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An alternative pathway of vitamin D₂ metabolism Cytochrome P450_{scc} (CYP11A1)-mediated conversion to 20-hydroxyvitamin D₂ and 17,20-dihydroxyvitamin D₂

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Abstract

We report an alternative, hydroxylating pathway for the metabolism of vitamin D₂ in a cytochrome P450 side chain cleavage (P450_{scc}; CYP11A1) reconstituted system. NMR analyses identified solely 20-hydroxyvitamin D₂ and 17,20-dihydroxyvitamin D₂ derivatives. 20-Hydroxyvitamin D₂ was produced at a rate of 0.34 mol·min⁻¹·mol⁻¹ P450_{scc}, and 17,20-dihydroxyvitamin D₂ was produced at a rate of 0.13 mol·min⁻¹·mol⁻¹. In adrenal mitochondria, vitamin D₂ was metabolized to six monohydroxy products. Nevertheless, aminoglutethimide (a P450_{scc} inhibitor) inhibited this adrenal metabolite formation. Initial testing of metabolites for biological activity showed that, similar to vitamin D₂, 20-hydroxyvitamin D₂ and 17,20-dihydroxyvitamin D₂ inhibited DNA synthesis in human epidermal HaCaT keratinocytes, although to a greater degree. 17,20-Dihydroxyvitamin D₂ stimulated transcriptional activity of the involucrin promoter, again to a significantly greater extent than vitamin D₂, while the effect of 20-hydroxyvitamin D₂ was statistically insignificant. Thus, P450_{scc} can metabolize vitamin D₂ to generate novel products, with intrinsic biological activity (at least in keratinocytes).

Keywords

cytochrome P450_{scc}; keratinocytes; skin; vitamin D₂

Vitamin D₂ (ergocalciferol) is a product of UVB-mediated transformation of ergosterol, a 5,7-diene phytosterol, which is synthesized by fungi and phytoplankton but not in the animal kingdom [1]. The physicochemical reactions that generate vitamin D₂ are similar to those involved in the generation of vitamin D₃ from 7-dehydrocholesterol: UVB energy converts ergosterol into previtamin D₂, while thermal energy (at 37 °C) converts previtamin D₂ into

vitamin D₂ [1]. Vitamin D₂ differs from vitamin D₃ in exhibiting a lesser hypercalcemic effect [2,3], making it a potential precursor for effective drugs in therapy for cancer [1,3–5], or for proliferative cutaneous diseases [1,6]. Such use is based on the non-metabolic actions of vitamin D apart from its effect on calcium. These include modulation of immune and neuroendocrine activities, cellular proliferation, differentiation and apoptosis in cells of different lineages, and protection of DNA against oxidative damage and action as a cell membrane antioxidant [1,3,6,7].

Structurally, vitamin D₂ differs from vitamin D₃ in that its side chain has a C24 methyl group and a C22–C23 double bond. These features are responsible for the differences in oxidative processes occurring on the side chain relative to those observed for vitamin D₃ [8,9]. However, the main steps of metabolic conversion of vitamins D₃ and D₂ *in vivo* are mediated by the same enzymes, with similar products that include 24- and 25-hydroxy derivatives [1,5,10,11]. These are further hydroxylated at position 1 to generate 1 α ,24-dihydroxyvitamin D₂ and the metabolite with the highest biological activity, 1 α ,25-dihydroxyvitamin D₂ [12]. Additional hydroxylations produce 1 α ,24(S),26-trihydroxyvitamin D₂ and 1 α ,24(R),25-trihydroxyvitamin D₂, and further hydroxylation at position 26 or 28 results in tetrahydroxyvitamin D₂ [12]. 24-Hydroxyvitamin D₂ and 25-hydroxyvitamin D₂ are inactivated through the transformations to 24(S),26-dihydroxyvitamin D₂ and 24(R),25-dihydroxyvitamin D₂, respectively [12]. Additional derivatives that have been identified are generated through other modifications of the side chain or of the A-ring [12].

Cytochrome P450scc (CYP11A1) catalyzes the first step in steroid synthesis, the cleavage of the side chain of cholesterol to produce pregnenolone [13–15]. This reaction proceeds via the enzyme-bound reaction intermediates 22R-hydroxycholesterol and 20 α ,22R-dihydroxycholesterol [13–15]. Recently, it has been demonstrated that in addition to cholesterol, P450scc can also use 7-dehydrocholesterol, vitamin D₃ and ergosterol as substrates [16–19]. P450scc cleaves the side chain of 7-dehydrocholesterol, producing 7-dehydropregnenolone [18]. With ergosterol and vitamin D₃, P450scc hydroxylates the substrate but cleavage of the side chain is not observed [17,19]. P450scc converts vitamin D₃ to 20-hydroxyvitamin D₃ and 20,22-dihydroxyvitamin D₃ [16,17] and metabolizes ergosterol to 17 α ,24-dihydroxyergosterol [19]. Thus, a new family of metabolites can be generated by the action of P450scc, with the nature of the modifications differing between substrates of animal (7-dehydrocholesterol and vitamin D₃) and plant (ergosterol) origin. To further characterize these novel metabolic pathways, we have investigated the action of mammalian cytochrome P450scc on vitamin D₂, utilizing both purified enzyme in a reconstituted system and adrenal mitochondria, with products being identified by MS and NMR.

Results and Discussion

Metabolism of vitamin D₂ by purified P450scc in a reconstituted system

Vesicle-reconstituted P450scc metabolized vitamin D₂ to two novel products as shown by TLC; these were not seen in control incubations where the electron source was omitted (Fig. 1). As expected, there was production of a little pregnenolone from cholesterol that copurified with bovine P450scc, confirming the side chain-cleaving activity of the enzyme. Following their elution from TLC plates, both vitamin D₂ metabolites displayed UV absorbance corresponding to an intact vitamin D chromophore (λ_{max} at 265 nm and λ_{min} at 228 nm). For metabolite 1, the molecular ion had m/z = 412 with fragment ions m/z = 394 (412–H₂O), m/z = 379 (394–CH₃), m/z = 376 (412–2H₂O) and m/z = 361 (379–H₂O). The molecular ion of metabolite 2 had m/z = 428, with fragment ions at m/z = 410 (428–H₂O), m/z = 392 (428–2H₂O), m/z = 395 (410–CH₃) and m/z = 377 (428–2H₂O–CH₃). Since vitamin D₂ has

$m/z = 396$, metabolite 1 was identified as hydroxyvitamin D₂, and metabolite 2 as dihydroxyvitamin D₂ (Fig. 1C).

Identification of the structure of vitamin D₂ metabolites

Incubation of P450_{scc} (2.0 μ M) with vitamin D₂ in phospholipid vesicles (40 mL) for 1 h produced 70 μ g of TLC-purified hydroxyvitamin D₂ (4% yield) and 60 μ g of TLC-purified dihydroxyvitamin D₂ (3.3% yield). Products from two 40 mL incubations were pooled and used for structural analysis by NMR.

Identification of metabolite 1 was accomplished by analysis of proton 1D, COSY and proton–carbon correlation spectroscopy (HSQC) spectra of this compound and of parent vitamin D₂ (Fig. 2). The high-order pattern in proton NMR of vitamin D₂ at 5.19 p.p.m. (22-CH) and 5.20 p.p.m. (23-CH) became separated to 5.54 p.p.m. (22-CH) and 5.42 p.p.m. (23-CH) in metabolite 1 (Fig. 2, projections on COSY spectra). The scalar coupling between 22-CH and 20-CH did not exist in this metabolite (Fig. 2B). At the same time, the doublet of the 21-methyl in vitamin D₂ (proton at 1.01 p.p.m. and carbon at 21.2 p.p.m.; Fig. 2C) became a singlet in metabolite 1 with a down-field shift (proton at 1.30 p.p.m. and carbon at 29.5 p.p.m.; Fig. 2D), also indicating the removal of scalar coupling from 20-CH. Other regions of the spectra are similar between vitamin D₂ and metabolite 1. All these changes can be readily explained by the presence of a 20-OH group in metabolite 1. The impurities present in metabolite 1 have strong NMR signals in the low chemical shift region but not in the high chemical shift region, and they probably derive from the TLC plate used in the purification process.

The HSQC spectrum of the methyl region in metabolite 2 was cleaner and similar to that of metabolite 1, indicating the presence of 20-OH and no other hydroxyl group on the side chain (Fig. 3D). The A-ring and double bond linker were also intact in this metabolite, indicating that the second hydroxylation is either at the B-ring or at the C-ring (Fig. 3). The well-isolated proton NMR signals of 9-CH₂ (1.68 p.p.m. and 2.82 p.p.m.) have very similar position and coupling patterns in vitamin D₂ and metabolite 2, indicating that the B-ring stays intact. Therefore, the second hydroxylation must occur in the C-ring. The 14-CH in this metabolite has a large downfield shift in its proton NMR (1.99 p.p.m. in vitamin D₂ and 2.68 p.p.m. in metabolite 2; Fig. 3A and Fig. 3B), while the proton NMR of the 17-CH in the vitamin D₂ standard at 1.32 p.p.m. disappeared. The shift of the 14-CH is caused by the formation of 17-OH in this metabolite. Hence, this dihydroxylated metabolite is most likely to be 17,20-dihydroxyvitamin D₂.

We have therefore shown that P450_{scc} hydroxylates vitamin D₂, and generates hydroxyvitamin D₂ and dihydroxyvitamin D₂ as main products in approximately equivalent amounts. NMR analysis further showed that these products correspond to 20-hydroxyvitamin D₂ and 17,20-dihydroxyvitamin D₂, and also revealed that the initial hydroxylation occurs at position 20, and is followed by a second hydroxylation at C17 (Fig. 4). The explanation for hydroxylation in these positions lies in the structure of vitamin D₂, which has a C22–C23 double bond that both prevents hydroxylation at C22 and apparently limits hydroxylation of the side chain to C20. Hydroxylation of the C-ring at position 17 indicates a shift in substrate orientation in the active site, as compared to cholesterol, vitamin D₃, or 24 α -methylcholesterol (campesterol), where P450_{scc} is free to hydroxylate at C20 and C22 [16,17,20]. Interestingly, ergosterol (provitamin D₂) is hydroxylated at C17 similar to vitamin D₂, but the second hydroxylation is at C24 rather than C20 [19]. The detected accumulation of 20-hydroxyvitamin D₂ (Fig. 1A) suggests that it can be released from the active site of P450_{scc}, with only a portion remaining bound or rebinding for subsequent hydroxylation at C17. This is again in contrast to the P450_{scc}-mediated metabolism of ergosterol, where the accumulation of monohydroxy product is only minor, and also in contrast to the conversion of cholesterol into pregnenolone, where hydroxycholesterol intermediates are not normally released [20,21].

The rate of vitamin D₂ metabolism by purified P450scc

To obtain an estimate of the initial rate of vitamin D₂ metabolism by P450scc, vitamin D₂ at a molar ratio to phospholipid of 0.4 was incubated with P450scc for 5 min at 35 °C. The 20-hydroxyvitamin D₂ and 17,20-dihydroxyvitamin D₂ products were extracted, purified by TLC and quantitated from their absorbance at 264 nm. 20-Hydroxyvitamin D₂ was produced at a rate of 0.34 mol·min⁻¹·mol⁻¹ P450scc, and 17,20-dihydroxy vitamin D₂ was produced at a rate of 0.13 mol·min⁻¹·mol⁻¹ P450scc. Under similar conditions, this preparation of P450scc converted cholesterol to pregnenolone at a rate of 14.4 mol·min⁻¹·mol⁻¹ P450scc. The rate of hydroxylation of vitamin D₂ by P450scc is slightly lower than the rate of hydroxylation of its precursor, ergosterol [19].

Vitamin D₂ metabolism by adrenal mitochondria

To evaluate the biological relevance of the above findings, we incubated purified adrenal mitochondria, which contain a high concentration of P450scc, with vitamin D₂. Tests were performed in the presence (experimental) or absence (control) of NADPH and isocitrate. When the reaction products were subjected to LC/MS or LC with UV spectral analysis, we detected six new products by monitoring at 265 nm of HPLC-separated fractions (Fig. 5). These products had UV absorbance spectra characteristic of the vitamin D triene, with λ_{max} at 265 nm and λ_{min} at 228 nm, and displayed a molecular ion $[M + 1]^+$ at $m/z = 413$ and a major fragment ion at $m/z = 395$ ($413 - \text{H}_2\text{O}$), indicating that they represent isomers of hydroxyvitamin D₂ (not shown). The molecular ion $[M + 1]^+$ for vitamin D₂ (not shown) had the expected $m/z = 397$.

To further study the possible involvement of P450scc in the formation of the vitamin D₂ metabolites, aminoglutethimide, a specific inhibitor of cytochrome P450scc in rat adrenal mitochondria [22,23], was added to the reaction mixture. The formation of the unknown metabolites 1, 2, 3, 5 and 6 decreased in a parallel fashion (Fig. 5C). More profound inhibition was observed in the case of metabolite 4, which suggests that it represents 20-hydroxyvitamin D₂. This provides further evidence that vitamin D₂ hydroxylation in adrenal mitochondria is catalyzed by P450scc, especially for production of metabolite 4 (putative 20-hydroxyvitamin D₂).

Initial tests for biological activity of vitamin D₂ metabolites

Cultured human epidermal HaCaT keratinocytes were incubated with HPLC-purified 20-hydroxyvitamin D₂ or 17,20-dihydroxyvitamin D₂ added to the culture media at a concentration of 10⁻¹⁰ M. This caused inhibition of DNA synthesis, significantly greater than that seen with vitamin D₂ itself (Fig. 6A). A similar inhibitory effect of both hydroxy metabolites was also seen in an additional independent experiment (not shown). We also tested for an effect of hydroxyvitamin D₂ products on keratinocyte differentiation, with vitamin D₂ and 5 mM Ca²⁺ as positive controls. This was done using the firefly luciferase reporter gene plasmid IVL-Luc, containing the involucrin gene promoter region (-668 bp to + 34 bp) (Fig. 6B). Since involucrin expression is characteristically proportional to keratinocyte differentiation [24–28], these assays are typically used in models testing for keratinocyte differentiation [24]. All of the tested compounds stimulated transcriptional activity of the involucrin promoter; the most significant effects were shown by Ca²⁺ and 17,20-dihydroxyvitamin D₂, which simulated luciferase activity 25-fold and 12-fold, respectively (Fig. 6B). The stimulatory effect of 17,20-dihydroxy-vitamin D₂ was significantly higher than that of vitamin D₂ ($P < 0.05$), while the effect of 20-hydroxyvitamin D₂ on involucrin promoter activity was statistically insignificant ($P > 0.05$). Thus, the data above indicate that vitamin D₂ can be converted to product(s) of higher biological activity by P450scc.

Conclusions

The novel hydroxylating activity of mammalian P450scc towards vitamin D₂ to generate 20-monohydroxyvitamin D₂ and 17,20-dihydroxyvitamin D₂ raises questions of a new role for this enzyme and on the biological activity of its products. It was previously shown that P450scc cleaves the side chain of 7-dehydrocholesterol to produce 7-dehydropregnenolone [16,18], that it hydroxylates vitamin D₃ to 20S-hydroxyvitamin D₃ and 20,22-dihydroxyvitamin D₃ [17], and that it hydroxylates ergosterol to 24-monohydroxyergosterol and 17 α ,24-dihydroxyergosterol [19]. Thus, it is becoming apparent that metabolism by mammalian P450scc opens novel pathways, where processing is determined by both substrate structure (5,7-dienes vs. secosteroids) and origin (animal kingdom vs. fungi or phytoplankton). The human disease, Smith Lemli Opitz Syndrome, illustrates the significance of the former pathway; defective 7-delta reductase leads to excessive accumulation of 7-dehydropregnenolone and its hydroxy derivatives, with characteristic pathologic features [29,30]. Conversely, in the case of vitamin D₃, P450scc action may prevent its sequential transformation to bioactive calcitriol, although the activity of the alternative products remains to be tested [17]. The transformation of ergosterol [19] results in distinct products that are also biologically active. Both 20-monohydroxyvitamin D₂ and 17,20-dihydroxyvitamin D₂ inhibit proliferation of human keratinocytes to a greater degree than vitamin D₂ itself, while only 17,20-dihydroxyvitamin D₂ is able to stimulate activity of the involucrin promoter (higher than vitamin D₂). Thus, both products show biological activity, differentially expressed depending on the phenotypic feature measured. Within the context of the recently described pleiotropic activity of vitamin D [1,4,5], the new hydroxy derivatives of vitamin D₂ could have a place in the management of epithelial hyperproliferative disorders or skin diseases. This may be particularly important when considering the limitation in clinical use imposed by the potentially toxic hypercalcemic action [1,4,5]. Since vitamin D₂ is absorbed by the alimentary tract, it could be metabolized in any organ expressing high levels of P450scc, such as adrenal glands (see above), gonads [31] or placenta [32], raising the possibility of additional systemic effects. Organs expressing low levels of P450scc, which include brain [33], gastrointestinal tract [34], kidney [35], and skin [18], could alternatively produce and use the same metabolites in local paracrine, autocrine or intracrine roles.

Our previous work [17–19] and current findings have clearly uncovered a new biological significance for an ancient enzyme, cytochrome P450scc. We have shown that P450scc opens new metabolic pathways, thus generating novel steroidal and secosteroidal derivatives. Of these, some have already been shown to possess biological activity (vitamin D₂ and ergosterol hydroxy derivatives), while for others the biological activity remains to be defined.

Experimental procedures

Enzymatic assays

Metabolism of vitamin D₂ by purified cytochrome P450scc—The detailed methodology has been described before [17]. Briefly, bovine cytochrome P450scc and adrenodoxin reductase were isolated from adrenals [36,37]. Adrenodoxin was expressed in *Escherichia coli* and purified as previously described [38]. The reaction mixture comprised 510 μ M phospholipid vesicles (dioleoyl PC plus 15 mol% cardiolipin) with a vitamin D₂/phospholipid molar ratio of 0.2, 50 μ M NADPH, 2 mM glucose 6-phosphate, 2 U·mL⁻¹ glucose-6-phosphate dehydrogenase, 0.3 μ M adrenodoxin reductase, 6.5 μ M adrenodoxin, 3.0 μ M cytochrome P450scc and buffer pH 7.4. After incubation at 35 °C for 3 h, the mixture was extracted with methylene chloride and dried under nitrogen. Products were analyzed and purified by preparative TLC on silica gel G with three developments in hexane/ethyl acetate (3 : 1, v/v). For NMR and MS analyses, they were eluted from the silica gel with chloroform/methanol (1 : 1, v/v), dried separately under nitrogen, and shipped on dry ice.

Metabolism of vitamin D₂ by adrenal mitochondria—Adrenals were obtained from male Wistar rats aged 3 months, killed under anesthesia. The animals were housed at the vivarium of the Department of Biotestings of Bio-organic Chemistry Institute, Minsk, Belarus. The experiments were approved by the Belarus University Animal Care and Use Committee. The reactions were run as described previously [17,18]. Briefly, mitochondria prepared from the adrenals were preincubated (10 min at 37 °C) with 100 μM vitamin D₂ (dissolved in 45% 2-hydroxypropyl-cyclodextrin) in buffer comprising 0.25 M sucrose, 50 mM Hepes pH 7.4, 20 mM KCl, 5 mM MgSO₄, and 0.2 mM EDTA. The reactions were started by adding NADPH (0.5 mM) and isocitrate (5 mM) to the samples, and after 90 min mixtures were extracted with methylene chloride and the organic layers combined and dried. The residues were dissolved in methanol and subjected to LC/MS analysis as detailed below.

NMR

Samples of the purified hydroxy metabolites of vitamin D₂ (the masses of the compounds were confirmed by MS) were dissolved in 40 μL of '100% D' CDCl₃ (Cambridge Isotope Laboratories, Inc., Andover, MA), and NMR spectra were acquired using a Varian Inova-500 M NMR spectrometer equipped with a 4 mm inverse gHX Nanoprobe (Varian NMR, Inc., Palo Alto, CA). The total volume in the NMR rotor was 40 μL, and all spectra were acquired at a temperature of 294 K with a spinning rate of 2500 Hz. Proton 1D NMR, COSY and HSQC spectra were acquired and processed with standard parameters. Possible positions of the hydroxyl groups in the metabolite were analyzed by comparing the acquired spectra with those of parent vitamin D₂.

MS

Products of vitamin D₂ metabolism by purified P450_{scc} were eluted from TLC plates and dissolved in ethanol, and electron impact (EI) mass spectra were recorded with a Micromass VG Autospec Mass Spectrometer (Waters, Milford, MA) operating at 70 eV with scanning from 800 to 50 at 1 s-per decade.

The products of mitochondrial transformation (see above) were dissolved in methanol and analyzed on an HPLC mass spectrometer (LCMS-QP8000α, Shimadzu, Kyoto, Japan) equipped with a Restec Allure C18 column (150 × 4.6 mm; 5 μm particle size, and 60 Å pore size), UV/VIS photodiode array detector (SPD-M10Avp) and quadrupole mass spectrometer. The LC/MS WORKSTATION CLASS 8000 software was used for system control and data acquisition (Shimadzu). Elution was carried out at a flow rate of 0.75 mL·min⁻¹ at 40 °C. The mobile phases consisted of 85% methanol and 0.1% acetic acid from 0 to 25 min, followed by a linear gradient to 100% methanol and 0.1% acetic acid from 25 to 35 min, and 100% methanol and 0.1% acetic acid from 35 to 50 min. The mass spectrometer was operated in atmospheric pressure chemical ionization (APCI) positive ion mode and nitrogen was used as the nebulizing gas. The MS parameters were as follows: nebulizer gas flow rate 2.5 L·min⁻¹; probe high voltage 4.5 kV; probe temperature 250 °C; curved desolvation line heater temperature 230 °C. Analyses were carried out in the scan mode from *m/z* 370 to 430 or in SIM mode.

Cell culture experiments

HaCaT keratinocytes were grown in DMEM with 5% fetal bovine serum and 1% antibiotic solution as described previously [39]. Vitamin D₂ metabolites, produced by purified P450_{scc} and isolated by TLC, were further purified by RP-HPLC through a Restec Allure C18 column (150 × 4.6 mm; 5 μm particle size; 60 Å pore size) following the procedure described for LC/MS (see above). For testing biological activity, vitamin D₂ and its metabolites were dissolved in cyclodextrin, as described previously [19].

DNA synthesis—Cells were seeded at 5000 per well into 96-well plates in growth medium. After 6 h, medium was discarded and serum-free Ham's F10 medium was added. After 12 h, this medium was changed to 5% fetal bovine serum Ham's F10 medium containing compounds to be tested at 10^{-10} and 10^{-12} M. After 12 h, medium was discarded and replaced with 5% fetal bovine serum Ham's medium containing test compounds and [^3H]thymidine ($1\ \mu\text{Ci}\cdot\text{mL}^{-1}$), and incubated for an additional 12 h. After treatment, media were discarded, cells were detached with trypsin and harvested on a glass fiber filter, and radioactivity proportional to methyl- ^3H thymidine incorporated into DNA was counted with a Packard direct beta counter (Packard, Meriden, CA).

Reporter gene assay—The effect of vitamin D₂ metabolites on the transcriptional activity of the involucrin promoter was assessed with a reporter gene assay. Cells were seeded at 20 000 per well in 24-well plates in growth medium. After 6 h, cells were transfected using transfection reagents (sc-29528 and sc-36868) from Santa Cruz Biotechnology Inc., Santa Cruz, CA in serum-free F10 medium with firefly luciferase reporter gene plasmid IVL-Luc containing the involucrin gene promoter region (−668 bp to + 34 bp; added at $1\ \mu\text{g}$ per well) and with phRL-TK (expresses Renilla luciferase and serves as normalization control; Promega, Madison, WI; added at $1\ \mu\text{g}$ per well). IVL-Luc and p-Luc (control without promoter, empty vector) plasmids were constructed as described previously [18]. Twelve hours after transfection, the medium was changed to 5% fetal bovine serum Ham's F10 medium containing vitamin D₂ and its hydroxy derivatives. Compounds were added again after 12 h. After another 12 h (entire incubation with compounds lasted 24 h), cells were lysed with passive lysis buffer and luciferase, and Renilla luciferase signals were recorded after sequential addition of Luciferase Assay Reagent II and Stop-Glo Reagent (Promega, Madison, WI) using a TD-20/20 luminometer (Turner Designs, Sunnyvale, CA). After subtraction of background, the specific signal was divided by the Renilla signal. Resulting values were divided by the mean value for controls (cells transfected with IVL-Luc construct and incubated without compounds).

Statistical analysis

Data are presented as mean $\pm$ SEM ($n = 3\text{--}8$) and analyzed with Student's *t*-test. Each experiment was performed independently two times.

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Abbreviations

APCI	atmospheric pressure chemical ionization
EI	electron impact
P450scc	cytochrome P450 side chain cleavage
HSQC	proton–carbon correlation spectroscopy

Fig 1. Metabolism of vitamin D₂ by purified bovine cytochrome P450 side chain cleavage (P450scc). Incubations were carried out in a reconstituted system comprising purified P450scc (3 μ M), adrenodoxin reductase, adrenodoxin and phospholipid vesicles containing vitamin D₂ at a molar ratio to phospholipid of 0.2. (A) Reaction products were analyzed by TLC and visualized by charring. Lane 1, Experimental incubation with NADPH; lane 2, control incubation without NADPH; lane 3, pregnenolone (P) and vitamin D₂ standards. Metabolite 1 (M1) and metabolite 2 (M2) are marked by arrows. (B) Electron impact MS of metabolite 1. (C) Electron impact MS of metabolite 2.

Fig 2.

NMR spectra of vitamin D₂ metabolite 1 identified as 20-hydroxyvitamin D₂. (A) Proton-proton COSY of vitamin D₂ standard. (B) COSY of vitamin D₂ metabolite 1. (C) Proton-carbon correlation spectroscopy (HSQC) of vitamin D₂ standard. (D) HSQC of vitamin D₂ metabolite 1. The separation of 22/23 proton signals in metabolite 1 and the lack of scalar coupling between 20-CH and 22-CH at 5.54 p.p.m. (circle in (B)) clearly indicates hydroxylation at 20-C. The doublet-to-singlet transition of proton NMR with concurrent downfield shift of the 21-methyl signal (1.01 p.p.m. and 21.2 p.p.m. to 1.3 p.p.m. and 29.5 p.p.m.) confirms hydroxylation at the 20 position. Impurities in the methyl regions are probably from the TLC purification process.

Fig 3.

NMR spectra of vitamin D₂ metabolite 2 identified as 17,20-dihydroxyvitamin D₂. (A) Proton spectra of metabolite 2. (B) Proton spectra of vitamin D₂. (C) COSY of metabolite 2. (D) Proton-carbon correlation spectroscopy (HSQC) of methyl regions of metabolite 2. Numbers in (B) indicate proton positions in the vitamin D₂ standard. In metabolite 2, the 20-hydroxyl is clearly present and there are no other changes in the side chain as indicated by COSY and HSQC. The large downfield shift of 14-CH from 1.99 p.p.m. to 2.68 p.p.m. with disappearance of the 17-CH signal at 1.32 p.p.m. indicates that hydroxylation has occurred at the 17-C position.

Fig 4. Proposed sequence for the cytochrome P450 side chain cleavage (P450_{scc})-catalyzed transformation of vitamin D₂ with chemical structures of the reaction products.

Fig 5. RP-HPLC separation of products of vitamin D₂ metabolism by adrenal mitochondria. (A) Incubation of mitochondria in the absence of NADPH and isocitrate. (B) Experimental incubation with NADPH and isocitrate. (C) Experimental incubation with 200 μ M aminoglutethimide. The HPLC elution profile was monitored by absorbance at 265 nm. 1–6, novel vitamin D₂ metabolites; 7, vitamin D₂.

Fig 6.

Metabolites of vitamin D₂ inhibit DNA synthesis and stimulate differentiation in human HaCaT keratinocytes. (A) HaCaT keratinocytes were synchronized and incubated for 24 h in Ham's F10 medium containing serum and vitamin D₂ or its metabolites, and [³H]-thymidine. (B) HaCaT keratinocytes were transfected with a construct containing the involucrin promoter (IVL-Luc) or with empty (promoter-free) construct, synchronized and incubated for 24 h in Ham's F10 medium containing serum and vitamin D₂ or its metabolites. Data are shown as mean ± SEM (*n* = 3–8).