Genistein inhibits Vitamin D hydroxylases CYP24 and CYP27B1 expression in prostate cells

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Abstract

In human prostate cancer cells, the availability of the steroid hormone 1,25-dihydroxyvitamin D3 for antimitotic action is determined through the activity of the two enzymes CYP24 and CYP27B1, viz. 25-hydroxyvitamin D-24-hydroxylase and 25-hydroxyvitamin D-1α-hydroxylase. High performance liquid chromatography (HPLC) analysis of [3H]25(OH)D3 metabolism in human prostate cancer DU-145 cells revealed that genistein and other isoflavonoids, such as dihydrogenistein and daidzein, as well as the antiestrogenic compound ICI 182,780, inhibited Vitamin D metabolizing enzyme activities. Reverse transcriptase-polymerase chain reaction (RT-PCR) showed that only in case of genistein this was due to transcriptional inhibition of CYP24 and CYP27B1 gene expressions. In case of CYP27B1, reduction of gene activity involves histone deacetylation because genistein was inactive in the presence of the histone deactylase inhibitor trichostatin A. In contrast, under the same condition, CYP24 gene activity was largely suppressed. In summary, our results suggest that a combined effect of genistein and trichostatin A could increase the responsiveness of human prostate cancer cells to the antiproliferative action of 1,25-dihydroxyvitamin D3.

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1. Introduction

The active metabolite of Vitamin D3, 1,25-dihydroxyvitamin D3 (1,25-(OH)2D3) exerts antimitotic and prodifferentiating actions in many types of malignant cells [1], including prostate cancer cells [2–4]. 1,25-(OH)2D3 acts by binding to the Vitamin D receptor (VDR), which is a ligand-activated transcription factor [5]. 1,25-(OH)2D3 is produced mainly in the kidney through hydroxylation of its precursor, 25-(OH)D3, by the cytochrome P450 enzyme CYP27B1, viz. 25-hydroxyvitamin D3-1α-hydroxylase (1α-OHase). When sufficient 1,25-(OH)2D3 is available, CYP27B1 expression is low, while, conversely, expression of CYP24, the gene coding for 25-hydroxyvitamin D3-24-hydroxylase (24-OHase), is up-regulated resulting in an increased rate of side-chain oxidation at C24 and C23 of the active hormone 1,25-(OH)2D3 as well as of its precursor 25-(OH)D3 [6].

It has to be also noted that several types of human tumor cells express the 1α-OHase [7,8]. From our studies on the expression of this enzyme in human colon carcinoma cells, we had put forward the notion that tumor-localized conversion of 25-(OH)D3 into the active hormonal metabolite could constitute an autocrine/paracrine defense mechanism against hyperproliferation and tumor progression [9]. However, at the same time high or aberrant expression of the 24-OHase at the tumor site [10] could cause rapid metabolism of 1,25-(OH)2D3 into less active Vitamin D compounds, and could thus counteract any of its inhibitory actions on tumor growth. In this respect, it is of interest that the 24-OHase was recently suggested to be a potential oncogene [11].

Since a high risk for prostate cancer is apparently associated with low ultraviolet radiation and low serum levels of 1,25-(OH)2D3 [12,13], the potential relevance of 1,25-(OH)2D3 or, respectively, of certain of its non-hypocalcemic synthetic analogs for therapy and prevention of prostate cancer was explored in several animal studies (see, e.g. [14,15]) and is under investigation in ongoing clinical trials [16].

The significantly lower prevalence of prostatic malignancies in Asian as compared to Western industrialized
countries [17] has been linked to the consumption of the typical Asian diet, which contains high amounts of soy products and thus is rich in phytoestrogens, particularly in isoflavonoids. One major isoflavonoid is genistein, which inhibits growth of prostate cancer cell lines [18] and angiogenesis in vitro [19], probably through its negative effects on tyrosine kinases [20] and topoisomerase [21]. It is conceivable that genistein and other phytoestrogens through their potential to act as selective estrogen receptor modulators could have an effect on Vitamin D-related inhibition of tumor growth, since interactions in this respect between Vitamin D and estrogen have been observed in human breast cancer cells [22] and in murine colon carcinoma [23]. Since genistein is an inhibitor of several members of the cytochrome P450 enzyme family [24–26], a likely site of interaction with the Vitamin D system is at regulation of expression and activity of the Vitamin D-metabolizing cytochrome P450 enzymes, 1α-OHase and 24-OHase. We therefore initiated a study to address the question whether selected isoflavonoids, viz. genistein, dihydrogenistein and daidzein, in comparison with 17β-estradiol (17β-E2) and with the antiestrogen ICI 182,780, were able to modulate Vitamin D metabolism in the DU-145 and PC-3 human prostate cancer cell lines.

2. Materials and methods

2.1. Chemicals

Genistein and 17β-E2 were purchased from Sigma. Daidzein and dihydrogenistein were provided by Dr. K. Wihulä (Department of Chemistry, University of Helsinki, Helsinki). ICI 182,780 was purchased from Tocris Cookson (Bristol, UK). Trichostatin A and actinomycin D were from Calbiochem (Merck, Darmstadt, Germany). 25-Hydroxy[26,27-methyl-3H]cholecalciferol (30 Ci/mmol) were from Calbiochem (Merck, Darmstadt, Germany). 25-Hydroxy[26,27-methyl-3H]cholecalciferol (30 Ci/mmol) was purchased from Amersham Pharmacia Biotech (Buckinghamshire, UK).

2.2. Cell culture

DU-145 and PC-3 prostate cell lines are both androgen receptor-negative. The former was derived from a brain metastasis, whereas the latter was from a bone metastasis of a prostate primary tumor. Both cell lines were cultured in RPMI-1640 medium supplemented with 10% fetal calf serum, glutamine (4 mM), penicillin (50 U/ml) and streptomycin (50 μg/ml) (all from Invitrogen, Paisley, UK). Media were changed every second day. For experiments, cells were grown to confluency and, prior to experiments, cultured for 48 h in six-well plates in phenol red-free Dulbecco’s modified Eagle’s medium containing 10 μg/ml transferrin (Sigma), 5 ng/ml sodium selenite (Merck, Darmstadt, Germany) and antibiotics. Then treatments in fresh medium were started for indicated time periods.

2.3. High performance liquid chromatography (HPLC) analysis of 25-(OH)-D3 metabolism

During previous time course experiments we had established that evaluation of both 24-hydroxylation and 1α-hydroxylation of 25-(OH)-D3 was possible after 3 h of incubation with the precursor. Therefore, 25-(OH)-D3 together with 0.5 μCi/ml 25-(OH)-[26,27-methyl-3H]-D3 (specific activity 30 Ci/mmol) as tracer was added at a total concentration of 16.6 nM for the last 3 h of culture. Incubations were stopped by addition of 1.0 ml methanol to each well. Plates were stored at −20°C with UV light protection until further use. Lipids were extracted from cultured cells and medium as described previously [10]: 30 μl of 25-(OH)-D3 and 1, 25-(OH)-D3 (each at 10−4 M) were added to each culture well as internal standard to evaluate efficiency of the extraction procedure. Recovery of Vitamin D metabolites was between 85 and 92%

HPLC was performed with a system consisting of two pumps (Model 515, Waters). A photodiode array detector (model SPD-M10Avp, Shimadzu, Kyoto, Japan) was used to monitor UV absorption of added standards. 25-(OH)-[26,27-methyl-3H]-D3 metabolites were detected with a flow radiocromatography detector (C5o5STR, Packard Instruments Company, Meriden, CT) on an Ultra-sphere Silica 5 μm, 4.6 mm × 250 mm column (Beckman Instruments, Fullerton, CA) using an isocratic method 94.6 hexane:2-propanol at a flow rate of 2 ml/min. 25-(OH)-D3 metabolites were identified by matching their elution rates to comigration with known standards (generously supplied by Dr. G.S. Reddy, Brown University, Providence, RI).

2.4. Semi-quantitative reverse transcriptase-polymerase chain reaction (RT-PCR)

Multiple RT-PCR, i.e. simultaneous amplification of a transcript specific for either 1α-OHase or 24-OHase mRNA, and a transcript specific for the epithelial cell marker cytokeratin (CK) 8 as loading control, was carried out for semi-quantitative evaluation of mRNA expression levels as described previously [10].

To amplify a 440 base pair segment of 1α-OHase cDNA, primers were: 5′-CAG AGG CAG CCA TGA GGA AC-3′ (sense); and 5′-GGG TCC CTG GAA GTG GCA TAG-3′ (antisense). To identify a 507 bp fragment of 24-OHase the following primers were used: 5′-CCC ACT AGC ACC TTCG TAC CAA C-3′ (sense); and 5′-CGT AGC CTT TCT TGG GGT AGT C-3′ (antisense). A 300 base pair segment of CK8 was co-amplified (primers: sense 5′-AGT GGG CAG CAG CAA CTT TCG-3′; and antisense 5′-TTC AGC TTC TCC TGG CCC AGA G-3′). PCR products were checked for correct size and fragment length by multiple digestion with restriction enzymes.

Five microliters of PCR product were loaded on a 2% agarose gel containing ethidium bromide and were separated at 70 V, 250 mA. Gels were scanned and analyzed with
2.5. Statistical analyses

Data were obtained from at least three different experiments and are presented as means ± S.D. Student’s t-test was used for statistical group analysis. Statistical significance was considered at P < 0.05.

3. Results

3.1. Action of isoflavonoids, estrogen and antiestrogen on 24-oxidation and 1α-hydroxylation of Vitamin D in DU-145 cells

Fig. 1A shows that by HPLC analysis a number of side-chain metabolites of 25-(OH)D₃, viz. 24,25-(OH)₂D₃, 23,25-(OH)₂D₃, and 25-(OH)-24-oxo-D₃, as well as 1,25-(OH)₂D₃ were identified in lipid extracts of DU-145 cells, providing unequivocal evidence that this type of prostate cancer cells express both 24-OHase and 1α-OHase activity. An inhibitory effect of the isoflavonoid genistein on both enzymes is illustrated in Fig. 1B. When cultured in the presence of 50 μM of the compound, only minute amounts of both types of metabolites of 25-(OH)D₃ were isolated from DU-145 cells.

From the cumulative data shown in Fig. 1C, it is obvious that genistein, in a dose-dependent manner (between 5 and 50 μM), inhibits 24-OHase-mediated formation of side-chain metabolites as well as 1α-hydroxylation of 25-(OH)D₃. In contrast, other isoflavonoids such as the natural metabolite dihydrogenistein or the structurally related daidzein, only inhibited 1α-OHase activity but had no effect on the 24-OHase.

Interestingly, 17β-E₂, when present in DU-145 cell cultures at 1.0 or 10 nM, respectively, affected neither Vitamin D-metabolizing enzyme activity, whereas the classical estrogen receptor antagonist ICI 182,780 strongly reduced activities of both hydroxylases at 10 and 50 μM (Fig. 1C).

When we studied the time course of the inhibitory effects of genistein and of ICI 182,780 (Fig. 2), we noticed a gradual decrease over 48 h in the extent of side-chain modification and of 1α-hydroxylation of 25-(OH)D₃ when DU-145 cells were exposed to genistein (Fig. 2A), whereas in case of ICI 182,780 the maximum of its inhibitory effects was seen already at 4 h culture time (Fig. 2B).

3.2. Action of phytoestrogens on 24-OHase and 1α-OHase mRNA

To investigate a potential transcriptional control by genistein we performed multiplex RT-PCR for 24-OHase or 1α-OHase with CK8 as an internal loading control. Results of representative RT-PCR runs and of densitometric

![Graph](image-url)
evaluations of the amplification procedure are shown in Fig. 3A and B, indicating that treatment of DU-145 cells with 50 μM of genistein for 8 h reduced mRNA levels of the 24-OHase to one-third of control values, whereas in case of the 1α-OHase, only a 25% inhibition was observed (Fig. 3B). In contrast, dihydrogenistein treatment of DU-145 cells did not reduce expression levels of either mRNA species (Fig. 3A and B).

3.3. Effect of genistein on induction of 24-OHase mRNA expression in PC-3 cells

To address the question, whether genistein could also interfere with induction of 24-OHase expression by 1,25-(OH)2D3, we used the prostatic cancer cell line PC-3, which under normal growth conditions is negative for 24-OHase mRNA (Fig. 4). When PC-3 cultures were treated with 10 nM 1,25-(OH)2D3 for 6 h, 24-OHase mRNA was strongly expressed. After pretreatment with 50 μM genistein for 24 h however, 1,25-(OH)2D3-mediated induction of 24-OHase message was largely abolished (Fig. 4A and B).

3.4. Genistein and trichostatin A interact in regulation of 24-OHase and 1α-OHase mRNA expression

From the results obtained so far it is apparent that of all compounds tested, only genistein inhibits the Vitamin D-metabolizing hydroxylases 24-OHase and 1α-OHase at the mRNA level. Since transcriptional repression is
known to frequently involve histone deacetylation, we tested the possibility that the suppressive effect of genistein on 24-OHase and 1α-OHase could be reversed by inhibition of histone deacetylase (HDAC) activity. We therefore co-incubated DU-145 cells for 24 h with 50 μM genistein and 1.0 μM trichostatin A (TSA), a potent inhibitor of all known mammalian HDAC [27,28]. TSA, which alone had no effect on 1α-OHase mRNA, reversed the respective inhibitory effect of genistein almost completely (Fig. 5A and B). In contrast, TSA alone reduced levels of the 24-OHase transcript similar to genistein highly significantly, and in combination with genistein completely abolished transcription of the CYP24 gene (Fig. 5A and B).

3.5. Genistein does not affect mRNA stability

In order to evaluate whether genistein affected 24-OHase and 1α-OHase expression by reducing mRNA half-life, we studied the effect of genistein in DU-145 cells, in which transcription had been blocked by pre-treatment with 2.0 μg/ml actinomycin D for 2 h. Fig. 6 shows that 50 μM genistein caused a more than 50% reduction of 24-OHase mRNA levels in transcriptionally active control cultures, whereas no change in 24-OHase transcripts was observed when transcription was blocked before treatment with genistein (Fig. 6A and B). We made similar observations with respect to 1α-OHase mRNA (data not shown).

4. Discussion

Variant levels of expression of the 24-OHase, which is considered a potential oncogene [11], have been observed in human carcinoma cells. Whereas, for example, in colon carcinoma Caco-2 cells or in the prostate cancer cell line PC-3, respectively, 24-OHase expression without stimulation by 1,25-(OH)2 D3 is low or almost undetectable [29] (cf. Fig. 4), DU-145 prostate cancer cells, as can be inferred from HPLC and RT-PCR analysis of 25-(OH)D3 metabolism (Figs. 1–3), constitutively express high levels of the 24-OHase enzyme. It has been suggested that the 24-OHase, through limiting the intracellular availability of 1,25-(OH)2 D3, confers Vitamin D resistance to DU-145 cells [30].

In the present study, we demonstrate that genistein is a potent inhibitor of the 24-OHase expression, because the isoflavonoid suppresses not only induction by 1,25-(OH)2 D3 of the CYP24 gene in 24-OHase-negative PC-3 cells, but importantly, also inhibits transcription of the gene when highly expressed in DU-145 cells (Figs. 1, 3, and 4). In contrast, other isoflavonoids such as dihydrogenistein, a natural metabolite of genistein, or daidzein, did not alter CYP24 activity significantly (Fig. 1C). Typically for advanced prostate cancer cells, DU-145 cells are endowed with low 1α-OHase activity, which is inhibited by genistein but also by the other isoflavonoids tested (Figs. 1–3).

An estrogenic mode of action of genistein with respect to inhibition of the two Vitamin D-metabolizing enzymes seems unlikely, since 17β-E2 did not affect either 24-OHase or 1α-OHase activity (Fig. 1C). Although it is apparent that the classical estrogen receptor antagonist ICI 182,780 mimicked the effect of genistein on hydroxylase activity, there is no effect on the levels of 24-OHase and 1α-OHase transcripts after 8 h incubation (not shown), which may be...
considered further evidence against involvement of the estrogen receptor in the mechanism mediating inhibition of the two Vitamin D hydroxylases.

Taken together, the inhibitory effect of genistein on the Vitamin D-metabolizing hydroxylases is unique inasmuch as of all compounds tested only genistein inhibits 24-OHase and 1α-OHase expression at the transcriptional level. With respect to 1α-OHase inhibition, the mechanism of action of genistein obviously involves histone deacetylation, since the effect of the phystostegin is precluded in the presence of the HDCA inhibitor TSA (Fig. 5). In contrast, this cannot be said unequivocally for 24-OHase, since TSA per se also blocked transcription of this potential oncogene (Fig. 5).

In our study, rather high levels of genistein, namely 25-50 μM, produced the most significant effects. In several previous investigations, human plasma concentrations of genistein never exceeded 10 μM (see, e.g. [31]). Interestingly, Morton et al. [32] reported much higher levels of isoflavonoids in the prostatic fluid than in plasma. We were recently able to demonstrate in a mouse model that after gavage with genistein intraprostatic levels of the isoflavonoid genistein, in combination with 1,25-(OH)2 D3, was able to rescue 1α-OHase expression (Fig. 5) would minimize the extent of side-chain oxidation of 1,25-(OH)2D3 and thereby increase the availability of the hormone, regardless whether from endogenous or exogenous sources. In addition, at the same time, TSA would rescue 1α-OHase expression, which often is low in prostate cancer cells [36], from further down-regulation by genistein (Fig. 5). In both cases, genistein when administered in combination with TSA could enhance the responsiveness of prostate cancer cells to 1,25-(OH)2D3 and thereby augment the growth inhibitory action of the secoestrogenic hormone.

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References


