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The Flavonoid Apigenin Suppresses Vitamin D Receptor Expression and Vitamin D Responsiveness in Normal Human Keratinocytes

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Apigenin, a flavonoid with chemopreventive properties, induces cellular growth arrest, with concomitant inhibition of intracellular signaling cascades and decreased proto-oncogene expression. We report that apigenin potently inhibited vitamin D receptor (VDR) mRNA and protein expression in human keratinocytes without changes in VDR mRNA half-life. Concurrently, downregulation of retinoid X receptor α, a dramatic loss of c-myc mRNA, and upregulation of $p21^{WAF1}$ took place. Furthermore, a nearly complete suppression of vitamin D responsiveness was observed as estimated by induction of 24-hydroxylase mRNA. The apigenin effect on VDR expression was shared by some other (quercetine and fisetine) but not all tested flavonoids. Interestingly, the apigenin-mediated VDR suppression was counteracted by the NFκB inhibitors sodium salicylate and caffeic acid phenethyl ester. The presented results propose suppression of nuclear receptor levels as a novel mechanism whereby flavonoids exert their pleiotropic effects. This study may also contribute to the understanding of the regulation of VDR expression in epidermal keratinocytes.

Apigenin belongs to a large group of chemically related compounds called flavonoids. These are dietary derived, polyphenolic plant pigments with anti-oxidative properties that exhibit a variety of biological activities including anti-proliferative and anti-inflammatory effects (1, 2). Some of these actions probably arise from the inhibition of enzymes involved in signal transduction such as tyrosine kinases (3–5), protein kinase C (4–7), phosphatidylinositol 3-kinase (5, 8) and extracellular signal regulated kinase (9) and from the suppression of proto-oncogenes downstream of these signaling cascades (7, 9). Apigenin exhibits potent anti-proliferative effects on cultured keratinocytes (10) and blocks ultraviolet- or chemically-induced skin carcinogenesis in mice (11, 12). These properties make apigenin an appealing compound for cancer chemoprevention (1, 7).

Nuclear receptors are transcription factors, which are activated by small lipophilic ligands such as steroids, retinoic acid, $1,25(OH)_2D_3$ and fatty acids (13). Some of these steroid receptors, the so-called class II nuclear receptors form heterodimers with retinoid X receptor (RXR) to regulate the transcription of target genes (13, 14). Several lines of evidence indicate that some of these class II receptors, including vitamin D receptor (VDR), retinoic acid receptor and peroxisome proliferator-activated receptor are implicated in the control of epidermal proliferation, differentiation and carcinogenesis (15–18). The VDR ligand, $1,25(OH)_2D_3$, exerts potent anti-proliferative effects on cultured keratinocytes (15, 19) and can influence the epidermal carcinogenesis process (20, 21). The regulation of nuclear receptor abundance is an important means to control the action of $1,25(OH)_2D_3$ and other steroids (22). We have recently shown that VDR expression in cultured keratinocytes is extensively regulated by environmental factors that determine the proliferation and differentiate state of the cells such as interaction with the extracellular matrix (23), cell density, extracellular calcium concentration, growth factors (24), and ultraviolet B (UVB) irradiation (25). In these experiments, VDR levels fluctuated in concert with the expression of proliferation markers such as c-myc and cyclin D1 (23–25). Hence the potent growth-inhibitory action of apigenin in cultured keratinocytes urged us to investigate its effects on VDR abundance. We describe that apigenin and some other flavonoids are very powerful inhibitors of VDR and RXRα expression and vitamin D responsiveness in keratinocytes.

Abbreviations used: $1,25(OH)_2D_3$, 1α,25-dihydroxyvitamin D_3; RXR, retinoid X receptor; UVB, ultraviolet B; VDR, vitamin D receptor.

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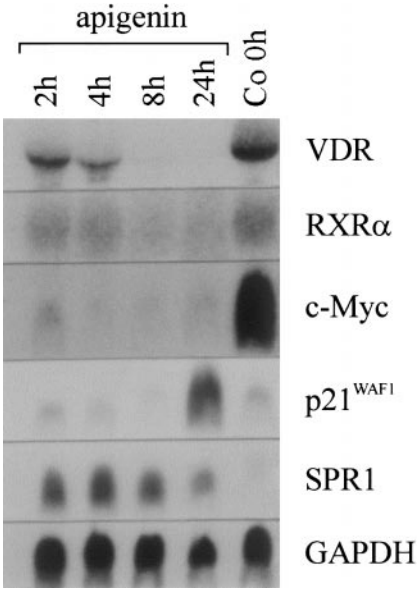

FIG. 1. Apigenin suppresses VDR and RXRα mRNA expression. Subconfluent normal human epidermal keratinocytes (Co 0 h) were treated for the indicated time (in hours) with apigenin (20 μM). Total RNA (12 μg) was isolated for Northern blot analysis followed by sequential hybridization with ^{32}P-labeled probes for VDR, RXRα, c-myc, SPR1, p21^{WAF1}, and GAPDH.

MATERIALS AND METHODS

Cell culture and reagents. Normal human epidermal keratinocytes were isolated from foreskins as described (19) and propagated in Keratinocyte Serum-Free Medium containing 0.09 mM calcium and supplemented with bovine pituitary extract and epidermal growth factor (Gibco-BRL, Gaithersburg, MD). Apigenin and other flavonoids were purchased from Sigma (St. Louis, MO) and dissolved in DMSO. Apigenin was used at a concentration of 20 μM throughout the experiments, which was the lowest dose to exhibit full inhibition of VDR expression (data not shown). Sodiumsalicylate (Sigma) was used at a concentration of 20 mM from a stock in PBS. Caffeic acid phenethyl ester (Calbiochem, La Jolla, CA) was added at 25 μg/ml from an ethanol stock. Actinomycin D was dissolved in DMSO and used at a concentration of 10 μg/ml.

Northern blot analysis. Northern blot experiments were performed as described (19). The following cDNA probes were used: human VDR cDNA (American Type Culture Collection, Rockville, MD), human 1,25-dihydroxyvitamin D_3 24-hydroxylase cDNA (26), human WAF1 cDNA (27), a 0.479 kb human c-myc probe prepared with a RT-PCR amplimer set (Clontech Laboratories Inc., Palo Alto, CA) and a small proline rich protein 1 (SPR 1) probe (28). For verification of even loading we used a probe for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) or 18S RNA.

Western blot analysis. 100 μg total protein samples were subjected to immunoblotting using standard procedures (19). We used a mouse monoclonal antibody to chicken VDR (29) and a monoclonal anti-β-actin antibody (Sigma) as a control. Quantification of protein or RNA bands on Western or Northern blots was performed using a laser densitometric scanner (Pharmacia Biotech Inc., Piscataway, NJ).

RESULTS

Apigenin was previously shown to provoke cell cycle arrest in human keratinocytes (10). Accordingly, the mRNA for the proto-oncogene c-myc was most rapidly (less than 2 h) and dramatically (more than 98%) suppressed by the flavonoid at a concentration of 20 μM (Fig. 1). In addition, the transcript for the cyclin-dependent kinase inhibitor p21^{WAF1}, a negative regulator of cell cycle progression, was markedly (10-fold) induced, though with a slower time course (Fig. 1). Besides these molecular markers for growth arrest, apigenin-treated cells displayed a tendency towards the differentiated keratinocyte phenotype with a 15-fold induction of the cornified envelope precursor small proline rich protein 1 (SPR1) (Fig. 1). The treated keratinocytes also adopted a different morphological aspect characterized by closely juxtaposed cells with a polygonal shape (not shown). These effects were accompanied by spectacular changes in the expression of the investigated nuclear receptors: apigenin caused a huge, rapid and sustained 98% suppression of VDR mRNA (Fig. 1). Transcript levels of its partner nuclear receptor RXRα were also downregulated but to a lesser extent (65%; Fig. 1). The aforementioned changes in gene expression as well as the morphological changes were fully reversible by washing the cells thoroughly and incubating them with fresh growth medium not containing the flavonoid (not shown). Immunoblot analysis essentially confirmed the Northern blot data with a profound drop of VDR protein levels by 95% and an 80% decrease of RXRα following apigenin addition (Fig. 2).

The half-life of VDR mRNA was equal (about 3 h) in apigenin-treated or untreated keratinocytes (Fig. 3). In addition, the time course of VDR mRNA downregulation remained unchanged in apigenin-treated cells with or without actinomycin D (Fig 3). These data imply that apigenin suppressed the transcription of the VDR gene without affecting VDR mRNA stability. Because the pattern of VDR and RXRα gene expression elicited by apigenin resembled the UVB effects we described previously (25), we investigated the effect of sodiumsalicylate and caffeic acid phenethylester. These are inhibitors of the transcription factor NFκB (30, 31) that were able to block UVB-mediated suppres-

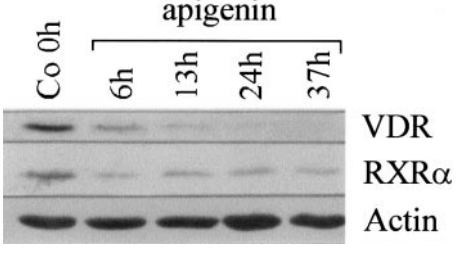

FIG. 2. Decreased VDR and RXRα protein levels following apigenin treatment. Keratinocytes (Co 0 h) were treated for the indicated time (in hours) with 20 μM apigenin. Total protein samples (100 μg) were subjected to immunoblot analysis with specific antibodies against VDR, RXRα, and β-actin.

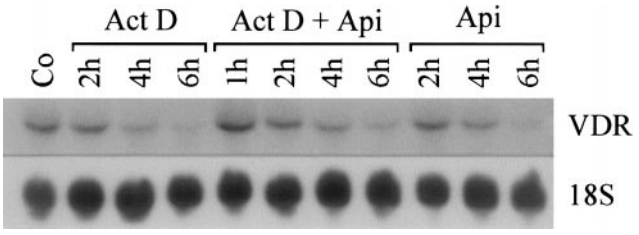

FIG. 3. Apigenin does not affect VDR mRNA half-life. Subconfluent keratinocytes (Co 0 h) were treated with actinomycin D (Act D, 10 μg/ml), apigenin (api, 20 μM), or both for the indicated time in hours. VDR mRNA was investigated by Northern blot analysis.

sion of VDR (25). Both compounds equally and strongly counteracted the apigenin effects on VDR, RXRα and c-myc expression (Fig. 4), suggesting that a similar mechanism may be involved in the UVB and apigenin effects on VDR expression. Sodiumsalicylate or caffeic phenethylester did not affect VDR mRNA levels by themselves (data not shown).

To assess whether the suppression of VDR was a unique feature of apigenin we tested some other related flavonoids (fisetin, genistein, luteolin, myricetin and quercetin). The apigenin effect on VDR expression was shared by some of these to a varying degree (fisetin > quercetin > genistein) whereas other compounds (luteolin, myricetin) exerted only marginal effects (Fig. 5). Fisetin, luteolin, quercetin and to a lesser extent genistein also suppressed RXRα mRNA levels (Fig. 5). Previously, we observed a striking correlation between c-myc and VDR mRNA levels in keratinocytes (23–25). This remarkable association was reported before in osteosarcoma cells (32). Interestingly, some of the flavonoids (luteolin, myricetin and to a certain degree genistein and quercetin) seemed to affect c-myc and VDR expression in a dissociated way.

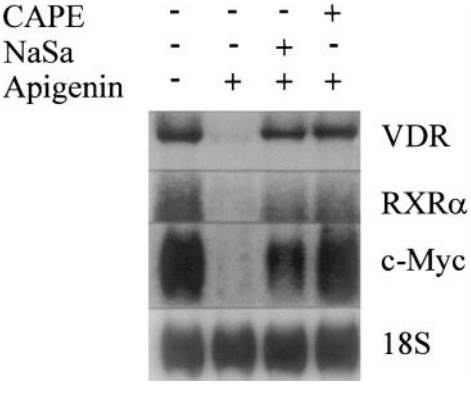

FIG. 4. Suppression of VDR mRNA by apigenin is abolished by sodium salicylate (NaSa) or caffeic acid phenethyl ester (CAPE). Keratinocytes were pretreated for 1 h with 20 mM sodium salicylate or 25 μg/ml caffeic acid phenethyl ester and then treated with 20 μM apigenin for 7 h. Total RNA (12 μg) was used to detect the indicated mRNAs by Northern analysis.

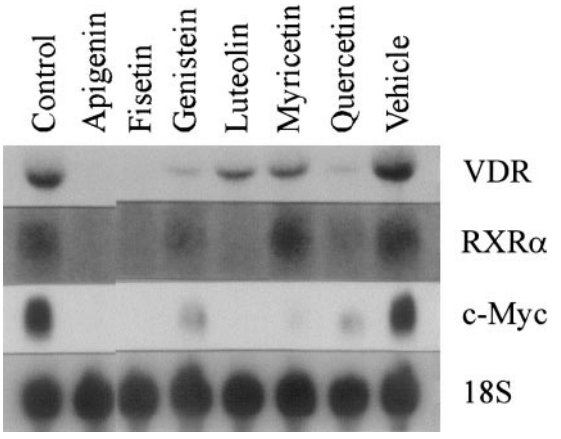

FIG. 5. VDR expression is differentially affected by different flavonoids. Keratinocytes (control) were treated with 20 μM apigenin or with fisetin, genistein, luteolin, myricetin, or quercetin (100 μM) for 24 h. Northern blot analysis was used to visualize the indicated mRNAs.

Apigenin and some other selected flavonoids thus brought about a dramatic fall in VDR (and RXRα) abundance. To determine the functional repercussion of this effect, we studied 1,25$(OH)_2D_3$ induced 24-hydroxylase mRNA levels in the presence or absence of flavonoids. The 24-hydroxylase protein is a catabolic enzyme for 1,25$(OH)_2D_3$ and its gene promoter contains two collaborative vitamin D response elements (33). Therefore it represents an excellent biomarker to assess vitamin D responsiveness (23–24). As shown in Fig. 6, the decreased VDR levels in apigenin-treated keratinocytes almost completely abrogated the response to 1,25$(OH)_2D_3$. For the other flavonoids there was also a straightforward correlation between the effects on VDR expression and vitamin D responsiveness, with the exception of luteolin (compare Fig. 5 and Fig. 6).

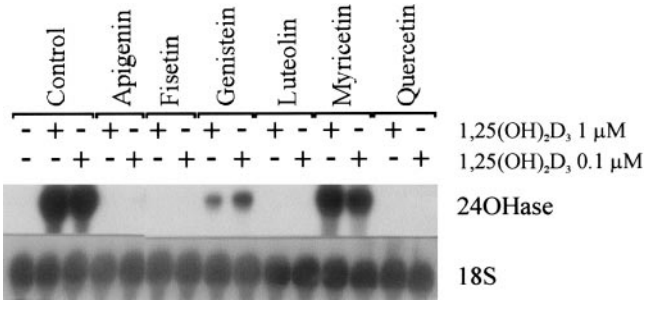

FIG. 6. Apigenin and some other flavonoids suppress vitamin D responsiveness. Keratinocytes were treated with vehicle (control), 20 μM apigenin, or fisetin, genistein, luteolin, myricetin, or quercetin (100 μM) for 24 h and then stimulated for an additional 20 h with 1,25$(OH)_2D_3$ at 0.1 or 0.04 μM. Total RNA samples were subjected to Northern blot analysis to visualize 24-hydroxylase mRNA induction as a sensitive marker for vitamin D responsiveness.

DISCUSSION

Apigenin provoked a very strong, rapid and sustained suppression of VDR levels and (to a lesser extent) of RXRα levels in our model of cultured human keratinocytes. The effect on VDR expression appeared to involve transcriptional suppression and led to a drastically decreased vitamin D responsiveness. Some other flavonoids exerted similar effects. The biological relevance of this marked regulation of VDR expression by apigenin remains elusive. Substantial amounts of flavonoids are taken in via fruit and vegetables and their beneficial effects in the prevention of cancer and coronary heart disease have raised considerable interest (34). These favorable actions are presumably based on their anti-oxidative properties (35) and their ability to inhibit signaling molecules and proto-oncogene expression (5, 9). Apigenin and related products have also been shown to exert some endocrinological effects: they inhibit aromatase activity (36), interfere with thyroid function (37) and exhibit estrogenic or progestational properties by activation of the respective steroid receptors (38, 39). Our observations indicate that regulation of nuclear receptor levels is an additional mechanism whereby these agents may impact the endocrine system. However, it remains to be determined whether these effects can also be triggered in vivo by dietary ingestion of flavonoids and whether vitamin D target tissues such as bone or gut could be affected on the long term in populations that consume a diet rich in flavonoids (like in Western-Africa where the anti-thyroid effect of flavonoids is thought to contribute to the occurrence of endemic Goiter) (37). Moreover, molecules that inhibit VDR expression *in vivo* could be useful as a novel therapeutic approach of hypercalciuria, caused by intestinal calcium hyperabsorption due to hyperresponsiveness of VDR gene expression to calcitriol (40).

The impressive effects of flavonoids in our experimental model may also prove valuable to dissect the signaling pathways involved in the regulation of VDR in keratinocytes. Similarities in the effect of apigenin and UVB (25) on VDR expression suggest a common underlying mechanism. Indeed, the unrelated NFκB inhibitors sodiumsalicylate and caffeic acid phenethylester blocked the downregulation of VDR by UVB (25) or apigenin, suggesting the involvement of NFκB in the regulation of VDR expression. NFκB is a well known mediator of the UVB response (41) and the observation of decreased IκBα protein levels in apigenin-treated keratinocytes (our unpublished results) also points to NFκB activation. VDR transcript levels were regulated by apigenin in an apparently coordinate way with mRNA levels of the proliferation marker c-myc. These data confirm an earlier report on this issue (32) and support the alleged association between VDR expression and the proliferative keratinocyte phenotype (23–24). In this context, it is of great interest that NFκB was recently implicated in cellular growth arrest in general (42) and in epidermal growth control in particular (43). The presence of a consensus binding sequence for NFκB in the 5′-flanking region of the VDR (44) and c-myc (45) genes, indicates that both genes may be jointly regulated by NFκB. Direct regulation of the VDR promoter by c-myc (46) may further contribute to their concordant expression. However, treatment with some flavonoids was accompanied by discordance of c-myc and VDR mRNA levels in our experiments. Therefore it seems that additional signaling pathways are involved in VDR and c-myc regulation, which may be differentially affected by distinct flavonoids.

Taken all together, our results demonstrate that modulation of nuclear receptor expression belongs to the pleiotropic biological actions of flavonoids. It remains to be determined which mechanisms are involved and how these effects relate to the growth suppressive properties of apigenin (and related compounds).

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