Soy isoflavonoids and cancer — metabolism at the target site

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Abstract

Isoflavonoids are members of the broad class of plant polyphenols that have been shown in vivo to have benefit in the prevention of a wide variety of chronic diseases, including cancer. For genistein (5,7,4′-trihydroxyisoflavone) (GEN), the major isoflavone in soy, reported mechanisms for these biological activities are numerous and include regulation of estrogen-mediated events, inhibition of tyrosine kinase and DNA topoisomerase activities, synthesis and release of TGFβ, and modulation of apoptosis. However, the biochemical effects of GEN in cell culture occur at concentrations in the micromolar range, far above the circulating levels of the unconjugated GEN. This may point to the limitations of cell culture for the evaluation of the activity and mechanisms of potential anti-carcinogens. GEN is extensively metabolized in vivo, with only about 14–16% excreted in an unmodified form. Metabolism may also occur because of interaction between GEN (as well as other polyphenols) and oxidants produced by inflammatory cells (HOCl, HOBr and ONOO$^-$). These react with GEN to form brominated, chlorinated and/or nitrated GEN. Emerging evidence indicates that these modifications may substantially increase the biological activities of the parent compound. Future investigations of GEN and other polyphenols must, therefore, take into account metabolism at the tissue site. © 2001 Elsevier Science B.V. All rights reserved.

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1. Introduction

The risk of prostate cancer is much lower in the peoples of SE Asia compared to Americans [1]. This difference rapidly disappears on immigration of SE Asian men to the USA [2] and has been attributed in part to changes in specific dietary components [3]. One dietary factor that is associated with a reduced risk of prostate cancer is soy [4–6]. In USA, among adventists, consumption of soy milk (>1 serving per day) is associated with a 70% lower risk of prostate cancer [7].

Soy is unique among most common foods in that it contains 1–3 mg/g of isoflavones, a particular class of the bioflavonoids [8]. SE Asians consume on average 40 mg of isoflavones each day, at least 10 times more than Americans do [9–11]. One of these isoflavones, genistein (5,7,4′-trihydroxyisoflavone) (GEN) (Fig. 1), has been shown to have several biological and biochemical effects. These range from being an inhibitor of protein tyrosine phosphorylation [12] and tumor necrosis factor alpha activation [13], as an agonist and
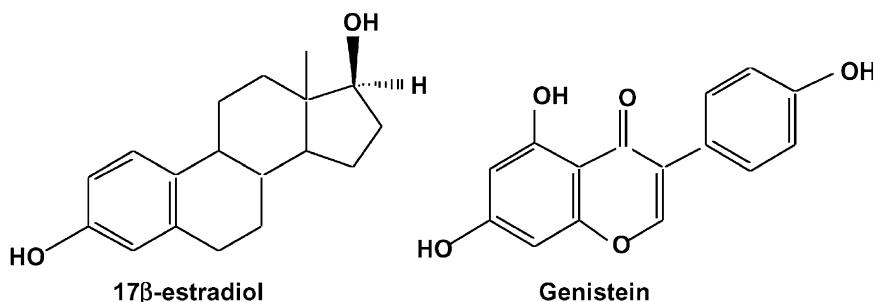
Fig. 1. The chemical structures of 17β-estradiol and the isoflavone genistein.

antagonist of the estrogen receptors, α and β [14], and as an anti-oxidant [15].

In the past 2 years, there have been a limited number of studies using animal models of prostate cancer to explore the chemopreventive effects of soy, extracts of soy and purified isoflavones. In each case, a protective effect of isoflavones has been observed. One study examined the role of soy and soy extracts on the growth of a transplantable prostate tumor in rats [16]. In a similar animal model, dietary genistein was also shown to inhibit prostate tumor growth [17]. The carcinogen 3,2′-dimethyl-4-aminobiphenyl can be used to induce prostate cancer when it is injected subcutaneously into male rats. Animals consuming genistein in their diet had a small but significant reduction (29%) in prostate tumor number [18]. In a recent development, the chemopreventive effect of genistein has been examined using the TRAMP mouse model of prostate cancer. Genistein both reduced the number of prostate tumors and the size of the tumors [19].

Using cell culture approaches with established human prostate cancer cell lines, it was shown that the soy isoflavone genistein inhibits both serum and growth factor-stimulated proliferation of these cells [20]. This result has been confirmed by others, however, the concentrations needed to achieve this inhibition in cell culture ($>50\,\mu M$) are two to three orders of magnitude greater than those observed in Asian men eating soy as a significant part of the diet [21]. This suggests that if isoflavones have an effect in vivo, then they do so via secondary mechanisms. These could include regulation of growth factor

secretion by neighboring cells in the prostate or by growth factor receptors in the tumor cell itself [22], or subtleties in the metabolism of genistein that are not modeled in cell culture. A possible example of the latter is the recent discovery in the rat that the principal metabolite of genistein that accumulates in the prostate is 2-(4-hydroxyphenyl)-propionic acid (2-HPPA) [23] (Fig. 2). This compound is not formed in cell culture experiments and, therefore, must be investigated directly.

Another important consideration in vivo is the localized metabolism of isoflavones that may occur in the vicinity of tumor cells. In previous studies, we have shown that genistein is sulfated rapidly by certain human breast cancer cell lines (e.g. ZR-75-1 cells), but not others (BT-20 cells) [24,25]. In tumors, there is often invasion of inflammatory cells. Eosinophils are frequently associated with breast tumors. Eosinophils are unique in that they utilize hydrogen peroxide as a precursor to form the hypohalogenous acid HOBr [26]. A similar reaction is catalyzed by myeloperoxidase in neutrophils to produce HOCl [27]. These proinflammatory oxidants react with tyrosine residues on proteins [28,29]. We hypothesized that they would also react with the isoflavones.

The goal of this study was to evaluate whether evidence could be obtained for the formation of these novel metabolites of isoflavones in humans. Two physiological sites were used in this assessment — urines from men undergoing a clinical trial of the effect of soy protein on plasma biomarkers and freshly isolated human neutrophils.
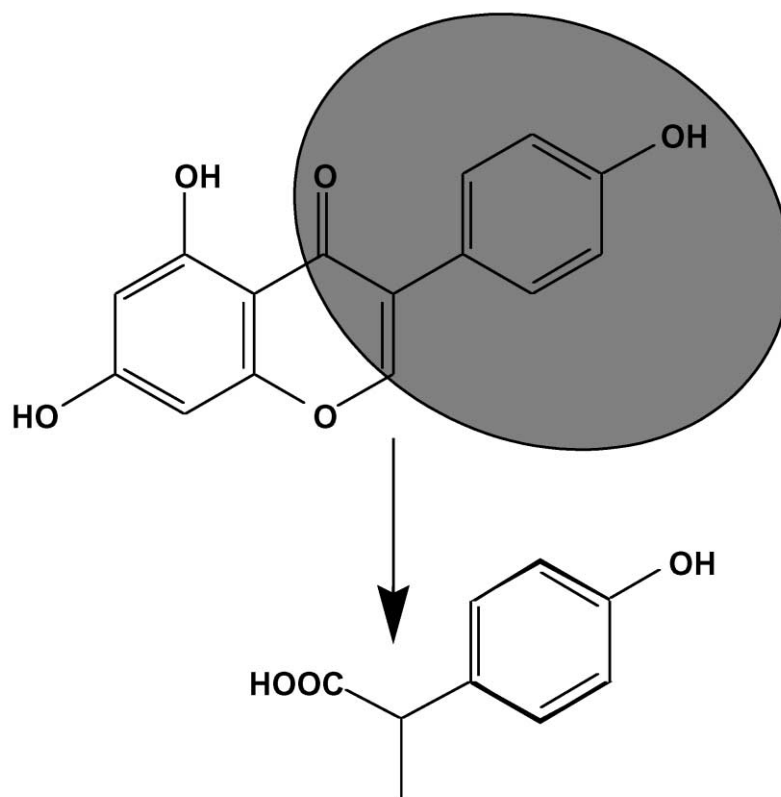
Fig. 2. The conversion of genistein to the metabolite found in the prostate, 2-HPPA.

2. Materials and methods

2.1. Materials

Isoflavones were obtained as described previously [30]. 2-(4-Hydroxyphenyl)-propionic acid was a gift of Dr. Nick Coldham (Addlestone, Surrey, UK). 4-Methoxyphenylacetic acid and phorbol myristoyl acetate (PMA) were purchased from Aldrich Chemical Co. (Milwaukee, WI). All other reagents were the best grades available.

2.2. Methods

2.2.1. Clinical

Urine specimens used in this study were from elderly male subjects (>55-year-old) who consumed two soy protein beverages (containing 20 g of soy protein per serving) on a daily basis for 6 weeks. The urines were collected prior to the study and at 2, 4 and 6 weeks after the start of the study. This study was approved by the UAB Institutional Board for Human Use. The methods for isolation and treatment of isoflavones and their metabolites from the urines in this study have been published elsewhere [31,32].

2.2.2. Cell culture

Experiments with inflammatory cells were carried out with freshly isolated human polymorphonuclear cells (PMNs) from healthy volunteers. The isoflavone biochanin A (5,7-dihydroxy-4′-methoxyisoflavone) (50 μM) was chosen as the substrate since it is a poor inhibitor of protein tyrosine kinases and myeloperoxidase activity [15]. Cells were suspended in phosphate-buffered saline and incubated at 37°C. An oxidative burst of HOCl was created by adding

PMA (10 μM). After incubation for 30 min, the cells were removed by centrifugation. The supernatant was extracted with two volumes of diethyl ether and the ether extract evaporated to dryness. The reaction mixture was dissolved in 80% aqueous methanol prior to analysis.

2.2.3. LC–mass spectrometry analysis

Reaction mixtures were separated by HPLC using a 10 cm × 4.6 mm i.d., C-8 Aquapore reverse-phase column with isocratic elution with the mobile phase 40% aqueous acetonitrile:10 mM ammonium acetate at a flow rate of 1 ml/min. Part of the column eluent (10 μl/min) was passed into the IonSpray™ interface of a PE-Sciex (Concord, Ont., Canada) API III triple quadrupole mass spectrometer. The voltage on the spraying needle was −2700 V and the orifice potential was set at −60 V. Negative ion spectra were recorded over a m/z range of 200–400. Selected $[M-H]^-$ molecular ions were analyzed by collision-induced dissociation with 90% argon–10% nitrogen, and the daughter ion mass spectra recorded over a range from m/z 20 to the m/z of the selected parent ion. In experiments to quantitatively measure the isoflavones and their metabolites, specific molecular ion/daughter ion combinations were recorded in the multiple reaction

ion monitoring (MRM) mode. Data were analyzed using software provided by the manufacturer on Macintosh Quadra 950 and PowerPC 9500 computers (Apple Computers, Cupertino, CA).

3. Results and discussion

Initial LC–MRM–MS of the isoflavones and their metabolites in the urine of the elderly male subjects suggested that a compound with a $[M-H]^-$ ion of m/z 165 and a resulting daughter fragment ion of m/z 119 was present. The amount of this compound was substantial in two of the subjects studied but did not show any relationship to the amount of genistein (its presumed precursor) (Fig. 3). However, MS–MS analysis of the m/z 165 molecular ion revealed that it was different from authentic 2-(4-hydroxyphenyl)-propionic acid (Fig. 4). The presence of the m/z 106 ion suggested that it might have a methoxyl group. However, a potential alternative metabolite, 4-methoxyphenylacetic acid, although having the same molecular weight and m/z 106 fragment ion, did not have the m/z 119 ion. The identity of the human metabolite was therefore not established — as it eluted very rapidly, it could in
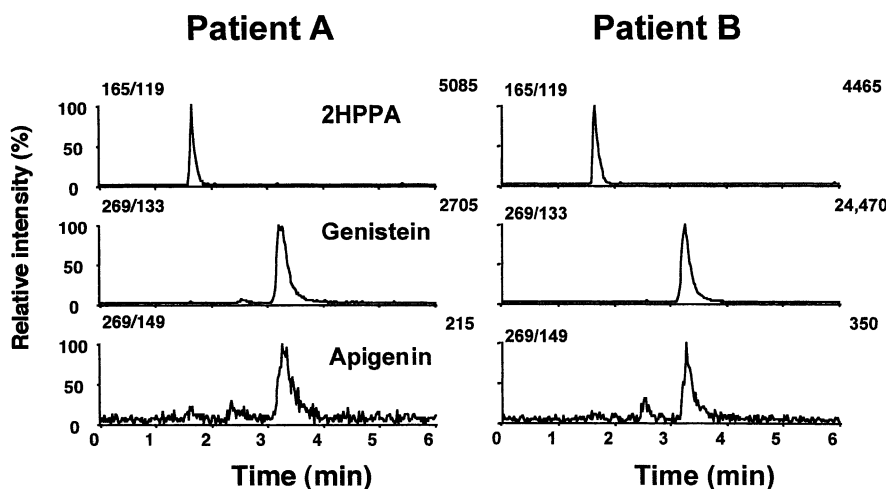

Fig. 3. LC–MS analysis of the isoflavone genistein and its metabolite 2-HPPA from urines of two men taking two soy protein beverages (total of 40 g) per day. The ion chromatograms are for parent ion/daughter ion combinations that are specific for 2-HPPA (m/z 165/119), genistein (m/z 269/133) and apigenin (m/z 269/149) (added as the internal standard). The intensities are given by the value in the top right hand corner of each ion chromatogram.
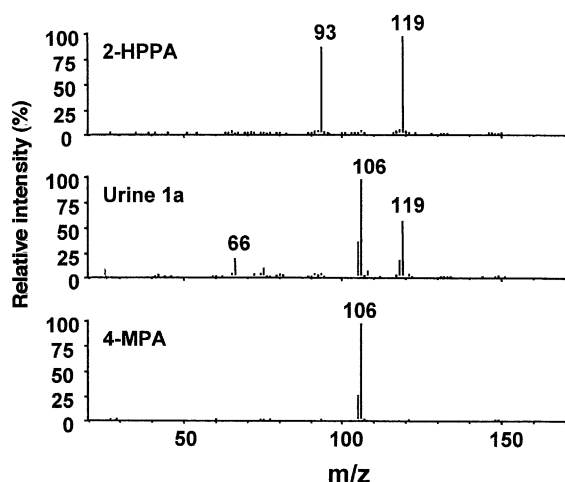
Fig. 4. Daughter ion mass spectra of authentic 2-(4-hydroxy-phenyl)-propionic acid, the metabolite from human urine (see Fig. 3), and authentic 4-methoxyphenylacetic acid.
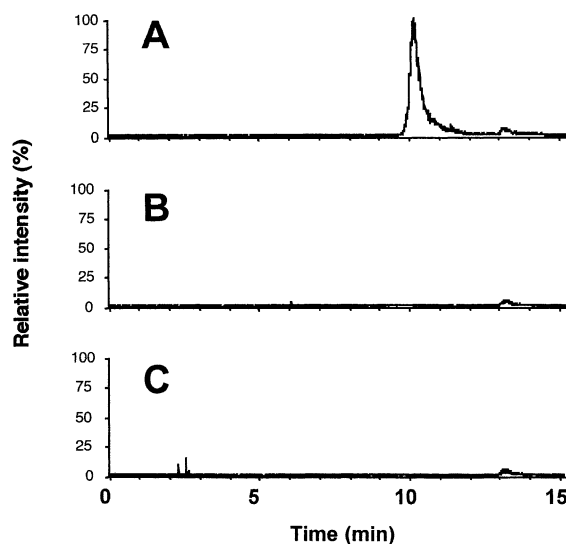

Fig. 5. Human PMNs generate chlorobiochanin A when stimulated by a phorbol ester. LC–MS analysis using reaction ion monitoring for chlorobiochanin A (*m/z* 317/301). (A) Chlorobiochanin A is formed only when biochanin A is added to PMNs in the presence of the phorbol ester. Deleting biochanin A (B) or the phorbol ester (C) eliminated the formation of chlorobiochanin A.

fact be a mixture of more than one compound. These data suggest that 2-HPPA contrary to the rat is only found in very low concentrations in humans.

We then turned to a reaction that may occur in the region of inflammatory cells. This type of cell is commonly found in the region of tumors, as well as in atherosclerotic lesions. As substantial amounts of chloro- [28] and bromo-tyrosine [29] residues are present in the proteins in an atherosclerotic lesion, it should be anticipated that a similar situation should occur in tumors. The similarity of the isoflavone B-ring to tyrosine suggests that chloro- and bromo-isoflavone derivatives should be formed under these circumstances.

When genistein was reacted with HOCl in the absence of cells, the 6-, 8- and 3′-isomers of chlorogenistein as well as an unidentified dichlorogenistein isomer were formed [33]. Similar reaction products were formed from HOBr [34]. This result was similar to that reported for iodination of genistein by thyroid peroxidase which generates HOI from H_2O_2 and iodide [35].

To determine whether cells can form chlorinated isoflavones, biochanin A (4′-methoxygenistein) was incubated with human PMNs in the presence of PMA. LC–MS analysis revealed that chlorinated biochanin A was detected only when PMA was added (Fig. 5).

The specificity of this observation was confirmed as no peak was observed when biochanin A was omitted (with the addition of PMA).

From these data, we conclude that the formation of isoflavone metabolites is becoming increasingly complex, albeit that the discovery of new metabolites remedies in part the large difference in intake and excretion of the isoflavone genistein. Although evidence was not obtained for the presence of the rat genistein metabolite HPPA in human urine, a similar, but unidentified phenolic acid was detected.

The demonstration that human PMNs stimulated to have a respiratory burst can produce halogenated isoflavones strongly suggests that this may occur in vivo. The consequence of that is that these locally formed metabolites may have unique biochemical properties and may account for the anti-cancer effects of isoflavones. These changes may involve differences in anti-oxidant properties of isoflavones that although relatively weak, may be increased by synergistic interactions of other dietary anti-oxidants [36]. The presence of a chlorine group, particularly in the 3′-position on the B-ring, may augment the

anti-oxidant properties further. In addition, there may be altered interactions of isoflavones with enzymes catalyzing the synthesis and metabolism of estrogens, with the estrogen receptor system, and with specific protein kinases involved in signal transduction.

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