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In vivo production of novel vitamin D2 hydroxy-derivatives by human placentas, epidermal keratinocytes, Caco-2 colon cells and the adrenal gland

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Abstract

We investigated the metabolism of vitamin D2 to hydroxyvitamin D2 metabolites ((OH)D2) by human placentas *ex-utero*, adrenal glands *ex-vivo* and cultured human epidermal keratinocytes and colonic Caco-2 cells, and identified 20(OH)D2, 17,20(OH)₂D2, 1,20(OH)₂D2, 25(OH)D2 and 1,25(OH)₂D2 as products. Inhibition of product formation by 22R-hydroxycholesterol indicated involvement of CYP11A1 in 20- and 17-hydroxylation of vitamin D2, while use of ketoconazole indicated involvement of CYP27B1 in 1 α -hydroxylation of products. Studies with purified human CYP11A1 confirmed the ability of this enzyme to convert vitamin D2 to 20(OH)D2 and 17,20(OH)₂D2. In placentas and Caco-2 cells, production of 20(OH)D2 was higher than 25(OH)D2 while in human keratinocytes the production of 20(OH)D2 and 25(OH)D2 were comparable. HaCaT keratinocytes showed high accumulation of 1,20(OH)₂D2 relative to 20(OH)D2 indicating substantial CYP27B1 activity. This is the first *in vivo* evidence for a novel pathway of vitamin D2 metabolism initiated by CYP11A1 and modified by CYP27B1, with the product profile showing tissue- and cell-type specificity.

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Keywords

Vitamin D; CYP11A1; 20-hydroxyvitamin D₂; keratinocytes; placenta; adrenals

1. Introduction

The vast majority of circulating vitamin D₃ (D₃) in humans originates from UVB induced epidermal photo-transformation of 7-dehydrocholesterol (7DHC, cholesta-5,7-dien-3 β -ol) that involves the opening of the B-ring giving the pre-vitamin D₃ intermediate, followed by its slow temperature-dependent isomerization to the final vitamin D₃ product (Holick, 2003, Holick and Clark, 1978, Holick et al., 1995). Similarly, vitamin D₂ (D₂) is produced by the action of UVB irradiation on ergosterol, a 5,7-diene phytosterol that is synthesized by fungi and phytoplankton but not in the animal kingdom (Bikle, 2011b, Holick, 2003). D₂ enters the systemic circulation through the alimentary tract and it represents a major form of dietary vitamin D in humans (Bikle, 2011b, Holick, 2003). At the systemic level, both vitamins D₃ and D₂ are hydroxylated sequentially at position C25 in the liver and C1 in the kidney to produce biologically active 1,25(OH)₂D₃ and 1,25(OH)₂D₂ (Holick, 2003, Holick et al., 1975, Zhu and DeLuca, 2012). However, both reactions also occur in peripheral organs including the skin (Bikle, 2011b, Bikle et al., 1986, Holick, 2003, Zhu and DeLuca, 2012). Both 1,25(OH)₂D₃ and 1,25(OH)₂D₂ regulate systemic calcium and phosphate homeostasis, play an important role in the functioning of the musculo-skeletal system, display developmental activity, are involved in the regulation of endocrine, immune and cardiovascular systems, and control formation of the skin barrier and its adnexal structures (Bikle, 2008, Bikle, 2011a, Bikle, 2011b, Holick, 2003, Holick, 2007, Plum and DeLuca, 2010). They also have a wide variety of ameliorating effects on cancer, proliferative, inflammatory and other diseases (Bikle, 2008, Bikle, 2011a, Bikle, 2011b, Holick, 2003, Holick, 2007, Plum and DeLuca, 2010).

Recently, it was discovered that CYP11A1 hydroxylates the side chain of vitamin D₃ (Guryev et al., 2003, Slominski et al., 2005) and D₂ without its cleavage (Nguyen et al., 2009, Slominski et al., 2006). In the case of vitamin D₃, it is hydroxylated *in vitro* by CYP11A1 in a sequential manner at positions C17, C20, C22 and C23 to produce at least 10 metabolites with 20(OH)D₃, 20,23(OH)₂D₃, 20,22(OH)₂D₃, 17,20(OH)₂D₃ and 17,20,23(OH)₃D₃ being the major products (Slominski et al., 2005, Tuckey et al., 2011, Tuckey et al., 2008a). Consistent with experimental results, preferential hydroxylation of vitamin D₃ at position C20 was predicted by molecular modeling using the crystal structure of human CYP11A1 (Strushkevich et al., 2011). The products of the above pathway that lack a 17 α -hydroxyl group can also be hydroxylated *in vitro* in position 1 α by CYP27B1 (Tang et al., 2013, Tang et al., 2010). These novel vitamin D₃ hydroxy-derivatives are biologically active with a potency defined by the cell lineage (Slominski et al., 2010, Slominski et al., 2013c), and are at least as potent as the classical 1,25(OH)₂D₃ (calcitriol) in skin cells with anti-proliferative, pro-differentiation, anti-cancer and anti-inflammatory properties (Janjetovic et al., 2011, Janjetovic et al., 2010, Janjetovic et al., 2009, Kim et al., 2012, Slominski et al., 2013a, Slominski et al., 2012a, Zbytek et al., 2008). Most recently, we have shown *in vivo* production of 20(OH)D₃, 22(OH)D₃, 20,23(OH)₂D₃,

20,22(OH)₂D₃, 1,20(OH)₂D₃, 1,20,23(OH)₃D₃ and 17,20,23(OH)₂D₃ in placentas, adrenal glands and epidermal keratinocytes when vitamin D₃ was added to the incubation media (Slominski et al., 2012b).

The fungal-derived vitamin D₂ is also hydroxylated *in vitro* by bovine CYP11A1 with production of 20(OH)D₂, 17,20(OH)₂D₂ and 17,20,24(OH)₃D₂ (Nguyen et al., 2009, Slominski et al., 2006). The 20(OH)D₂ can be further hydroxylated by recombinant CYP27B1 to 1,20(OH)₂D₂ (Slominski et al., 2011). Moreover, 20(OH)D₂ stimulates keratinocytes differentiation and inhibits proliferation of epidermal melanocytes, and melanoma and leukemia cells, while being non-toxic in rodents at doses as high as 4 µg/kg, defining it as a potential therapeutic agent for hyperproliferative disorders, or as an adjuvant in cancer therapy (Slominski et al., 2011). However, it is unknown whether vitamin D₂ can be metabolized *in vivo* to produce the above hydroxy-derivatives detected during *in vitro* enzymatic reactions. Therefore, we tested whether human placentas, rat and bovine adrenal glands, human epidermal keratinocytes, and colon cancer Caco-2 cells can metabolize vitamin D₂ to the novel hydroxy-derivatives. The involvement of CYP11A1 was demonstrated and the relative production of the classical 25(OH)D₂ versus the novel 20(OH)D₂ was compared.

2. Materials and methods

2.1. Human and animal protocols

All studies involving specimens from humans were obtained through protocols approved by Institutional Review Boards at participating institutions. All studies involving animals followed protocols approved by Institutional University Animal Care and Use Committees at the participating institutions.

2.2. Ex-utero incubations with human placentas

For ex-utero incubations, term human placentas (37–42 weeks) were obtained from MedPlex in Memphis, TN. The incubations of placental fragments followed protocols described in (Slominski et al., 2012b, Slominski et al., 2012c). Briefly, the placental fragments were incubated with vitamin D₂ at concentrations of 10, 100 or 500 µM at 37°C for 20 h as described previously (Slominski et al., 2012b). In control experiments, substrates were omitted from the incubation mixture or placental fragments were boiled 5 min prior to addition of substrates. After placing the tubes on ice, secosteroids were extracted with dichloromethane and dried under nitrogen. The samples were further analyzed by HPLC and mass spectrometry as described below.

2.3. Incubations with cultured keratinocytes and Caco-2 cells

Human epidermal HaCaT keratinocytes, neonatal keratinocytes (passage 3) and colonic Caco-2 cells were cultured as described in (Slominski et al., 2006). Cells were detached from plates as described previously and washed twice in ice cold PBS (Slominski et al., 2012a). The cells were suspended in tris-buffered medium (110 mM NaCl, 5 mM KCl, 2.5 mM CaCl₂, 1 mM MgSO₄, 1 mM KH₂PO₄, 33 mM Tris-HCl, pH7.4) containing 5 mM isocitrate, 0.5 mM NADPH, 0.2% glucose and 1% BSA, at a concentration of 3×10^6

cells/mL or 0.25×10^6 cells/mL (neonatal keratinocytes), and incubated in the presence of 0, 50 or 500 μM vitamin D2 for 16 h at 37°C. The products were extracted twice with 2.5 volumes of dichloromethane and dried under N_2 gas. For analytical procedures the samples were redissolved in methanol and subjected to HPLC or LC-MS analyses as described below (section 2.6).

To inhibit CYP27B1 activity, HaCaT keratinocytes were cultured with and without 20 μM ketoconazole (Sigma, St. Louis, MO) for 2 days in DMEM media containing 5% FBS. The cells were collected and washed with PBS followed by HEPES-buffered medium pH 7.4 (120 mM NaCl, 5 mM KCl, 1 mM EDTA, 1 mM MgSO_4 , 15 mM sodium acetate, 100 mM HEPES, 10 mM glucose and 1% BSA). The cells (2×10^6) were suspended in HEPES-buffered medium with or without 20 μM ketoconazole in the absence of vitamin D2 or with 500 μM vitamin D2. Reactions were started by adding 2 mM isocitrate and 0.2 mM NADPH and samples incubated for 16 h at 37°C. The products were extracted and prepared for LC-MS as above.

2.4. Incubations of mitochondria from placenta and adrenal glands with vitamin D2

Mitochondria were isolated from human placentas obtained from King Edward Memorial Hospital for Women (Subiaco, WA, Australia) or bovine adrenals obtained from the slaughterhouse and processed following protocols described previously (Slominski et al., 2012c). Briefly, mitochondria (1.76 mg/mL, disrupted by sonication) were incubated in buffer consisting of 50 mM HEPES (pH 7.4), 0.25 M sucrose, 20 mM KCl, 5 mM MgSO_4 , 10 μM human adrenodoxin, 0.4 μM human adrenodoxin reductase, 5 mM isocitrate, 0.5 mM NADP, secosteroid (typically 50–200 μM in 0.45% 2-hydroxypropyl- β -cyclodextrin) and 0.5 mg/mL bovine serum albumin (fatty acid free), in a final volume of 12.0 mL. Initially, mitochondria, substrates and the other components except isocitrate and NADP^+ , were preincubated at 37°C for 8 min. The reaction was then started by the addition of the isocitrate and NADP^+ . Control incubations contained all reaction components except the isocitrate and NADP^+ . Incubations were carried out for 4 h at 37°C and reactions terminated by the addition of ice-cold dichloromethane and vortexing. Samples were extracted twice with dichloromethane and dried under N_2 at 30°C. The resulting residue was re-dissolved in chloroform and applied as a band across a 20 cm $\times$ 20 cm $\times$ 0.2 mm TLC silica gel 60 plate. Authentic standards were spotted either side of the test extract for identification purposes using a similar procedure to that described before (Tuckey et al., 2011). TLC plates were developed 3 times in hexane/ethyl acetate (1:1 v/v). Sections of the plate containing standards were removed from the main plate and sprayed and charred as before (Tuckey et al., 2011). The resulting standard spots were then aligned with the remainder of the plate and areas corresponding to authentic standards were removed and secosteroids extracted from the silica with three 15 mL aliquots of dichloromethane/methanol (1:1). The solvent was removed under N_2 at 30°C and the resulting residue dissolved in 15 mL dichloromethane/methanol (1:1) and passed through a 3 μm nylon filter to remove any remaining silica particles. The solvent was removed as before and the residue dissolved in 200 μL of ethanol for HPLC and LC-ESI/MS/MS analysis, see below.

2.5. Metabolism of vitamin D2 by purified CYP11A1

Human CYP11A1, adrenodoxin and adrenodoxin reductase were expressed in *Escherichia coli* and purified as described before (Tuckey et al., 2011, Woods et al., 1998). Purified CYP11A1 (2 μ M) was incubated with 200 μ M vitamin D2 in 2-hydroxypropyl- β -cyclodextrin at a final concentration of 0.45%. The incubation also contained 10 μ M adrenodoxin, 0.3 μ M adrenodoxin reductase and 10 μ M NADPH, and was carried out for 2 h at 37°C, as before (Tuckey et al., 2011). The reaction was terminated and products extracted with dichloromethane as described above and subjected to HPLC analysis, see below. Products were identified from their retention times (RT) compared to authentic standards, originally synthesized enzymatically using bovine CYP11A1 and identified by NMR (Nguyen et al., 2009, Slominski et al., 2006).

2.6. Reverse phase-liquid chromatography (RP-HPLC) and mass spectrometry analyses (MS)

RP-HPLC and MS analyses for extracts obtained after incubations with placentas or cells followed the protocols described in (Slominski et al., 2012b). Briefly, vitamin D hydroxy-metabolites, were separated on a gradient of methanol in water (85%–100%) for 20 min at 0.5 mL/min using a dual pump chromatography system (Waters 2695 Alliance, Milford, MA) equipped with a Waters Atlantis dC18 column (100 $\times$ 4.6 mm, 5 μ m particle size). Fractions were monitored with a photodiode array or UV detector at 265 nm (Waters 996, Milford, MA) and collected manually for further MS analyses.

To quantify and to confirm the identity of mono- and dihydroxy-vitamin D2 metabolites, the collected fractions with RT of the corresponding standards were analyzed under isocratic conditions using 96% methanol in water at a flow rate of 0.1 mL/min for 10 min on a Zorbax Eclipse Plus C18 column (2.1 $\times$ 50 mm, 1.8 μ m) (Agilent Technology, Santa Clara, CA) using an API-3000 LC-MS/MS mass spectrometer (Applied Biosystems, Toronto, Canada) equipped with an ESI source in the positive mode at the condition of declustering potential: 80 V; entrance potential: 10 V; ion spray voltage 4.5 kV. The ion at $m/z = 435.4$ $[M + Na]^+$ or $m/z = 451.4$ $[M + Na]^+$ was used to identify mono- or dihydroxyderivatives, respectively. The relative concentrations of products were calculated from LC-MS peaks area in relation to standards curves generated using the corresponding authentic standards. The values are presented as means $\pm$ SD (n=3).

In addition, vitamin D2 metabolites in tissue and cell extracts were further analyzed on a Waters AcquityTM Ultra Performance Liquid Chromatography (UPLC) system (Milford, MA, USA) equipped with Waters XevoTM G2-S qTOF MS and an ESI source. The separation was carried out on an Agilent Zorbax Eclipse Plus C18 Column (2.1 $\times$ 50 mm, 1.8 μ m) (Agilent Technology, Santa Clara, CA) with a flow rate of 0.3 mL/min maintained at 35°C. 95% methanol (0.1% formic acid) was used as mobile phase in the isocratic condition, or an Atlantis dC18 column (100 $\times$ 4.6 mm, 5 μ m particle size) with a gradient of methanol in water (85%–100%) for 20 min at a flow rate of 0.5 mL/min. In addition, to separate 1,20(OH)₂D2 and 1,25(OH)₂D2, a Waters C18 column (250 $\times$ 4.6 mm, 5 μ m particle size) was used with a gradient of acetonitrile in water (40%–100%) at a flow rate 0.5 mL/min (15 min), followed by a wash with 100% acetonitrile for 30 min at flow rate 0.5

ml/min and for 20 min at a flow rate 1.5 ml/min. The separated 1,20(OH)₂D₂ (collected from 20.3 to 20.9 min of RT) and 1,25(OH)₂D₂ (collected from 21.6 to 22.2 min of RT) were subjected to LC-MS using the same equipment and conditions mentioned above. For MS, the scan range was set at 50 to 1200 *m/z* in positive mode, the capillary and cone voltage were 3.0 kV and 30 V, respectively. The desolvation gas was set to 1000 L/h at 500°C; the cone gas was set at 100 L/h with a source temperature of 150°C. Leucine-enkephalin was used as the lockmass solution at a concentration of 100 ng/mL with a flow rate of 5 µL/min. All data were collected in centroid mode and controlled by Waters MassLynx v4.1 software. The mono- and dihydroxyvitamin D₂ masses were extracted using ions at *m/z* = 435.4 [M + Na]⁺ for 20(OH)D₂ and 25(OH)D₂ or *m/z* = 451.4 [M + Na]⁺ for 1,20(OH)₂D₂, 1,25(OH)₂D₂ and 17,20(OH)₂D₂.

Vitamin D₂ hydroxy-metabolite samples obtained from incubations with mitochondria or reconstituted purified CYP11A1 were analyzed by HPLC using a quaternary pump system (Agilent 1100; Palo Alto CA, USA) equipped with an Agilent Zorbax Stablebond C18 column (250 × 4.6 mm, 3.5-µm particle size) and a Phenomenex Security Guard C18 cartridge pre-column connected to an online Waters YMC 3 µm filter. Separations were performed at 25°C with an injection volume of 25 µL. The mobile phase consisted of a linear gradient from 35% acetonitrile plus 0.1% formic acid in water to 100% acetonitrile plus 0.1% formic acid over 60 min at a flow rate of 1 mL/min. The 100% acetonitrile condition was held for 5 min. Detection was achieved using UV and a photodiode array (Agilent 1100) while monitoring at a wavelength of 265 nm. The relative concentrations of products were calculated from the areas of the HPLC peaks in relation to 1-point standard curves generated using the corresponding authentic hydroxyvitamin D₂ standards.

To further verify the RP-HPLC speciation, the same samples were analyzed using an API-4000 QTrap LC-ESI-MS/MS mass spectrometer (Applied Biosystems, Toronto, ON, Canada) utilizing the same liquid chromatography methodology as the RP-HPLC analysis, coupled with an electrospray ionization (ESI) source in the positive ion mode at the condition of ion spray voltage, 5.5 kV; declustering potential, 60–90 V; entrance potential, 10 V; collision energy, 10–19 mV; collision energy exit potential, 10–29 mV; dwell time, 30 ms; ESI temperature, 700°C; source curtain gas, 20; nebulizer source gas, 70; auxiliary source gas, 70. All analyte hydroxyvitamin D₂ derivatives used a [M-H₂O+H]⁺ Q1 ion *m/z* of 411.2 amu with the exception of 20(OH)D₂ analytes which used a [M+H]⁺ Q1 ion *m/z* of 413.2 amu. The transitions used to monitor products for all hydroxyvitamin D₂ derivatives in the multiple reaction monitoring (MRM) mode with the exception of 20(OH)D₂, were Q1–Q3 *m/z* of 411.2 → 393.3 amu. The Q1–Q3 transition used for 20(OH)D₂ in the MRM mode was 413.2 → 71.1 amu. These Q1–Q3 transitions represent the major/specific transitions seen in corresponding authentic standards during LC-ESI-MS/MS analysis.

To measure vitamin D₂ metabolism in adrenal glands ex-vivo, female Wistar rats (180–200 g) were used and incubations carried out as described in (Slominski et al., 2012b, Slominski et al., 2012c). Briefly, adrenal glands fragments were incubated with vitamin D₂ at concentrations of 200 µM at 37°C for 4 h and after extractions (Slominski et al., 2012b), the residues were analysed using an LC–MS QP8000a (Shimadzu, Japan) equipped with diode array and single quadrupole mass-spectrometric detectors using an EC 50/4.6 Nucleodur

C18 ISIS column with 1.8 μm particle size (Macherey-Nagel GmbH & Co. KG, Germany). Elution was carried out at a flow rate of 0.5 mL per min and temperature of 40°C with a linear gradient from 65 to 100% of methanol (0–7 min), followed by 100% methanol from 7 to 10 min. The MS was operated with atmospheric pressure chemical ionization, in the positive ion mode with nitrogen as the nebulizing gas. The MS parameters were as follows: nebulizer gas flow rate 2.5 L/min; probe high voltage 2.5 kV; probe temperature 400°C; curved desolvation line (CDL) heater temperature 230°C; CDL voltage –60V and voltage of all deflectors 30V. Analyses were carried out in the SIM or scan mode from m/z 250–450.

2.7. Molecular modeling of the binding of vitamin D2 and its hydroxyderivatives to CYP11A1

Molecular modeling was carried out using the crystal structure of human CYP11A1 in complex with cholesterol (Protein Data Bank code 3N9Y) (Strushkevich et al., 2011) and the Schrodinger Molecular Modeling Suite 2011 (Schrodinger Inc., Portland, OR). Procedures are similar to those described previously (Chen et al., 2011, Slominski et al., 2012c). The structure of 3N9Y complex was prepared using the Protein Preparation wizard and the ligand binding site in the prepared complex was defined using the Glide Grid Generation module. The native ligand (cholesterol) or the ligands of interest (vitamin D2 or its hydroxyderivatives) were built using the Ligprep module before they were docked into the active site of CYP11A1 using the Glide ligand docking module in Schrodinger Suite. The Glide docking score obtained from this modeling approach is an estimation of the binding energy (kcal/mol) when a ligand binds to CYP11A1. Lower (more negative) numbers suggest more favorable binding interactions between the ligand and CYP11A1. Data analyses were performed using the Maestro interface of the software.

3. Results

To define the *in vivo* capability of tissues expressing CYP11A1 to metabolize vitamin D2, we incubated human placenta fragments *ex-utero* with this secosteroid. HPLC analysis revealed the presence of 4 peaks that were absent in negative controls incubated either without substrate or after boiling of placental fragments (Fig. 1). These peaks had identical retention times (RT) and mass spectra to the corresponding standard 20(OH)D2, 17,20(OH)₂D2, 1,20(OH)₂D2 and 25(OH)D2 (Fig. 1A, B). The identity of the secosteroid corresponding to each peak was further confirmed by LC-MS analysis of each peak separately in the SIM mode, using $m/z = 435.4$ $[\text{M} + \text{Na}]^+$ for (OH)D2 and $m/z = 451.4$ $[\text{M} + \text{Na}]^+$ for (OH)₂D2. This confirmed the identification of species corresponding to the authentic standards of 20(OH)D2, 25(OH)D2, 1,20(OH)₂D2 and 17,20(OH)₂D2 (Fig. 1C). Of note, when peak 3 from Fig. 1 was further analyzed using LC-MS in the SIM mode it revealed an additional mono-hydroxy D2 species besides the expected 25(OH)D2, in which the position of the hydroxyl group is unknown. We repeated the analyses of placental extracts using UPC LC-QTOF MS with masses for dihydroxyvitamin D2 determined in the SIM mode using $m/z = 451.4$ $[\text{M} + \text{Na}]^+$ and found that the relative concentrations of 1,25(OH)₂D2 and 1,20(OH)₂D2 in tests samples were similar (inset in Fig. 1A).

To further investigate the ability of placentas and adrenals to metabolize vitamin D2, incubations were carried out with mitochondria isolated from human placentas (Figs 2 and

3) and the bovine adrenal cortex (Supplemental Figs 1 and 2). The CYP11A1 reaction products of vitamin D₂ metabolism, 20(OH)D₂ and 17,20(OH)₂D₂, were clearly identified following their preliminary separation by thin-layer chromatography, from their HPLC retention times, UV spectra (not shown), spiking with authentic standards and their mass transitions by LC-MS/MS in the MRM mode (Fig. 2 and Supplemental Fig. 1). Progesterone, produced from endogenous cholesterol in the mitochondria from the actions of CYP11A1 and 3 β -hydroxysteroid dehydrogenase, was also detected. The production of 1,20(OH)₂D₂ from 20(OH)D₂ was similarly demonstrated for placental (Fig. 3) and bovine (Supplemental Fig. 2) mitochondria while conversion of 25(OH)D₂ to 1,25(OH)₂D₂ served as a positive control. LC-MS analysis of samples obtained from fragments of rat adrenal glands incubated *ex-vivo* with vitamin D₂ revealed the presence of a peak with a RT and mass spectrum identical to those of authentic 20(OH)D₂, which was absent in negative controls incubated either without substrate or after boiling of the tissue (Supplemental Fig. 3). This not only substantiates the results of experiments performed with isolated mitochondria (Figs 4, 5), but also demonstrates the ability of adrenal glands maintained *ex-vivo* to transform vitamin D₂ to 20(OH)D₂ (Supplemental Fig. 3).

The ability of human CYP11A1 to metabolize vitamin D₂ was confirmed by incubating purified recombinant human CYP11A1 with vitamin D₂. Like bovine CYP11A1 (Nguyen et al., 2009, Slominski et al., 2006), human CYP11A1 transformed vitamin D₂ to 20(OH)D₂ and 17,20(OH)₂D₂, with the former being the most abundant product (Supplemental Fig. 4). To further support the involvement of CYP11A1 in the *in vivo* metabolism of vitamin D₂, we tested the ability of the tight-binding intermediate of cholesterol metabolism by CYP11A1, 22R-hydroxycholesterol (Hume et al., 1984, Tuckey, 2005, Tuckey and Cameron, 1993), to inhibit vitamin D₂ metabolism. This intermediate efficiently and specifically competes with other CYP11A1 substrates to block their metabolism (Slominski et al., 2005, Slominski et al., 2012b). Production of 20(OH)D₂ and 17,20(OH)₂D₂ by placental mitochondria was almost completely inhibited by 22R-hydroxycholesterol (Fig. 4). The conversion of 20(OH)D₂ to 17,20(OH)₂D₂ by mitochondria isolated from the human placenta (Fig. 5A–E) and bovine adrenal glands (Supplemental Fig. 5) was also markedly inhibited by 22R-hydroxycholesterol. Interestingly, mitochondria isolated from human placenta (Fig. 5F–J) and bovine adrenal glands (Supplemental Fig. 6) converted 1 α (OH)D₂ into 1,20(OH)₂D₂ and this reaction was also inhibited by 22R-hydroxycholesterol, indicating CYP11A1 involvement.

Further support for the involvement of CYP11A1 in the hydroxylation of the side chain of vitamin D₂ was provided by molecular modeling using the crystal structure of human CYP11A1 reported by Strushkevich et al. (Strushkevich et al., 2011). Modeling of vitamin D₂ and its hydroxyderivatives into the active site (Fig. 6) revealed excellent docking scores of –10.99, –10.66 and –10.83 for D₂, 20(OH)D₂ and 17,20(OH)₂D₂, respectively, which were similar to those of native substrate cholesterol (–11.54). Analysis of the distances between the heme iron and the carbons in the ligand indicated very good correlations to the hydroxylation preferences among the different ligands. For the native ligand cholesterol, the distance from the heme iron to C17, C20, and C22 is 6.13 Å, 4.14 Å, and 3.63 Å, respectively (Figure 6A). This is in agreement with the first hydroxylation preferentially

occurring at C22 of cholesterol (Tuckey, 2005). For vitamin D2, these distances become 5.23 Å, 4.57 Å, and 4.57 Å, respectively, suggesting either hydroxylation at C20 and C22 would have comparable preference (Figure 6B). However, at least two factors in the hydrogen abstraction step in this hydroxylation process would strongly favor C20 over C22 (Olsen et al., 2006). First, the energy required to dissociate the C20 (an sp^3 -hybridized carbon) and its attached hydrogen will be much lower than the energy required to dissociate C22 (a sp^2 -hybridized carbon) and its attached hydrogen. Second, when the hydrogen is abstracted from the carbon, the resulted radical centered at C20 can conjugate with the double bond electrons between C22 and C23 in the vitamin D2 side chain, making the transition state more stable compared with a radical centered at C22. Therefore, even though the similar distance between the heme iron and the carbon would suggest similar preference for hydroxylation, the structure of the D2-side chain would strongly prefer hydroxylation at C20 over C22. In addition, when we modeled the binding of 20(OH)D2, the product of 20-hydroxylation of vitamin D2, (Figure 6C), the distance between the heme iron and C20 was reduced by 0.3 Å to 4.27 Å, while the distance to C22 is increased by 0.5 Å to 5.06 Å. This indicates a possible slight slide during the process of 20-hydroxylation by CYP11A1 that was also observed by Strushkevich et al. (Strushkevich et al., 2011), and is consistent with experimental data obtained with purified enzyme (Supplemental Fig. 4) (Nguyen et al., 2009, Slominski et al., 2006). This potential slide also put C17 in a much closer position (4.67 Å) to the heme iron compared with C22 and is consistent with the preferred formation 17,20(OH)₂D2 (Figure 6D) as the major dihydroxy-product of vitamin D2 metabolism by the purified enzyme (Supplemental Fig. 4 and (Slominski et al., 2006)).

The production of 20(OH)D2, 17,20(OH)₂D2, 1,20(OH)₂D2 and 25(OH)D2 by placental tissue was dependent on the concentration of vitamin D2 in the incubation mixture, with production of 20(OH)D2 and 1,20(OH)₂D2 being observed at the lowest concentration tested (10 µM) (Fig. 7). Importantly, production of 20(OH)D2 was significantly higher ($p < 0.0001$) than that of 25(OH)D2 at each concentration of D2 tested. Interestingly, production of 1,20(OH)₂D2 was higher than that of 17,20(OH)₂D2 at the lower concentrations of vitamin D2 substrate used (10 and 100 µM), indicating efficient hydroxylation at C1 under *in vivo* conditions (Fig. 7). We previously showed that placental mitochondria metabolize vitamin D3 to 20(OH)D3 (Slominski et al., 2012b). We therefore compared the relative abilities of placental mitochondria to metabolize vitamins D2 and D3. Using 200 µM vitamin D2 or D3 as substrate, vitamin D2 was metabolized at a rate of 192 pmol/mg protein/h, slightly lower than the rate of 245 pmol/mg protein/h for vitamin D3 metabolism.

Both HaCaT keratinocytes and colon Caco-2 metabolized vitamin D2. We detected production of 20(OH)D2, 17,20(OH)₂D2, 1,20(OH)₂D2, 25(OH)D2 and 1,25(OH)₂D2 by these cells Using LC-MS analyses in the SIM mode (Fig. 8). This production was dose and cell-type dependent (Table 1). The production of 20(OH)D2 was higher than that of 25(OH)D2 in Caco-2 cells but was similar in HaCaT cells (Table 1). We also noted cell type differences with significantly higher production of 1,20(OH)₂D2 and lower accumulation of 20(OH)D2 in HaCaT keratinocytes than in Caco-2 colon cells ($p < 0.0001$, Table 2). Production of 17,20(OH)₂D2 was also higher ($p < 0.0001$) in Caco-2 cells than in

keratinocytes after incubation with 500 μ M vitamin D₂, while production of 25(OH)D₂ was higher ($p < 0.0001$) in HaCaT keratinocytes than the colon cells with 50 μ M vitamin D₂, but not different with 500 μ M D₂ (Table 1).

To further confirm the involvement of CYP27B1 in the 1 α -hydroxylation of 20(OH)D₂, we added ketoconazole, a CYP27B1 inhibitor (Hong et al., 2008, Lehmann et al., 2003), to HaCat keratinocytes. The ketoconazole markedly inhibited transformation of vitamin D₂ to 1,20(OH)₂D₂. The expected inhibition of 1,25(OH)₂D₂ production served as a positive control (Supplemental Fig. 7).

Finally, using LC-QTOF MS we also detected the transformation of vitamin D₂ to 20(OH)D₂, 17,20(OH)₂D₂, 1,20(OH)₂D₂, 25(OH)D₂ and 1,25(OH)₂D₂ in neonatal human primary keratinocytes at vitamin D₂ concentrations of 500 μ M (Fig. 9) or 50 μ M (not shown). Interestingly, the relative production of 25(OH)D₂ as indicated by the area under the peak was higher than that of 20(OH)D₂.

4. Discussion

In this paper we demonstrate for the first time the *in vivo* transformation of D₂ to 20(OH)D₂, 17,20(OH)₂D₂ and 1,20(OH)₂D₂ using placentas and adrenals which express high levels of CYP11A1 (Miller and Auchus, 2011, Tuckey, 2005), and epithelial cells (epidermal keratinocytes and colonic Caco-2 cells) which express relatively low levels of this enzyme (Sidler et al., 2011, Slominski et al., 2013b, Slominski et al., 2004, Taves et al., 2011). Notably, the amount of 20(OH)D₂ produced was higher than that of the initial product of the classical activation pathway, 25(OH)D₂, in placenta and Caco-2 cells, but similar or lower than for 25(OH)D₃ in human epidermal keratinocytes. The present data clearly show that after reaching these cells or tissues, D₂ can be transformed in a sequential manner to 20(OH)D₂ and 17,20(OH)₂D₂ through the action of CYP11A1. Similarly the prodrug, 1 α (OH)D₂, can be transformed to 1,20(OH)₂D₂.

The identification of the major metabolites of vitamin D₂ metabolism as being products of CYP11A1 action is based on several lines of evidence. First, the observed 20(OH)D₂ and 17,20(OH)₂D₂ are the major products of both purified bovine (Slominski et al., 2006) and human CYP11A1 (this study) on vitamin D₂, and CYP11A1 is the only known route for the production of these metabolites. Second, the conversion of D₂ to 20(OH)D₂, 20(OH)D₂ to 17,20(OH)₂D₂ and 1 α (OH)D₂ to 1,20(OH)₂D₂ by placental and adrenal mitochondria was almost completely inhibited by 22R-hydroxycholesterol. This reaction intermediate in the conversion of cholesterol to pregnenolone, acts as a specific competitive substrate for CYP11A1 which is the only enzyme reported to metabolize this sterol (Miller and Auchus, 2011, Tuckey, 2005, Tuckey and Cameron, 1993). Third, metabolism of the closely related vitamin D₃ by placental and adrenal tissue has been shown to be mediated by CYP11A1 (Slominski et al., 2012b). Finally, molecular modeling using the crystal structure of human CYP11A1 bound to cholesterol (Strushkevich et al., 2011), predicts that vitamin D₂, 20(OH)D₂ and 17,20(OH)₂D₂ are good substrates for this enzyme, having similar docking scores to cholesterol. The distances from C22 and C20 to the heme iron for vitamin D₂ and 20(OH)D₂, and the presence of the C22–C23 double bond, are consistent with initial

hydroxylation occurring at C20, as experimentally demonstrated for human CYP11A1 (Supplemental Fig. 4) and reported previously for bovine CYP11A1 (Nguyen et al., 2009, Slominski et al., 2006), as well as for vitamin D3 (Guryev et al., 2003, Slominski et al., 2005, Strushkevich et al., 2011). Thus, in the light of the above analyses we conclude that CYP11A1 mediates the *in vivo* hydroxylations of vitamin D2 and the prodrug 1 α -hydroxyvitamin D2, as depicted in Fig. 10.

Our studies with placental mitochondria show that the rate of metabolism of vitamin D2 is slightly less than that from an equal concentration of vitamin D3. This is consistent with studies with purified bovine CYP11A1 incorporated into phospholipid membranes which show that vitamin D3 is metabolized with a catalytic efficiency approximately twice that of vitamin D2 (Nguyen et al., 2009, Tuckey et al., 2008b). The relative metabolism of vitamins D2 and D3 by CYP11A1 *in vivo* should also depend on their relative concentrations, reflecting both dietary intake including supplements, and exposure to UVB with vitamin D3 usually predominating (Bikle, 2011b, Holick, 2007).

Placental fragments incubated *ex-utero* not only metabolized vitamin D2 via CYP11A1, but also converted D2 to 25(OH)D2, consistent with the reported expression of genes encoding 25-hydroxylase (CYP27A1 and CYP2R1) in the human placenta (Slominski et al., 2012b). Experiments with placental fragments, mitochondria isolated from placentas and adrenal glands, and cultured skin cells clearly demonstrated their ability to 1 α -hydroxylate 20(OH)D2 to 1,20(OH)₂D2 and 25(OH)D2 to 1,25(OH)₂D2. This 1 α -hydroxylation is attributed to the action of CYP27B1 as first, it is the only known enzyme with appreciable 1 α -hydroxylase activity on 25(OH)D3 (Bikle, 2011b, Holick, 2003) and other hydroxyvitamin D derivatives in humans (Tang et al., 2013). Second, it is expressed in the placenta and adrenals (Slominski et al., 2012b, Zehnder et al., 2002), and skin cells (Bikle et al., 1986, Lehmann, 1997) where it has been shown to 1 α -hydroxylate products of vitamin D3 metabolism (Bikle, 2011b, Holick, 2003, Lehmann et al., 2001) including 20(OH)D3 (Slominski et al., 2012b). Third, 1,20(OH)₂D2 is the known sole product of the action of purified recombinant human CYP27B1 on 20(OH)D2 (Slominski et al., 2011) and fourth, ketokonazole, a known inhibitor of CYP27B1 (Hong et al., 2008, Lehmann et al., 2001) inhibited the production of both 1,25(OH)₂D3 and 1,20(OH)₂D2 by cultured keratinocytes.

HaCaT keratinocytes and Caco-2 cells represent recognized models of normal human epidermal keratinocytes (Lehmann, 1997) and colonic and intestinal cells (Fleet et al., 2003), respectively. Both epidermal keratinocytes (Slominski et al., 2004) and Caco-2 cells (Sidler et al., 2011, Taves et al., 2011) express CYP11A1, however, at relatively low levels (Slominski et al., 2013b). Both cell types metabolized vitamin D2 through the classical (25-hydroxylase-dependent) and novel (CYP11A1-dependent) pathways, however, in a cell type dependent manner. Thus, there was significantly higher production of 20(OH)D2 than 25(OH)D2 in Caco-2 cells, which was similar to the pattern shown in the placenta and adrenals (see above), indicating a role of CYP11A1 in vitamin D2 metabolism in colonic cells. Interestingly, production of 20(OH)D2 and 17,20(OH)₂D2 was higher in Caco-2 cells than in HaCaT keratinocytes. The difference between colonic and epidermal epithelial cells is emphasized by similar (HaCaT) or higher (neonatal keratinocytes) production of 25(OH)D2 in comparison to 20(OH)D2 in epidermal keratinocytes, suggesting a similar

contribution of CYP11A1 and 25-hydroxylases to the vitamin D2 metabolism in epidermal cells. In addition, higher production of 1,20(OH)₂D2 in HaCaT cells than in Caco-2 cells suggests that there is a higher activity of CYP27B1 in epidermal keratinocytes.

After the initial indication that 20(OH)D2 and 17,20(OH)₂D2 were biologically active on HaCaT keratinocytes (Slominski et al., 2006), detailed studies showed that 20(OH)D2 can inhibit the proliferation of normal and malignant melanocytes, keratinocytes and leukemia cells (Slominski et al., 2011). 20(OH)D2 also induces the differentiation program in keratinocytes and leukemia cells, but was without measurable effect on the melanocyte differentiation program (Slominski et al., 2011). Most recently, we also demonstrated that 20(OH)D2 inhibits the proliferation of human dermal fibroblasts in a similar manner to 20(OH)D3 (Slominski et al., 2013a). The potencies of 20(OH)D2 and 1,20(OH)₂D2 were similar and their biological activities were mediated, at least in part, through activation of the vitamin D receptor (Slominski et al., 2011). However, 20(OH)D2 was non-calcemic in rats at doses up to 4 µg/kg, which is a highly toxic dose for the 1α-hydroxy-derivatives of vitamin D. Thus, 20(OH)D2 is an excellent candidate for further pre-clinical testing for its suitability as a potential drug for the treatment of hyperproliferative skin disorders, fibrosing diseases and cancer. The current findings that tissues expressing CYP11A1 can metabolize vitamin D2 to 20(OH)D2 with further metabolism to 17,20(OH)₂D2 and 1,20(OH)₂D2, open a new exciting opportunity to define a role for this new pathway in physiology and pathology.

In conclusion, we provide evidence for novel pathways of vitamin D2 metabolism *in vivo* that are initiated by CYP11A1 and modified by CYP27B1, and show product profiles that are tissues- and cell-type specific. The significance of these pathways cannot be overestimated, because they lead to production of biologically active vitamin D2 hydroxy-derivatives.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

20(OH)D2	20-Hydroxyvitamin D2
17, 20(OH)₂D2	17,20-dihydroxyvitamin D2
1, 20(OH)₂D2	1α,20-dihydroxyvitamin D2
25(OH)D2	25-hydroxyvitamin D2
1, 25(OH)₂D2	1α,25-dihydroxyvitamin D2

7DHC	7-dehydrocholesterol
7DHP	7-dehydropregnenolone
CDL	curved desolvation line
ESI	electrospray ionization
MRM	multiple reaction monitoring
MS	mass spectrometry
RP-HPLC	reverse phase-liquid chromatography
RT	retention time
SIM	selective ion monitoring
UPLC	ultra performance liquid chromatography

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Highlights

- A novel pathways of vitamin D2 metabolism is discovered in the placenta and adrenals
- This pathway is initiated by CYP11A1 and modified by CYP27B1
- This pathway also operates in epidermal keratinocytes and epithelial colon cells
- The product profile of the pathway shows tissue- and cell-type specificity
- Molecular modeling supports the role of CYP11A1 in vitamin D2 metabolism

Figure 1.

Detection of novel vitamin D₂ hydroxy-derivatives in the human placenta incubated ex utero with vitamin D₂. (A) HPLC chromatogram with peaks detected at 265 nm. Left panel: test incubation; right upper panel: negative control comprising boiled placenta incubated with vitamin D₂ substrate; right lower panel: control where placenta was incubated without the substrate. Numbers show retention times (RT) corresponding to the authentic standards: 1, 17,20(OH)₂D₂; 2, 1,20(OH)₂D₂; 3, 25(OH)D₂; 4, 20(OH)D₂. (B) Mass spectra of metabolites 1–4 collected after HPLC separation obtained using an API-3000 LC-MS/MS mass spectrometer. (C) LC-QTOF MS analysis of collected peaks 1–4 using EIC (extracted ion chromatogram) at m/z = 435.4 [M + Na]⁺ for 20(OH)D₂ and 25(OH)D₂ and m/z = 451.4 [M + Na]⁺ for 1,20(OH)₂D₂ and 17,20(OH)₂D₂. The identified

compounds with RT corresponding to the authentic standards are marked by arrowheads. Inset in A shows Waters UPLC-qTOF MS analysis of cell extracts with masses for dihydroxyvitamin D2 determined using $m/z = 451.4$ $[M + Na]^+$ for EIC.

Figure 2.

LC-MS analysis of $17\alpha,20(\text{OH})_2\text{D}_2$ (A–D) and $20(\text{OH})\text{D}_2$ (E–H) produced by placental mitochondria. Mitochondria were incubated with 200 μM vitamin D_2 for 4 h and the major products separated by TLC prior to RP-HPLC analysis, as described in the Materials and Methods. A) Chromatogram of the $17\alpha,20(\text{OH})_2\text{D}_2$ TLC zone for the control reaction with NADP^+ and isocitrate omitted. B) Test reaction showing the $17\alpha,20(\text{OH})_2\text{D}_2$ TLC-zone products. C) Test reaction sample spiked with authentic $17\alpha,20(\text{OH})_2\text{D}_2$. D) Analysis of the test reaction sample *via* LC-MS/MS in the MRM mode for the parent to product transition m/z 411.2 $\rightarrow$ 393.3 for $17\alpha,20(\text{OH})_2\text{D}_2$. E) HPLC chromatogram of the $20(\text{OH})\text{D}_2$ TLC zone for the control reaction with NADP^+ and isocitrate omitted. F) Test reaction showing the $20(\text{OH})\text{D}_2$ TLC-zone products. G) Test reaction sample spiked with authentic $20(\text{OH})\text{D}_2$. H) Analysis of the test reaction sample by LC-MS/MS in the MRM mode for the parent to product transition m/z 413.2 $\rightarrow$ 71.1 for $20(\text{OH})\text{D}_2$.

Figure 3.

Placental mitochondria metabolise 20(OH)₂D₂ to 1 α ,20(OH)₂D₂, with the expected transformation of 25(OH)₂D₂ to 1 α ,25(OH)₂D₂ serving as a positive control. Placental mitochondria were incubated with 50 μ M 20(OH)₂D₂ (A–D) or 50 μ M 25(OH)₂D₂ (E–H) for 4 h at 37° C, products partially purified by TLC and analyzed by RP-HPLC, as described in Materials and Methods. A) Chromatogram for the control reaction with NADP and isocitrate omitted. B) Test reaction showing the chromatogram of the 1 α ,20(OH)₂D₂ TLC-zone products. C) Test reaction sample spiked with authentic 1 α ,20(OH)₂D₂. D) Analysis of the test reaction sample *via* LC-MS/MS in the MRM mode for the parent to product transition m/z 411.2 $\rightarrow$ 393.3 for 1 α ,20(OH)₂D₂. E) Chromatogram for the control reaction with NADP⁺ and isocitrate omitted. F) Test reaction showing the chromatogram of the 1 α ,25(OH)₂D₂ TLC-zone products. G) Test reaction sample spiked with authentic 1 α ,25(OH)₂D₂. H) Analysis of the test reaction sample by LC-MS/MS in the MRM mode for the parent to product transition m/z 411.2 $\rightarrow$ 393.3 for 1 α ,25(OH)₂D₂.

Figure 4.

$22R$ -hydroxycholesterol inhibits production of $17\alpha,20(\text{OH})_2\text{D}_2$ and $20(\text{OH})\text{D}_2$ by placental mitochondria. Placental mitochondria were incubated with $200\ \mu\text{M}$ vitamin D_2 for 4 h at 37°C , products partially purified by TLC and analyzed by RP-HPLC. A) Chromatogram for the test reaction showing the $17\alpha,20(\text{OH})_2\text{D}_2$ TLC-zone products. B) Test reaction carried out in the presence of $100\ \mu\text{M}$ $22R$ -hydroxycholesterol showing the $17\alpha,20(\text{OH})_2\text{D}_2$ TLC-zone products. C) Test reaction showing the chromatogram of the $20(\text{OH})\text{D}_2$ TLC-zone products. D) Test reaction carried out in the presence of $100\ \mu\text{M}$ $22R$ -hydroxycholesterol showing the $20(\text{OH})\text{D}_2$ TLC-zone products.

Figure 5.

Placental mitochondria transform 20(OH)D2 to 17α,20(OH)₂D2 and 1α(OH)D2 to 1α,20(OH)₂D2. Placental mitochondria were incubated with 50 μM 20(OH)D2 (A–E) or 120 μM 1α(OH)D2 (F–J) for 4 h at 37°C, products partially purified by TLC and analyzed by RP-HPLC as described in the Materials and Methods. *A, F*) Chromatogram for control reactions with NADP⁺ and isocitrate omitted. *B, G*) Chromatogram for the test reaction with 20(OH)D2 (*B*) or 1α(OH)D2 (*G*). *C, H*) Test reactions carried out in the presence of 100 μM 22*R*-hydroxycholesterol. *D, I*) Sample of the test reaction spiked with authentic 17α, 20(OH)₂D2 (*D*) or 1α,20(OH)₂D2 (*I*). *E, J*) Analysis of the test reaction samples by LC-MS/MS in the MRM mode for the parent to product transition m/z 411.2 → 393.3 for dihydroxyvitamin D2.

Figure 6.

Docking of cholesterol (native substrate) and vitamin D2 derivatives into the active site of human CYP11A1 (PBD entry: 3N9Y). The docking score for each ligand is indicated in brackets. A, general view of the cholesterol binding pocket with a molecular surface limited to 3Å around cholesterol. B to D, close views of vitamin D2 and its hydroxyderivatives (brown thick tube) binding positions relative to the heme group (blue thin tube). Distances between ligand carbons and iron in the heme group were measured and labeled as colored dash lines (red: distance to C22; green: distance to C20; violet: distance to C17).

Figure 7.

Production of vitamin D₂ hydroxy-derivatives by human placental fragments after incubation for 16 h with different concentrations of vitamin D₂. The analysis was performed using an API-3000 LC-MS/MS mass spectrometer equipped with ESI source in the SIM mode with $m/z = 435.4$ $[M+Na]^+$ for mono-hydroxyvitamin D₂ and 451.4 $[M+Na]^+$ for di-hydroxyvitamin D₂. The relative concentrations of products were calculated from LC-MS peaks areas in relation to standards curves generated using the corresponding authentic standards. The vitamin D₂ concentrations used were 10, 100 and 500 μM and data are presented as means $\pm$ SEM (n=3).

Figure 8.

LC-MS analysis of vitamin D₂ hydroxy-derivatives produced by HaCaT epidermal keratinocytes (A–E) and Caco-2 colon cells (F–J) following incubation with 50 μ M vitamin D₂. (A–D) After RP-HPLC separation (see Materials and Methods) the peaks with RT corresponding to the standards were collected and further analyzed by an API-3000 LC-MS mass spectrometer equipped with an ESI source using the SIM mode to monitor $m/z = 435.4$ $[M + Na]^+$ (A, B, F, G) and $m/z = 451.4$ $[M + Na]^+$ (C, D, H, I) for respective detection of monohydroxy- or dihydroxyvitamin D₂ derivatives. (E, J) UPLC Waters XevoTM G2 QTOF MS analysis of cell extracts with masses for dihydroxyvitamin D₂ determined using $m/z = 451.4$ $[M + Na]^+$ for EIC. Arrows to 20(OH)D₂, 25(OH)D₂, 1,20(OH)₂D₂, 1,25(OH)₂D₂ and 17,20(OH)₂D₂ show masses and RT corresponding to the authentic standards.

Figure 9.

UPLC-qTOF MS identification of 20(OH)D₂, 25(OH)D₂ (A) and 1,20(OH)₂D₂, 1,25(OH)₂D₂ and 17,20(OH)₂D₂ (B) produced by neonatal epidermal keratinocytes incubated for 16 h with 500 μM vitamin D₂. The chromatograms show $m/z = 435.4$ $[M + Na]^+$ for mono-hydroxyvitamin D₂ and 451.4 $[M + Na]^+$ for di-hydroxyvitamin D₂ derivatives. Arrows show the corresponding RT of 20(OH)D₂, 25(OH)D₂, 1,20(OH)₂D₂, 1,25(OH)₂D₂ and 17,20(OH)₂D₂ authentic standards.

Figure 10.
Pathway for the *in vivo* metabolism of vitamin D₂ by CYP11A1

Table 1

Production of vitamin D2 hydroxy-derivatives by HaCaT keratinocytes and Caco-2 cells after a 16 h incubation with different concentrations of vitamin D2. Data are expressed as ng/10⁶ cells.

Vitamin D2		0 μM		50 μM		500 μM	
Cell type		HaCaT	Caco-2	HaCaT	Caco-2	HaCaT	Caco-2
20(OH)D2		0	0	9.5 ± 0.9	22.8 ± 0.2 *****	10.6 ± 0.9	24.5 ± 0.8 *****
25(OH)D2		0	0	9.3 ± 1.7 *****	4.2 ± 0.3 #####	7.9 ± 1.6	8.6 ± 0.17 #####
1,20(OH) ₂ D2		0	0	126.9 ± 4.0 #####	11.6 ± 0.1 *****	235.4 ± 8.7 *****	34.7 ± 0.9 #####
17,20(OH) ₂ D2		0	0	11.7 ± 0.7	12.7 ± 3.7	30.5 ± 3.4 #####	90.1 ± 4.2 *****

***** p < 0.0001 in student t-test on HaCaT vs. Caco-2;
p < 0.0001 in student t-test on 20(OH)D2 vs. 25(OH)D2 or 1,20(OH)₂D2 vs. 17,20(OH)₂D2