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Isoflavonoids inhibit catabolism of vitamin D in prostate cancer cells

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Abstract

The high ingestion of soybean products in Asian countries has been suggested to be responsible for a reduced incidence of prostate cancer. The mechanism of action, however, is unknown. Our data demonstrate that genistein and some isoflavone metabolites reduce the activity of 25-D3-24-hydroxylase (CYP24) in the human prostate cancer-derived cell line DU-145. CYP24 is also responsible for degradation of the active vitamin D metabolite 1,25-dihydroxyvitamin D3 which is known to be antimitotic and prodifferentiating in prostate cancer cells. High levels of CYP24 frequently found in prostate cancer cells may thus degrade the active metabolite. This could be prevented by ingestion of genistein-containing food such as soybeans.
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Keywords: Prostate cancer prevention; Isoflavonoids; Vitamin D

1. Introduction

A diet rich in soybeans, as it is consumed in some Asian countries (e.g. China and Japan), has been suggested to play a preventive role in the pathogenesis of many chronic diseases, for instance breast, colonic and prostatic malignancies as well as cardiovascular diseases and osteoporosis. The incidence of these diseases is significantly lower in those countries compared to the Western industrialised world.

Soybeans are rich in isoflavonoids which belong to the group of phytoestrogens. They are thought to be, at least in part, responsible for the above mentioned effects. One of these compounds, genistein, is in soy mainly in the glycone form, genistin, which is converted by the colonic microflora to its aglycone genistein, and further to dihydrogenistein and *p*-ethylphenol. Daidzin, the glycone form of daidzein, another major isoflavonoid contained in soybeans, is converted after hydrolysis to equol, O-desmethylangolensin and dihydrodaidzein [1]. In vitro and in vivo studies have demonstrated that isoflavonoids exert potent antineoplastic effects. Genistein has been shown to inhibit the activity of tyrosine kinase [2] and of topoisomerase [3], to inhibit angiogenesis [4], to induce apoptosis and a cell cycle block in the G2-phase [5]. Also, urinary excretion of isoflavonoids is lower in breast cancer patients than in healthy women [6]. Prostate cancer cells injected into nude mice were growth-arrested after feeding mice an isoflavonoid-rich diet [7]. However, in some

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instances these compounds do not show preventive action [8–10], and even cause enhancement of colonic tumor incidence in rats [11]. In the present report we describe a new mechanism of action for isoflavonoids, which may support their use in tumor prevention as well as in therapy of, for instance, early prostate malignancies.

Vitamin D3 is produced in the skin from 7-dehydrocholesterol by a photochemical reaction mediated by UV light. The vitamin D-25-hydroxylase (CYP27A1) in the liver converts vitamin D3 into 25-hydroxyvitamin D3 (25-D3), which in turn is hydroxylated by the 25-hydroxyvitamin D3-1α-hydroxylase (CYP27B1) in the kidney to 1α,25-dihydroxyvitamin D3 (1,25-D3), the active hormonal metabolite. Another major hydroxylation pathway is the 24-hydroxylation of 25-D3 by the 25-hydroxyvitamin D3-24-hydroxylase (CYP24) to 24,25-dihydroxyvitamin D3 (24,25-D3). CYP24 has a greater affinity to 1,25-D3 than to 25-D3 and thus also degrades the active hormone to mainly inactive compounds. Physiologically CYP24 expression is induced by 1,25-D3 to prevent accumulation of toxic doses of the hormone which would result in severe hypercalcemia. Additionally, as part of a negative feedback mechanism, 1,25-D3 reduces CYP27B1 expression (Fig. 1).

1,25-D3 has been demonstrated extensively to exert antineoplastic effects. It acts through binding to the vitamin D receptor (VDR), which is a ligand-activated transcription factor, and modulates expression of target genes. In vitro studies have shown that 1,25-D3 inhibits proliferation, promotes differentiation and induces apoptosis in many cancer cells [12]. In patients suffering from prostate cancer lower plasma levels of 1,25-D3 have been found [13] and a polymorphism of the VDR has been associated with an increased risk for prostate cancer development [14].

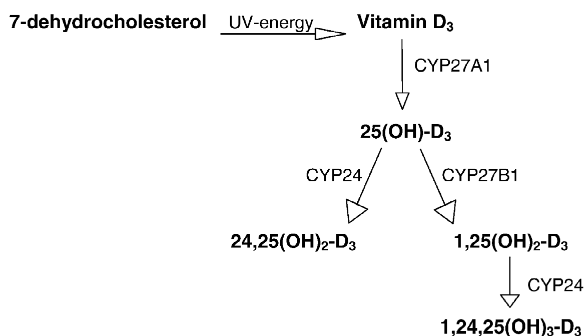

Fig. 1. In vivo vitamin D synthesis and metabolism.

CYP24 has been shown to be expressed in some prostatic tumor-derived primary cultures and cell lines [15]. It was suggested that this expression abrogates the growth regulatory effect of 1,25-D3. In this study we identified genistein as a potent inhibitor of 24-hydroxylation in the prostatic cancer cell line DU-145 and suggest a new mechanism for the tumor-preventive action of isoflavone compounds.

2. Materials and methods

2.1. Chemicals

Genistein (purity 98%) was purchased from Sigma (St. Louis, MO, USA). All other phytoestrogens (purity 98%) were provided by Dr. K. Wähälä (Department of Chemistry, University of Helsinki, Finland). All substances were prepared as stock solutions of 10^{-2} *M* in ethanol.

2.2. Cell culture

DU-145 cells were cultured in RPMI-1640 medium supplemented with 10% foetal calf serum (FCS), glutamine (4 m*M*), penicillin (50 U/ml) and streptomycin (50 μg/ml) (Gibco, Life Technologies, Gaithersburg, MD, USA). Media were changed every second day. For experiments, cells were grown to confluence and kept for 48 h in a phenol-red-free Dulbecco's modified Eagle's medium (DMEM) with transferrin (10 μg/ml, Sigma) sodium selenite (5 ng/ml, Merck, Darmstadt, Germany) and antibiotics. Then media were changed and the isoflavonoid treatment was begun: 5–50 μ*M* of genistein was used from 2 to 48 h and other substances were tested at concentrations of 5 and 50 μ*M* for 48 h. At 3 h before the end of culture, 25-D3 was added together with 0.5 μCi/ml 25(OH)-[26,27-methyl-^{3}H]-D3 (specific activity 30 Ci/mmol, Amersham Pharmacia Biotech, Buckinghamshire, UK) as tracer to yield a

final concentration of 16.6 n*M*. Incubations were stopped by addition of 1.0 ml methanol to each well. Plates were stored at −20 °C under UV light protection until further use. Vitamin D metabolites were subsequently extracted and HPLC analysis was performed.

2.3. Lipid extraction for HPLC

A 1-nmol amount of 25-D3 and 1,25-D3 each was added to culture wells as internal standard to evaluate the efficiency of the extraction procedure. After scraping off the cells, each well was rinsed once with 1 ml methanol and 0.8 ml H_2O. A 2-ml volume of dichloromethane was added and bottles were centrifuged at 1500 rpm at 4 °C for 10 min. The supernatant was reextracted twice with 1 ml dichloromethane. The extracts were dried under a gentle stream of nitrogen at 55 °C. Samples were resolved in the mobile phase [*n*-hexane–isopropanol (94:6, v/v)] and directly subjected to HPLC analysis. Recovery of vitamin D metabolites was between 85 and 90%.

2.4. High-performance liquid chromatography

For straight-phase HPLC a Waters 515 pump operating with an isocratic flow-rate of 2 ml/min was used. Vitamin D metabolites were separated on a silica-based column (Beckman Instruments, Fullerton, CA, USA) (4.6×250 mm, 5 μm). A photodiode array detection system was used to monitor the UV absorption of the added standards. Tritium-labeled metabolites were detected with a flow radiochromatography detector (Packard, Bioscience, Gronningen, The Netherlands). Eluted metabolites were identified by comigration with known standards (kindly provided by Dr. S. Reddy, Brown University, Providence, RI, USA).

2.5. Determination of tissue concentrations of genistein

Prostates were extirpated from mice 24 h after oral administration of 250 μg of genistein, were freeze-dried and stored at −70 °C. For experiments, 50 mg of the sample were dissolved in 300 μl H_2O at room temperature overnight. Next day samples were sonicated for 10 min. A 700-μl volume of methanol was added for precipitation overnight at −20 °C. After centrifugation and reprecipitation, methanol was evaporated and the water phase was extracted with hexane to remove lipids. The water phase was incubated with the hydrolysis mixture (ascorbic acid, charcoal-stripped *Helix pomatia* enzyme (Biosepra, France)) for 2 h at 60 °C. Isoflavonoids were extracted twice with diethyl ether. The ether phase was evaporated and samples were resolved in 300 μl of assay buffer (0.5% BSA–Tris, pH 7.76). Samples were analysed by time-resolved fluorescence immunoassay (TR-FIA).

2.6. Determination of plasma concentrations of genistein

Blood was collected from mice 24 h after oral administration of 250 μg of genistein. Plasma was prepared by conventional methods and was freeze-dried. For analysis, samples were redissolved in the same volume of distilled H_2O as the original plasma volume. Enzymatic hydrolysis was performed as described [16]. Briefly, β-glucuronidase (0.2 U/ml) and sulfatase (2 U/ml) were added and samples were incubated overnight at 37 °C. Isoflavonoids were extracted from plasma twice with diethyl ether. After evaporation of the ether phase, samples were suspended in 300 μl of assay buffer (0.5% BSA–Tris, pH 7.76). The levels of genistein were determined by TR-FIA.

2.7. Time-resolved fluoroimmunoassay

TR-FIA was performed as described previously [16]. Briefly, 15 μl of ^{3}H-estradiol glucuronide was added to tubes to measure the recovery. A 20-μl volume of hydrolysed plasma was pipetted into prewashed goat antirabbit IgG microtiterplate strips. To each were added 100 μl of antiserum and 100 μl of europium-labeled genistein. After incubation on a plate shaker at room temperature for 90 min, the strips were washed, 200 μl of enhancement solution was added. After agitation for 5 min and the strips were analysed in a Victor 1420 multilabel counter (Wallac Oy, Turku, Finland).

2.8. RNA extraction and RT-PCR

Total RNA extracts were prepared using Trizol reagent (Life Technologies) according to the manufacturers' instructions. A 2-μg amount of RNA was reverse-transcribed with a cDNA synthesis kit (SuperScript II, GibcoBRL, Life Technologies, San Diego, CA, USA).The cDNA was used for multiplex PCR of CYP24 and cytokeratin 8 (CK8) (as an internal gel loading control) using the Taq PCR core kit (Qiagen, West Sussex, UK).

Primers were selected using the cDNA as reference. To amplify a 506-base-pair fragment of CYP24 the following primers were used: 5′-CCCACTAG-CACCTCGTACCAAC-3′ (sense); and 5′-CGTAG-CCTTCTTTGCGGTAGTC-3′ (antisense). PCR conditions were: 15 s at 94 °C, 30 s at 59 °C, and 1 min at 72 °C for 28 cycles. CK 8 primers were: 5′-AGTGGGCAGCAGCAACTTTCG-3′(sense); 5′-TTCAGCTTGTCCTGGCCCAGAG-3′(antisense).

A 5-μl volume of PCR product was loaded onto a 2% agarose gel containing ethidium bromide and run at 85 V and 250 mA. Gels were scanned and analysed with a video camera imaging system (Herolab, Wiesloch, Germany), the density of the bands was measured under UV light.

3. Results

3.1. Concentration of genistein in prostatic tissue

In order to determine relevant prostate tissue concentrations which we could use for further in vitro investigations in prostate cells we administered one dose of 250 μg of genistein via a gastric tube to four mice. After 8 and 24 h blood was collected from the tail vein and mice were decapitated. The successful extirpation of the prostate gland was checked microscopically by staining with hematoxylin. We observed the classical rise in genistein concentration in plasma after 8 h (median range 180±20 n*M* versus 2 n*M* in the control mice) and the decline after 24 h (median range 40±4.5 n*M*). Concentrations of genistein in prostatic tissue however were about 10-fold higher when compared to plasma (Fig. 2).

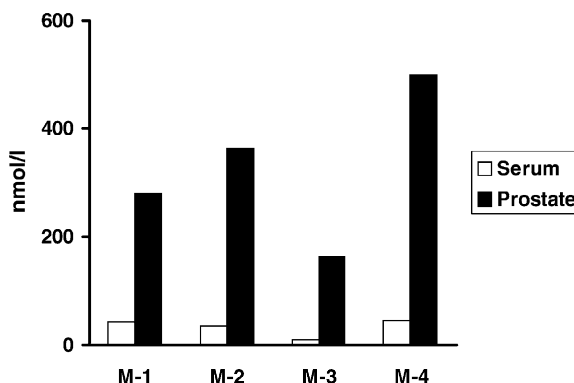

Fig. 2. Concentration of genistein in prostates and serum of 4 mice (M-1–M-4). Mice were administered 250 μg of genistein via a gastric tube; 24 h later genistein was measured by TR-FIA.

3.2. Genistein is a time- and dose-dependent inhibitor of 24-hydroxylation

Genistein and other isoflavones were previously reported to inhibit the activity of several cytochrome enzymes [17]. We used HPLC as an assay for CYP24 activity using 25-hydroxy[26,27^{3}H]cholecalciferol as a precursor. We incubated DU-145 cells with increasing concentrations of genistein (5–50 μ*M*) for 24 or 48 h. At a low concentration (5 μ*M*) genistein was unable to inhibit CYP24 activity (Fig. 3A), whereas at higher concentrations (25 and 50 μ*M*) genistein was a potent inhibitor (Fig. 3B and C) with almost no CYP24 activity detectable after 48 h of treatment with 50 μ*M* of the isoflavonoid (Fig. 3C).

3.3. Genistein reduces CYP24 gene expression

The above-mentioned effect of genistein on cytochrome enzymes was reported to be restricted to a direct inhibition of the enzymatic activity. We therefore wanted to investigate whether the observed effect is indeed a result of direct inhibition or whether transcriptional control is involved. We thus treated DU-145 cells with 50 μ*M* of genistein for 2–8 h and performed RT-PCR. Genistein strongly reduced CYP24 expression to 70% after only 2 h, and to more than 40% after 8 h (Fig. 4) indicating

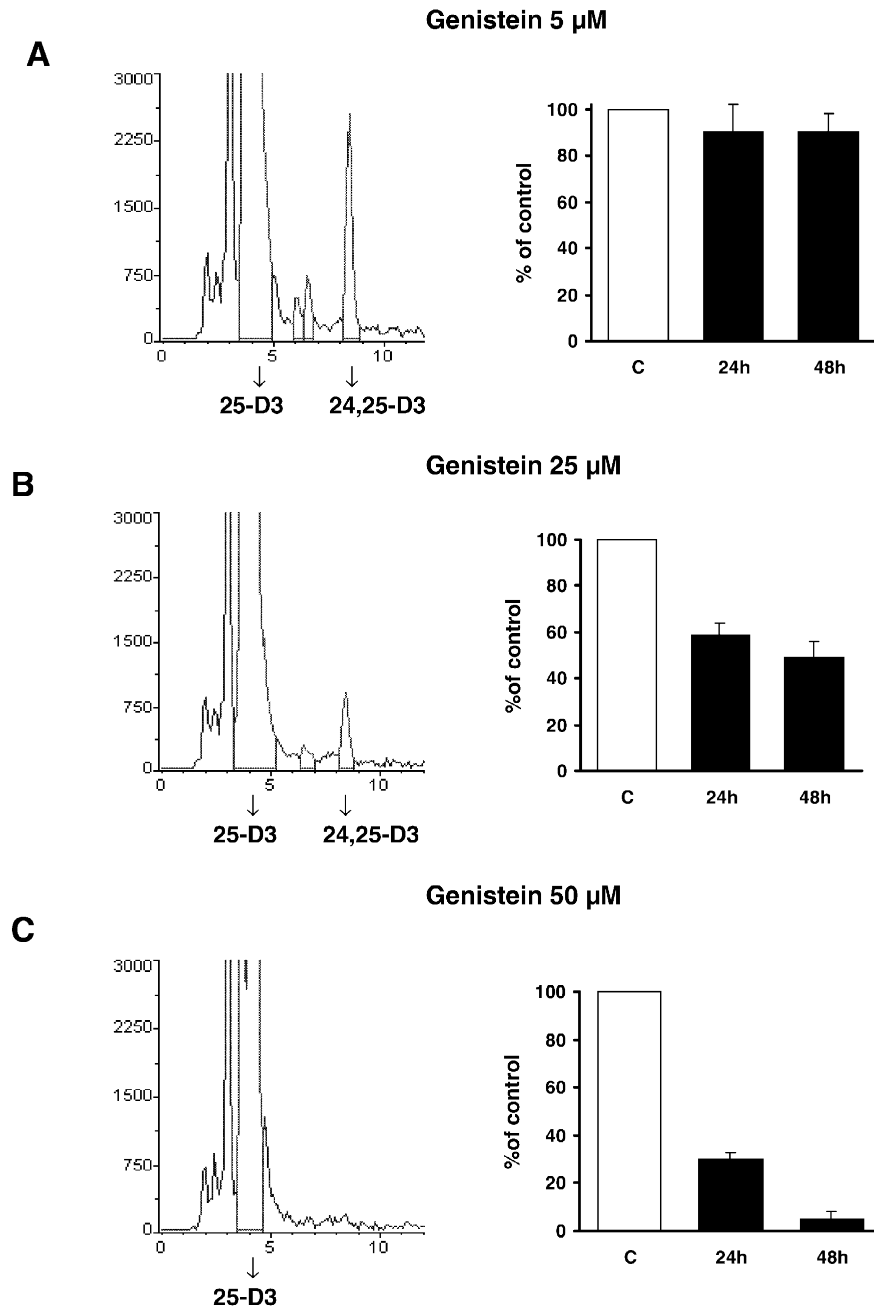

Fig. 3. Effect of genistein on CYP24 activity. DU-145 cells were treated with 5, 25 and 50 μ*M* genistein for 24 and 48 h. Tritiated 25-D3 was added for 3 h and metabolites were extracted as indicated in Materials and methods. (A) Treatment with 5 μ*M*; (B) 25 μ*M*; (C) 50 μ*M* genistein. Left panel: HPLC tracing. Right panel: quantitative evaluation from three different HPLC runs, in percent of solvent control.

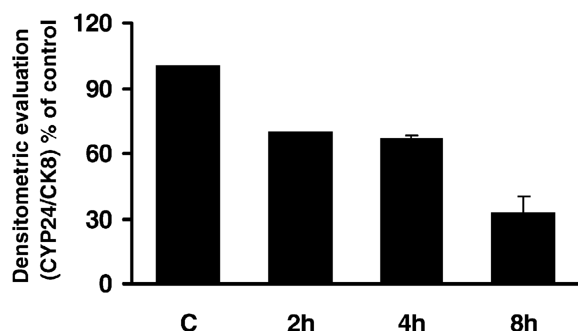

Fig. 4. Effect of 50 μ*M* genistein on CYP24 mRNA expression. Exposure time: 2–8 h. Quantitative densitometric evaluation from three different RT-PCR runs. CK8 mRNA was used as internal loading control.

that genistein indeed regulates CYP24 at the transcriptional level also.

3.4. Influence of daidzein and metabolites of genistein on CYP24 activity

In order to determine whether the observed effect is specific for genistein only, we tested dihydrogenistein, a known metabolite, and tetrahydrogenistein, a potential metabolite of genistein. We treated DU-145 cells for 48 h with 5 and 50 μ*M* of the metabolites and also with 5 and 50 μ*M* of daidzein, another major isoflavonoid, and determined CYP24 activity by HPLC. Though some inhibition was observed at concentrations of 50 μ*M* (Fig. 5), this was relatively weak when compared to that of genistein, which completely abrogated CYP24 activity (Fig. 3)

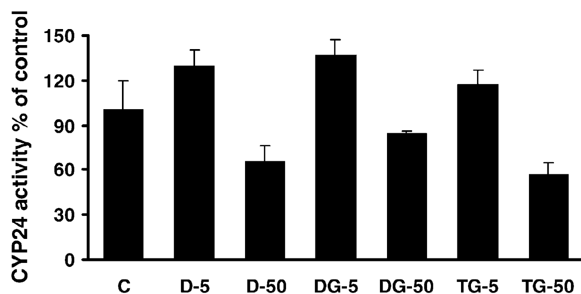

Fig. 5. Quantitative evaluation of CYP24 peak area from three different HPLC experiments. Cells were treated with 5 and 50 μ*M* daidzein (D), dihydrogenistein (DG) or tetrahydrogenistein (TG). CYP24 activity is expressed as percentage of the solvent control (C).

4. Discussion

Cancer prevention by nutritional means has been strongly advocated recently. Special attention has been given to phytoestrogens, since epidemiological studies indicate soy consumption as one of the principal factors in hormone-dependent tumor prevention. Though genistein, a major isoflavonoid present in soybeans, has been shown to inhibit protein tyrosine kinase activity which links its action to growth factor signaling pathways, a definite mechanism for cancer prevention in vivo has not been determined yet.

Epidemiological studies have also indicated the vitamin D system (vitamin D receptor and the active vitamin D metabolite 1,25-D3) as protective against a variety of tumors. Since in areas of high sun exposure the incidence of prostate cancer appeared to be reduced [18], and vitamin D synthesis in the skin is dependent on UV energy, it was suggested that the vitamin D system may also be preventive against prostate malignancies. We therefore investigated a possible interaction of genistein with the vitamin system, especially in view of the fact that genistein is also known to affect activity of a variety of cytochrome enzymes [17].

The catabolic pathway of 1,25-D3 involves activity of a hydroxylase, CYP24. This pathway present in the kidney prevents accumulation of toxic levels of 1,25-D3 in the blood. However, CYP24 activity is enhanced during aging [19] and could therefore conceivably also be responsible for a reduced antimitotic defence by 1,25-D3 against tumor growth in the elderly population. Prostate cancer is a disease typical of the elderly.

Interestingly, also prostatic cancer cell lines have been demonstrated to have CYP24 activity, to a varying extent. The CYP24 expressing prostatic cancer cell line DU-145 has been shown to be

unresponsive to 1,25-D3 due to the extremely high expression of this enzyme. This suggests that DU-145 cells may be typical for those prostate tumor cells, which have escaped the antimitotic influence of ambient 1,25-D3. We were able to demonstrate that genistein is a potent inhibitor of 24-hydroxylation in these cells. However, this is not only a direct enzymatic inhibition as has been demonstrated for genistein with respect to other cytochrome enzymes, but there is also strong transcriptional repression. Metabolites of genistein also repressed activity of CYP24, although to a much smaller extent.

The strongest inhibition that we observed was at a concentration of 50 μM. Similar concentrations in other studies have been suggested to be too high for physiological relevance, because of the much lower human plasma levels of genistein reported in the literature which never exceed 3 μM, even in the Asian population [21]. In animal experiments no more than 3.5 μM plasma genistein was reported [20,22]. Morton et al. [22] however demonstrated that the concentration of isoflavonoids is higher in prostatic fluid than in plasma. This led us to investigate the concentration of genistein in mouse prostate after one high dose (250 μg) of genistein. We were able to demonstrate that after only one bolus of genistein given orally, the substance accumulated at 10-fold higher levels in the prostatic tissue compared to serum concentrations. This result suggests that concentrations close to 50 μM of genistein could physiologically be reached in the tissue after chronic consumption of isoflavonoids and indicates a unique concentrative capability of prostate cells of which the mechanism of action is as yet unknown.

Neither of the metabolites of genistein nor daidzein were as effective as inhibitors of 24-hydroxylation as genistein was. This indicates a specific role for genistein in maintaining the antineoplastic activity of 1,25-D3 in the human prostate.

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