistein (B), biochanin A (C), sissotrin (D), prunetin (E), isoquercitrin (F) and tempol (G) on the impairment in mitochondrial respiration caused by hydrogen peroxide (columns on the left) or on the cellular viability (columns on the right). Reduction of mitochondrial respiration is expressed as reduction of MTT quantified by the measurement of absorbance at 550 nm. Values are the means ± s.e.m. from 4 determinations. $^{*}P < 0.05$, $^{**}P < 0.01$ vs control (0 mM) for each group of columns.

still exist about the potential dangers of consuming high levels of these compounds due to their oestrogenic and cytotoxic properties (Constantinou et al 1998; Cos et al 2003). In view of this we decided to assess the in-vitro effect of some common natural 5-OH-isoflavones on human endothelium, alone or in presence of a source of hydroxyl radicals, and so try to conclude on the potential antioxidant benefit and cytotoxic risk of these compounds to the human endothelium in a situation of a high oral intake of the 5-OH-isoflavones tested (Figure 1).

The degree of cellular injury was evaluated by the MTT assay, in which cell viability is assessed by mitochondrial-dependent reduction of MTT to formazan. Exposure of HUVEC to hydrogen peroxide (3 mM for 4 h) caused a substantial but submaximal impairment of mitochondrial respiration ($90.3 \pm 1.4\%$, $n = 6$). The natural antioxidant flavonoid isoquercitrin and the radical scavenger tempol were used as reference compounds (Pietta 2000; Cuzzocrea et al 2000).

The natural 5-OH-isoflavones tested did not prevent oxidative damage to endothelial cells at maximal extracellular concentrations of 1 mM (Figure 2A–E) but the reference antioxidant compounds used were able to protect the cells in a dose-dependent manner (Figure 2F, G). The flavonoid isoquercitrin reduced cell injury by 10.7%, 20.1% and 80.4% at concentrations of 100 μM, 300 μM and 1 mM, respectively (Figure 2F) and the radical scavenger tempol at 100 μM reduced cell injury by 13.9% (Figure 2G). Additionally, none of the natural 5-OH-isoflavones tested were able to reduce the radical DPPH up to a concentration of 80 μM (Figure 3), whereas the antioxidant flavonoid isoquercitrin reduced 53% DPPH radical absorbance at 5 μM and at 20 μM reached a plateau of about 96% reduction of DPPH radical absorbance. Isoquercitrin was found to be better radical scavenger than the food antioxidant BHT, which at a concentration of 5 μM reduced only 30% the DPPH radical absorbance and reached a plateau of activity (64% of DPPH reduction) at 40 μM (Figure 3). Despite our negative results in the two antioxidant assays performed, some of the tested isoflavones showed antioxidant properties in other in-vitro systems: genistein (1.5–12.4 μM) inhibited lipid peroxidation induced by different pro-oxidant agents (Kerry & Abbey 1998; Vedavanan et al 1999; Cos 2001; Lin et al 2002) and prunetin was as potent as genistein at inhibiting superoxide radical generation by xanthine/xanthine oxidase, whereas biochanin A exhibited no antioxidant effect in this assay (Wei et al 1995). Additionally, genistein, biochanin A and genistin were 2.9–1.2 times better radical scavengers than the aqueous soluble vitamin E analogue Trolox in the $ABTS^{\bullet+}$ cation method (Pietta 2000). Taking all this information together, one may conclude that these natural 5-OH-isoflavones are only weak antioxidants and so they may not be able to protect the vascular endothelium against severe oxidative stress, such as that mimicked by 3 mM of H_2O_2 for 4 h, which reduces cell viability by approximately 90%. Also, Kerry & Abbey (1998) reached the conclusion that genistein is not an effective physiological antioxidant in low-density lipoprotein (LDL) although they showed that this isoflavone was able to inhibit copper and peroxyl radical mediated LDL oxidation in-vitro. Our in-vitro results are also compatible with those reported by Hale et al (2002), who concluded that short-term oral isoflavone supplementation did not improve endothelial function in healthy menopausal women.

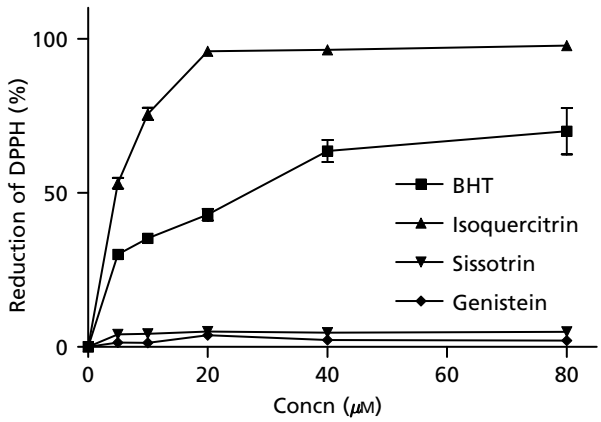


Figure 3 Radical DPPH scavenging capacity of 5-OH-isoflavones genistein, sissotrin and reference antioxidant compounds isoquercitrin and BHT, expressed as percentage of reduction of DPPH absorbance at 517 nm. Values are the means ± s.e.m. from three determinations.

Genistein and biochanin A, which have a free OH group at position 7, were shown to be toxic to human endothelial cells in a dose-dependent manner, at concentrations ≥ 300 μM ($\geq 25.9\%$ reduction in cellular viability) and ≥ 100 μM ($\geq 23.9\%$ reduction in cellular viability), respectively (Figure 2B, C). The range of cytotoxic concentrations (100–300 μM) is the same as that previously reported for genistein in cultured cancer cell lines (Constantinou et al 1998) and fibroblasts (Cos et al 2003), meaning that genistein cytotoxicity is non-specific and only occurs at high concentrations. The results also show, for the first time, that the cytotoxic effect of 5-OH-isoflavones is associated with the free 7-OH group, since methylation and glucosylation of this hydroxyl group (prunetin and genistin) results in the abolishment of cytotoxicity towards endothelial cells (Figure 2A, E). However, we think that minimal cytotoxic concentrations are not reachable in plasma via normal diet or even an isoflavone-rich diet, because sulfation or glucuronidation of the 7-OH group can occur after oral intake of isoflavones. Considering the work of Setchell & Zimmer-Nechemias (1997), who reported a mean plasma concentration of 2.53 μM for genistein and conjugated metabolites in 4-month-old infants fed an isoflavone-rich diet of exclusively soy milk formulas, and the work of Hendrich (2002), who reported that free aglycones can reach 30% of total isoflavone metabolites plasma concentration, it can be concluded that free genistein can reach plasma concentrations of 750 nM (30% of total genistein metabolites). According to our results, this value is non-cytotoxic to the healthy human endothelium.

Conclusions

Our in vitro results show that natural 5-OH-isoflavones are probably not able to protect the human endothelium against severe oxidative stress due to their low antioxidant capacity.

This study also allowed us to conclude that the non-specific cytotoxicity of some 5-OH-isoflavones, such as genistein, is due to the free 7-OH group. In our opinion, this finding is of some importance for the development of new drugs based on this chemical skeleton. However, considering the high minimal cytotoxic concentrations of 5,7-diOH-isoflavones (genistein and biochanin A), it can be concluded that consumption of diets rich in, or supplemented with, these isoflavones does not constitute a direct toxic risk to the human endothelium.

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