HHS Public Access

Author manuscript

Mol Cell Endocrinol. Author manuscript; available in PMC 2017 December 05.

Published in final edited form as:

Mol Cell Endocrinol. 2016 December 5; 437: 312–322. doi:10.1016/j.mce.2016.08.006.

Bioactive forms of vitamin D selectively stimulate the skin analog of the hypothalamus-pituitary-adrenal axis in human epidermal keratinocytes

Justyna M. Wierzbicka[a], **Michał A. Żmijewski**[a,*], **Anna Piotrowska**[a], **Boguslaw Nedoszytko**[b], **Magdalena Lange**[b], **Robert C. Tuckey**[c], and **Andrzej T. Slominski**[d,e,f]

[a]Department of Histology, Medical University of Gdańsk, Poland [b]Department and Clinic of Dermatology, Venereology and Allergology, Medical University of Gdansk, Poland [c]School of Chemistry and Biochemistry, The University of Western Australia, Crawley, Perth, WA 6009, Australia [d]Department of Dermatology, University of Alabama Birmingham, Birmingham, AL 35294, USA [e]Comprehensive Cancer Center, Cancer Chemoprevention Program, University of Alabama Birmingham, Birmingham, AL 35294, USA [f]VA Medical Center, Birmingham, AL 35294, USA

Abstract

Ultraviolet radiation B stimulates both the production of vitamin D_3 in the skin and the activation of the skin analog of the hypothalamic-pituitary-adrenal axis (HPA) as well as the central HPA. Since the role of vitamin D_3 in the regulation of the HPA is largely unknown, we investigated the impact of $1,25(OH)_2D_3$ and its noncalcemic analogs, $20(OH)D_3$ and 21(OH)pD, on the expression of the local HPA in human epidermal keratinocytes. The noncalcemic analogs showed similar efficacy to $1,25(OH)_2D_3$ in stimulating the expression of neuropeptides, CRF, urocortins and POMC, and their receptors, CRFR1, CRFR2, MC1R, MC2R, MC3R and MC4R. Interestingly, unlike other secosteroids, the activity of 21(OH)pD did not correlate with induction of differentiation, suggesting a separate but overlapping mechanism of action. Thus, biologically active forms of vitamin D can regulate different elements of the local equivalent of the HPA with implications for the systemic HPA.

Keywords

Vitamin D_3; Vitamin D_3 analogs; HPA axis; Corticotropin releasing factor; Keratinocytes differentiation; Calcium

[*]Corresponding author. Department of Histology, Medical University of Gdansk, ul. Debinki 1a, Room 230, 80-211 Gdańsk, Pomorskie, Poland. mzmijewski@gumed.edu.pl (M.A. Żmijewski).

Uncited references

Chakraborty et al., 1999; Ebeling, 2014; Elias, 2012; Holick, 1996; Holick et al., 1977; Loite et al., 2013; Mason et al., 2013; Paus, 2011; Pawelek et al., 1992; Pisarchik and Slominski, 2002; Quevedo et al., 2001; Quirk et al., 2016; Searing and Leung, 2010; Slominski et al., 1996; Tang et al., 2013; Vasiadi et al., 2012; Watt, 1983; Zbytek and Slominski, 2005; Zbytek and Slominski, 2007; Zbytek et al., 2002; Zbytek et al., 2004; Zbytek et al., 2005; Zmijewski and Slominski, 2009; Zouboulis, 2009.

1. Introduction

Skin responds to stress and possesses the cutaneous equivalent of the hypothalamic-pituitary-adrenal axis (HPA), referred to as the sHPA (reviewed in (Slominski et al., 2012; Paus et al., 2014). Its functions are coordinated by the corticotropin releasing factor (CRF) signaling system (reviewed in Slominski et al. (2013a)). An additional cutaneous regulatory system initiated by the action of ultraviolet B radiation (UVB) is the vitamin D_3 signaling pathway (reviewed in Bikle (2011a,b,c), Holick (2003)). After photochemical production, vitamin D_3 (which is a prohormone) (Holick et al., 1997; Holick, 1996), is activated by 25-hydroxylation catalyzed by CYP2R1 or CYP27A1 in the liver, then 1α-hydroxylation by CYP27B1 in the kidney, producing 1,25-diydroxyvitamin D_3 (1,25$(OH)_2D_3$) (Holick, 2011; Bikle, 2011a,b,c, 2012; Zhu et al., 2013). Local activation is also possible since these enzymes are present in the skin (Bikle, 2011a,b,c, 2012; Zhu et al., 2013; Bikle et al., 2004a,b), as is the vitamin D receptor (VDR) (Bikle, 2012; Reichrath et al., 1996), so that the skin is not only a source of active vitamin D_3 but also a target of its activity.

Recently, one of the key enzymes in steroidogenesis, CYP11A1 (also known as cytochrome P450scc), was shown to metabolize 7-dehydroholesterol and vitamin D_3 providing an alternative pathway of vitamin D activation (Slominski et al., 2005c; 2015b), with products including the low calcemic but biologically active secosteroids, 21(OH)pD which has a short (2C) side chain and 20*S*-hydroxyvitamin D3 (20(OH)D_3) (reviewed in Slominski et al. (2014, 2013c,d)).

Active forms of vitamin D_3 exhibit a broad spectrum of phenotypic effects on the skin such as inhibition of cell proliferation and stimulation of cell differentiation (reviewed in Bikle (2012), Oda et al. (2007), Bikle (2004), Holick (2014)). Furthermore, there is accumulating evidence that active forms of vitamin D possess anti-cancer (Holick, 2014; Bikle, 2008; Feldman et al., 2014), antifibrotic (Slominski et al., 2011; Slominski et al., 2013b,c,d; Bonventre, 2013), antioxidative (Slominski et al., 2015a; Tongkao-On et al., 2015; Gordon-Thomson et al., 2012) and anti-inflammatory properties (Cannell et al., 2014; Barragan et al., 2015; Wei and Christakos, 2015; Bikle, 2011a,b,c).

The skin equivalent of the HPA follows the basic scheme of the central HPA, with the expression of CRF, structurally related urocortins (UCN 1–3) and their corresponding receptors: CRFR1 and CRFR2 (Slominski et al., 2001; Slominski et al., 2004; Slominski et al., 2000; Slominski et al., 1998 Slominski etal., 2006b; Zmijewski and Slominski, 2009). Stimulation of the CRFR1 receptor in skin cells results in an induction of the expression of proopiomelanocortin (POMC) with the resulting generation of POMC-derived peptides, ACTH, α-MSH and β-endorphin (Slominski et al., 2005a,b; Rousseau et al., 2007). CRF and ACTH (interacting with the MC2R receptor) stimulate the production of glucocorticoids (cortisol and corticosterone) in skin cells (Slominski et al., 2005a,b; Cirillo and Prime, 2011; Hannen et al., 2011; Slominski et al., 2006a). Activation of the glucocorticoid receptor (GR) completes the stress response via inhibition of the HPA axis by attenuating CRF and POMC peptide production (Slominski et al., 2013a; Zmijewski and Slominski, 2011; Ito et al., 2005). Furthermore, locally produced glucocorticoids play an important role in the regulation of the skin inflammatory response (Sevilla et al., 2012; Slominski et al., 2013a,d).

UVB not only induces production of vitamin D_3, but also stimulates cutaneous production of a variety of classical hypothalamic and pituitary peptides involved in the HPA and increases the expression of their receptors (reviewed in Chakraborty et al. 1999; Pawelek et al., 1992; Slominski et al. (2012, 2013a,d)), and stimulates local steroidogenesis (Slominski et al., 2013a,d; Skobowiat et al., 2011; Skobowiat et al., 2013a,b; Talabér et al., 2013). UVB also upregulates the central HPA axis (Skobowiat and Slominski, 2015) and the POMC signaling system in the arcuate nucleus of the hypothalamus (Skobowiat and Slominski, 2016). Therefore, it is hypothesized that the selective expression of neuropeptides and their receptors not only contributes to the process of formation of the epidermal barrier, but also participates in skin stress and immune responses.

Vitamin D_3 and calcium are essential regulators of skin physiology including keratinocyte differentiation (Bikle, 2011a,b,c, 2012; Elias, 2012; Elias et al., 2013). Vitamin D and its analogs are known to modify expression of at least 3000 genes (Haussler et al., 2011), as shown by several transcriptome-wide arrays, however the effect on expression of the elements of HPA axis has not been investigated (Rid et al., 2013). Therefore, the major aim of this study was to determine whether $1,25(OH)_2D_3$ and its low calcemic analogs, $20(OH)D_3$ and 21(OH)pD, as well-studied inducers of keratinocytes differentiation, also modulate the expression of the sHPA.

2. Materials and methods

2.1. Cell culture

Pooled juvenile Human Epidermal Keratinocyte Progenitors (HPEKp) were acquired from CELLnTEC (Bern, Switzerland). Cells were cultivated in Epidermal Keratinocyte Medium (CnT-07, CELL-nTEC) containing low calcium (0.07 mM), supplement mix (A, B, C), bisphenol A (BPE) and gentamycin. This medium supports the retention of proliferative progenitors and reduces differentiation according to the manufacturer. HPEKp cells were cultured at 37 °C in a humidified 5% CO_2 incubator in T-75 culture flasks. Only cells from passages two to four were used for experiments. Cells were passaged at 80–90% confluency after trypsinization with TrypLE™ Express solution (Gibco, Life Technologies, USA).

2.2. Cell treatment

After 24 h of preincubation in supplemented CnT-07 medium, HPEKp cells were treated with vehicle, 0.1 μM $1,25(OH)_2D_3$ or 2.5 mM CaCl as a source of Ca^{2+}, alone or in combination with $1,25(OH)_2D_3$, for 4 or 24 h. Additionally, some cells were treated with the vitamin D_3 derivatives $20(OH)D_3$ or 21(OH)pD (0.1 μM) for 24 h. $1,25(OH)_2D_3$ was purchased from Pharmaceutical Research Institute, Warsaw, Poland. 21(OH)pD was synthesized according to a procedure described by Zmijewski et al. (2011) by ProChimia Surfaces Sp. Zo.o. (Poland), while $20(OH)D_3$ was enzymatically synthesized as described previously (Slominski et al., 2005c; Tuckey et al., 2011).

2.3. Proliferation assay

The degree of proliferation of cells following treatment was measured from their protein content using the sulforhodamine B assay (SRB). Measurements were made as described

previously (Wierzbicka et al., 2015), with some modifications. Cells were seeded at a density of ~12 × 10^3 cells per 100 µl in 96-well plates and allowed to attach for 24 h before treatment. The culture medium was replaced with fresh medium containing serial dilutions of 1,25$(OH)_2D_3$ or its analogs (0.01 nM–1 µM) in a volume of 100 µl/well. The plates were incubated at 37 °C for an additional 48 h, 100 µl of 10% TCA was added to each well and plates incubated for 1 h at 4 °C. Medium was removed and cells were washed 5 times with deionized water. Following overnight air drying, 100 µl of SRB solution [0.4% (w/v) in 1% acetic acid] was added to each well. After incubation for 15 min, plates were washed 5 times with 1% acetic acid and air-dried. The protein-bound dye was solubilized with 10 mM Tris-base solution (pH 10.5). The absorbance of the dye was recorded at 570 nm with a microplate reader. Results are expressed as percentage change in protein level

$$\left(\frac{\text{mean of treated group}}{\text{mean of control group}} \times 100\%\right).$$

2.4. Real-Time PCR

Total RNA was extracted from cell cultures or skin biopsies using the Total RNA MiniPLUS kit (A&A Biotechnology, Poland) according to the manufacturer's instructions. The concentration and purity of isolated RNA were measured with an Epoch spectrophotometer (BioTek, Winooski, USA). Two micrograms of total RNA were subjected to reverse transcription using a RevertAid™ First Strand cDNA Synthesis kit (Thermo Fisher Scientific Inc., USA.). The primers used for PCR amplification are listed in Table 1. The reactions were performed in duplicate for each primer set with Real Time HS 2× PCR Master Mix SYBR® kit (A&A Biotechnology, Poland). The data were collected using the StepOnePlus™ Real-Time PCR System (Life Technologies, USA). The amount of amplified product for each gene was compared to that for the reference gene (*RPL37*) using a comparative ΔΔCT method and presented as a fold change ± SD. In the initial stage of the project it was found that *RPL37*, a gene encoding a ribosomal 60s subunit protein, is a suitable reference gene for quantitative transcript analysis for human keratinocytes out of all the potential housekeeping genes that were tested (*RPL37, B2M* and *HPRT1*).

2.5. Immunofluorescence microscopy

Prior to immunofluorescence stainings, HPEKp keratinocytes were seeded in 8-well Lab-Tek II chamber slides (Nalge Nunc Inc., USA). At selected time points after treatment, cells were fixed with 4% paraformaldehyde (PFA) and then permeabilized in 0.2% TritonX-100 solution in PBS for 10 min. Blocking was performed with 1% BSA in PBS for 1 h at room temperature (RT). Following extensive rinsing in PBS, the primary antibodies diluted in the same blocking solution were added and incubated overnight at 4 °C. Next day, slides were rinsed with PBS and incubated with the corresponding Alexa Fluor® (AF488 or AF594)-conjugated secondary antibodies for 1 h at RT. Then slides were rinsed, counterstained with DAPI (Sigma Aldrich) and closed with a cover glass. Cells treated with only the secondary antibody were used as the negative control. Images of cells were collected with a Nikon Eclipse E800, and further analyzed with the use of ImageJ® software. All primary and secondary antibodies and their dilutions for use are listed in Table 2.

2.6. Flow cytometry

HPEKp cells were treated with 0.1 μM $1,25(OH)_2D_3$, $20(OH)D_3$ or 21(OH)pD for 24 h. Simultaneously control, unstimulated cells were cultured. After treatment cells were harvested and washed twice in PBS. Cells were fixed and permeabilized with Cytofix/Cytoperm Buffer (BD Biosciences, San Jose, CA, USA) and stained with selected antibodies (Table 2) according to the manufacturer's protocol. Samples were analyzed with a BD FACS Calibur flow cytometer. FL-1 and FL-4 signals (collected from 10,000 events in side scatter/forward scatter window after debris exclusion) were recorded. Forward (relative to cell size) and side (relative to cell granularity) scatter were also recorded. Data were further analyzed with BD CellQuest Pro software (BD Biosciences, San Jose, CA, USA).

2.7. Statistical analyses

Data are presented as mean ± SD, and were analyzed with a Student's *t*-test (for two groups) or one-way analysis of variance with appropriate post-hoc tests (for more than two groups) using Prism 4.00 (GraphPad Software, San Diego, CA). Statistically significant differences are denoted with asterisks: $*P < 0.05$, $**P < 0.01$, $***P < 0.005$.

3. Results

The general effects of $1,25(OH)_2D_3$ and its analogs on HPEKp primary keratinocytes were examined (Fig. 1). All secosteroids significantly inhibited cell proliferation in a dose-dependent manner with 21(OH)pD showing a similar IC_{50} to that of $1,25(OH)_2D_3$ (1.73 nM vs 1.69 nM, respectively), while $20(OH)D_3$ gave a higher value (12.5 nM) (Fig. 1A–C). However, $20(OH)D_3$ showed higher maximal inhibition (efficacy) in comparison to $1,25(OH)_2D_3$ and 21(OH)pD, reducing the cell number by 60–70% vs 20%, for $1,25(OH)_2D_3$ and 21(OH)pD. In addition, there was a significant difference ($**P < 0.01$) in the percentage of Ki67 positive cells (Fig. 1G) between control (71.3 ± 4.8%) and $1,25(OH)_2D_3$-treated cells (55.3 ± 7.8%). We investigated the expression of keratinocyte differentiation marker genes and found that expression of the early differentiation marker genes, cytokeratin 14 (*KRT14*) and cytokeratin 1 (*KRT1*), Fig. 1D, E was elevated in HPEKp keratinocytes treated with all secosteroids tested ($*P < 0.05$). *KRT14* expression was elevated by each secosteroid with the relative stimulation being 21(OH)pD > $20(OH)D_3$ > $1,25(OH)_2D_3$ (Fig. 1D). Only $1,25(OH)_2D_3$ and $20(OH)D_3$ treatment effectively increased the level of mRNA for the involucrin (*INV*) gene, a late differentiation marker, with the short side-chain analog, 21(OH)pD, having no effect (Fig. 1F). These results are consistent with previous data on induction of the *INV* gene expression in the HaCaT cell line treated with active forms of vitamin D_3 (Zbytek et al., 2008). The $1,25(OH)_2D_3$ effect was also confirmed by immunofluorescence staining for INV (Fig. 1H). $1,25(OH)_2D_3$ proved to be as strong an inducer of INV production as Ca^{2+}.

We also examined changes in the expression of genes involved in vitamin D_3 signaling following treatment with the secosteroids. There was a lack of effect of $1,25(OH)_2D_3$ and $20(OH)D_3$ on *VDR* gene expression with inhibition only occurring with 21(OH)pD (Fig. 2A). In contrast, the expression of the *PDIA3* gene which encodes a plasma membrane-bound receptor for vitamin D_3, was enhanced by $1,25(OH)_2D_3$ and $20(OH)D_3$, but not by

21(OH)pD (Fig. 2B). Only 1,25$(OH)_2D_3$ strongly induced the expression of *CYP24A1* which encodes the 24-hydroxylase that inactivates 1,25$(OH)_2D_3$, (Fig. 2C), while 20$(OH)D_3$ caused modest stimulation, in agreement with previous investigations (reviewed in Slominski et al. (2015a,b,c)). All secosteroids tested significantly stimulated the expression of the xenobiotic metabolizing enzyme, *CYP3A4,* at the mRNA level (Fig. 2E), and to a lesser extent stimulated expression of the 25-hydroxylase gene, *CYP2R1* (Fig. 2D). Interestingly, only 21(OH)pD significantly increased the expression of the 1α-hydroxylase gene, *CYP27B1* (Fig. 2F).

3.1. Vitamin D derivatives and calcium stimulate expression of sHPA axis

Previous studies have demonstrated that UVB can significantly stimulate the expression of HPA-related genes in epidermal keratinocytes and melanocytes (Zbytek et al., 2006a; Skobowiat et al., 2011 Slominski et al., 1996; Slominski et al., 2006c). To test whether these effects are secondary to the action of active forms of vitamin D, we investigated whether 1,25$(OH)_2D_3$ affects the expression of *CRF* and *CRF*-related genes [urocortins 1–3 (*UCN1–3*)] at the mRNA level (Fig. 3). HPEKp cells were treated with 0.1 μM 1,25$(OH)_2D_3$ or 2.5 mM calcium (Ca^{+2}) separately, or simultaneously with both (Fig. 3), for 4 or 24 h. Four hours after treatment *CRF* mRNA was significantly increased in all groups tested (Fig. 3A). By 24 h this stimulation decreased compared to 4 h, but still was higher than the control level. The strongest effect was seen for simultaneous treatment with 1,25$(OH)_2D_3$ and Ca^{+2}. The stimulation of the expression of *UCN1–3* was less pronounced or absent, and was predominantly seen at 24 h of treatment (Fig. 3B–D). These effects were also selective with weak but significant stimulation after a 4 h of treatment with 1,25$(OH)_2D_3$ (*UCN-2* and *3*), and 1,25$(OH)_2D_3$ plus Ca^{+2} (*UCN-3*), with strongest stimulation at 24 h by 1,25$(OH)_2D_3$ plus Ca^{+2} (*UCN1–3*). Significant stimulation of *UCN-1* and *3* expression by 1,25$(OH)_2D_3$ alone was only seen at 4 h (Fig. 3B–D).

The expression of the *POMC* gene, encoding the second element of the sHPA, was stimulated at 4 and 24 h of treatment with 1,25$(OH)_2D_3$ alone or in combination with Ca^{+2}, with the strongest stimulation being observed with 1,25$(OH)_2D_3$ plus Ca^{+2} for 24 h (Fig. 3E). 1,25$(OH)_2D_3$ and/or Ca^{+2} treatment of HPEKp cells also stimulated the expression of receptors for POMC-derived peptides (Fig. 3F–H). Incubation of cells with 1,25$(OH)_2D_3$ or 1,25$(OH)_2D_3$ plus Ca^{+2} resulted in increased expression of the *MC1R* gene at 4 and 24 h, with the highest stimulation seen for 1,25$(OH)_2D_3$ plus Ca^{+2} at 24 h, while Ca^{+2} alone had no effect (Fig. 3F). Stimulation of *MC2R* and *MC4R* gene expression was observed with all treatments, with the effects being greatest at 4 h (Fig. 3G and H). Finally, expression of the glucocorticoid receptor (GR) gene, *NR3C1* (Fig. 3I), was only slightly elevated in cells treated with 1,25$(OH)_2D_3$ or 1,25$(OH)_2D_3$ plus Ca^{+2}, at 24 h only.

The expression of selected elements of the sHPA in human primary keratinocytes (HPEKp) at the protein level was investigated by western blotting (Fig. 4) and immunofluorescence (IF) (Fig. 5). The level of CRF peptides in HPEKp cells was increased by treatment with 1,25$(OH)_2D_3$ and Ca^{+2} separately or together for 24 h (Fig. 4). Some processing of the 25 kDa CRF precursor to the 19 kDa form was apparent which is consistent with previous reports in the literature (Chretien and Seidah, 1984) and with data provided by the supplier

of the antiserum, with both forms displaying an increase with the treatments. The increase in CRF expression was accompanied by an increase in CRFR1 immunoreactivity as shown by western blotting (with a marked increase also occurring over 24 h without treatment) (Fig. 4) and by an elevated number of CRFR1 positive HPEKp cells (Fig. 5A). In addition, $1,25(OH)_2D_3$, Ca^{+2} or $1,25(OH)_2D_3$ plus Ca^{+2} stimulated the production of POMC-derived ACTH in cultured keratinocytes (Fig. 5B), consistent with stimulation of *POMC* gene expression. Finally, similar treatments increased the MC2R protein level as evaluated by western blotting and IF (Figs. 4 and 5C).

3.2. The effect of low-calcemic D_3 analogs on the expression of HPA axis elements

$20(OH)D_3$ and 21(OH)pD (0.1 μM) were tested for their ability to stimulate the expression of sHPA genes in comparison to $1,25(OH)_2D_3$. Both of these secosteroids stimulated the expression *CRF* similarly to $1,25(OH)_2D_3$ (Fig. 6A). Interestingly, the highest level of stimulation of the expression of *CRF* and urocortins was observed for 21(OH)pD (Fig. 6B–D). In addition, $1,25(OH)_2D_3$ and $20(OH)D_3$ did not affect the expression of the *UCN1* gene (Fig. 6B) and only moderately increased the expression of *UCN2* and *3* (Fig. 6C–D). The expression of the *POMC* gene was stimulated comparably by $1,25(OH)_2D_3$, $20(OH)D_3$ or 21(OH)pD (Fig. 6E). Furthermore, stimulation of the expression *MC1R* and *MC2R* genes followed the same trend, with comparable effects for all the secosteroids tested (Fig. 6F–G). *MC3R* mRNA was undetectable in primary keratinocytes from the control culture, but its expression was effectively induced by all the secosteroids tested, with the highest stimulation obtained with 21(OH)pD (compared to $1,25(OH)_2D_3$ treatment). Finally, $20(OH)D_3$ or $1,25(OH)_2D_3$ treatment, unlike 21(OH)pD, attenuated the expression of *CYP11A1* (Fig. 6I), while only 21(OH)pD moderately decreased the expression of *NR3C1* (Fig. 6J).

3.3. The degree of keratinocyte differentiation affects the expression of ACTH and MC2R in cells treated with vitamin D_3 derivatives

Since the differentiation of keratinocytes is accompanied by an alteration in the expression of several genes (Eckert and Rorke, 1989; Eckhart et al., 2013; Elias, 2012), we tested whether the degree of differentiation of keratinocytes, monitored by the level of expression of INV, affects the stimulation of ACTH and MC2R expression by vitamin D compounds. Using flow cytometry we tested two populations of keratinocytes, INV positive (INV^+, differentiated) and INV negative (INV^-, undifferentiated) cells (Fig. 7). $1,25(OH)_2D_3$ treatment increased the number of positive cells for ACTH (Fig. 7A) and its receptor (MC2R), in INV^+ keratinocytes (Fig. 7C), while their relative numbers decreased for INV^- cells (Fig. 7B, D). The treatment of HPEKp with $20(OH)D_3$ or 21(OH)pD resulted in a decrease in the % of cells co-expressing ACTH in INV^+ keratinocytes (Fig. 7A). Also, 21(OH)pD, but not $20(OH)D_3$, significantly decreased the % of cells co-expressing MC2R and INV^+ (Fig. 7C), while the % of cells positive for MC2R in INV^- cells was increased by 21(OH)pD and $20(OH)D_3$, but slightly decreased by $1,25(OH)_2D_3$ (Fig. 7D).

4. Discussion

Recently, we have reported increased expression of cutaneous elements of the HPA coincides with the keratinocyte differentiation program, with both processes being stimulated by calcium addition (Wierzbicka et al., 2016). Since active forms of vitamin D_3 protect the epidermis against UVB, a major environmental stressor (Slominski et al., 2015a,c; Gordon-Thomson et al., 2012; De Haes et al., 2005), clarification of the relationship between the cutaneous HPA axis and vitamin D_3-activated pathways is mandatory.

We observed that $1,25(OH)_2D_3$ and its non-calcemic analogs ($20(OH)D_3$ and 21(OH)pD) displayed antiproliferative properties, while only $1,25(OH)_2D_3$ and $20(OH)D_3$ stimulated differentiation of keratinocytes using *INV* as a marker. Previous studies on immortalized human epidermal keratinocytes have shown that $20(OH)D_3$ possesses antiproliferative activity similar to that of $1,25(OH)_2D_3$, with both compounds effectively stimulating *INV* expression (Zbytek et al., 2008). Interestingly, 21(OH)pD inhibited cell proliferation as effectively $1,25(OH)_2D_3$ and $20(OH)D_3$, but caused weaker induction of keratinocytes differentiation, with only a moderate effect on the expression of *KRT14* (an early differentiation marker) and no effect on *INV* expression (late differentiation marker). Treatment of primary keratinocytes with 21(OH)pD, which only has a 2C side chain, did not stimulate the expression of *CYP24A1*, a major target of the activated VDR, suggesting an alternative pathway may be activated by this secosteroid. This is consistent with previous studies showing that treatment of melanoma cells with 21(OH)pD does not stimulate the translocation of VDR to the nucleus (Zmijewski et al., 2011) nor cause stimulation of *CYP24A1* expression (Wasiewicz et al., 2015), despite inhibiting melanoma cell proliferation (Zmijewski et al., 2011; Wasiewicz et al., 2015). The lack of stimulation of VDR translocation and expression of *CYP24A1* and *INV* genes by 21(OH)pD can be explained by the recent *in silico* prediction that 21(OH)pD interacts poorly with the VDR, having the lowest docking score of the vitamin D analogs examined (Kim et al., 2012). Thus, the observed inhibition of cell proliferation by 21(OH)pD may be secondary to the interaction of this secosteroid, with an alternative receptor to the VDR.

$1,25(OH)_2D_3$ caused a massive increase in the expression of *CYP24A1*, with CYP24A1 catalyzing the inactivation of both $25(OH)D_3$ and $1,25(OH)_2D_3$ (Holick, 2003). As before (Zbytek et al., 2008), $20(OH)D_3$ caused very low stimulation of *CYP24A1* expression, with the gene product catalyzing the further activation of $20(OH)D_3$ instead of its inactivation, at least in melanoma cells (Tieu et al., 2012). $1,25(OH)_2D_3$, $20(OH)D_3$ and 21(OH)pD all caused a 3- to 3.5-fold increase in *CYP3A4* expression in keratinocytes at the mRNA level. CYP3A4, as well as acting on a wide range of xenobiotics, provides an alternate route to CYP24A1 for the metabolism of active forms of vitamin D_3, including $20(OH)D_3$ (Cheng et al., 2016). $20(OH)D_3$ and 21(OH)pD, but not $1,25(OH)_2D_3$, caused a small but significant stimulation of the expression of *CYP2R1* which encodes a vitamin D 25-hydroxylase. Only 21(OH)pD significantly stimulated expression of *CYP27B1* but it is unlikely that the encoded 1α-hydroxylase can act on vitamin D_3 analogs like 21(OH)pD, which possess a short side chain (Chen et al., 2014).

The current study shows that treatment of primary keratinocytes with $1,25(OH)_2D_3$ and calcium induces the expression, at least at the mRNA level, of all the elements of the HPA axis including *CRF, UNC1–3, POMC, MC1R* and *MC2R* and *NR3C1*. Furthermore, stimulation is generally greater than that by treatment with $1,25(OH)_2D_3$ alone, particularly at 24 h. We have previously shown that the process of keratinocytes differentiation, which is strongly induced by the cooperation of vitamin D_3 with Ca^{2+} (Bikle et al., 2004a,b), plays a crucial role in the stimulation of elements of the cutaneous HPA (Wierzbicka et al., 2016). However, the effect of incubation of calcium with primary keratinocytes for 4 or 24 h on the elements of the HPA was always weaker that caused by $1,25(OH)_2D_3$. This suggests that active forms of vitamin D_3 may act on the epidermal elements of the HPA through overlapping and distinct pathways to that induced by calcium.

21(OH)pD was the only secosteroid tested that was able to stimulate expression of urocortin 1 (*UCN 1*), and was the strongest inducer for *CRF, UCN2* and *UCN3* gene expression. It is well established that CRF and UCN1 enhance expression of *POMC* trough CRFR1 receptor-mediated activation of adenylate cyclase (Slominski et al., 2013a). It seems however, that the observed higher level of induction of *CRF* and *UCN1* by 21(OH)pD in comparison to other secosteroids, has no impact on POMC expression, since they all showed similar effects. 21(OH)pD was marginally better at stimulating the expression of receptors for melanocortins (except MC1R) than the other secosteroids. To our knowledge, this is the first study showing the stimulation of *MC3R* and *MC4R* expression by $1,25(OH)_2D_3$ or its non-calcemic analogs, $20(OH)D_3$ and 21(OH)pD. Recently, MC3R was proposed as an alternative target for corticotropins involved in immunomodulation (Zmijewski and Slominski, 2013). Although γ-MSH is the preferred ligand for MC3R, ACTH can bind to MC3R with comparable affinity (Bohm and Grassel, 2012). Moreover, MC3R may be involved in the activation of steroidogenesis (Harmer and Bicknell, 2005), but what is most interesting is the selective upregulation of IL-1β, IL-6, and NOS2 in $Mc3^{-/-}$ mice compared to wild type mice (Patel et al., 2010). Based on this, it was postulated that induction of MC3R contributes to the melanocortin-induced skin stress response.

The current study shows that ACTH and MC2R are preferentially found at the border of the *stratum spinosum* and *stratum granulosum* in full thickness skin biopsies. These results are supported by the observed induction of ACTH and MC2R expression in partially differentiated HPEKp keratinocytes (Wierzbicka et al., 2016). Using flow cytometry analysis and INV as a marker of moderate differentiation, we observed that $1,25(OH)_2D_3$ enhanced ACTH and MC2R production only in INV^+ cells, while $20(OH)D_3$ (moderately) and 21(OH)pD (more strongly) stimulated MC2R production in undifferentiated keratinocytes (INV^-). Unlike $1,25(OH)_2D_3$, treatment of primary keratinocytes with 21(OH)pD resulted in a significant decrease in MC2R immunoreactivity in differentiated (INV^+) cells but an increase in MC2R immunoreactivity in undifferentiated (INV^-) keratinocytes. Furthermore, in contrast to $1,25(OH)_2D_3$ there was not a significant effect of 21(OH)pD on ACTH immunoreactivity. This suggests that 21(OH)pD action on ACTH signaling can be separated from the induction of the keratinocyte differentiation program.

Finally, our data raise the intriguing question as to whether the stimulation of the cutaneous elements of the HPA by UVB is mediated, at least in part, by locally synthesized active

forms of vitamin D_3. Different elements of the HPA including POMC peptides, CRF and related peptides and glucocorticoids are involved in the regulation of the epidermal skin barrier and its immune activities, as described above. Therefore, our findings may not only explain a role of vitamin D in the regulation of epidermal neuroendocrine activities, but also may represent a dawn for studies on the mechanism of regulation of systemic HPA axis and/or of POMC and CRF activities in the brain (Skobowiat and Slominski, 2016; Slominski, 2015; Slominski et al., 2015a,b,c).

In summary, we have shown for the first time that active forms of vitamin D stimulate the expression of elements of the skin analog of the HPA axis. This process occurs rapidly, is enhanced by the induction of the keratinocyte differentiation program and may follow overlapping but different mechanisms defined by the length of side chain and location of hydroxyl groups on the vitamin D scaffold.

Acknowledgments

The project was supported by Grant from the Polish Ministry of Science and Higher Education, contract grant number: N402 662840 (to M.A.Z.), and in part by NIH grants R21AR066505, 1R01AR056666 and 2R01AR052190 to AS, and the University of Western Australia to RCT.

Abbreviations

INV	Involucrin
HPA	hypothalamic-pituitary-adrenal axis
sHPA	skin analog of HPA
HPEKp	human primary epidermal keratinocytes (pulled donors)
UVA/B	ultraviolet radiation A and B KRT1, KRT14, KRT15 keratin 1, 14, 15
CRF	corticotropin releasing factor
UCN1–3	urocortins 1–3
POMC	proopiomelanocortin
ACTH	adrenocorticotrophin
CRFR1	corticotropin-releasing hormone receptor 1
CRFR2	corticotropin-releasing hormone receptor 2
MC1-5R	melanocortin receptors 1–5
GR	glucocorticoid receptor
1α,25(OH)$_2$D$_3$	1α,25-dihydroxyvitamin D_3 (calcitriol)
20(OH)D$_3$	20*S*-hydroxyvitamin D_3

21(OH)pD	21-hydroxypregnacalciferel
25(OH)D3	calcifediol (25-hydroxyvitamin D3)
7-DHC	7-dehydrocholesterol (provitamin D3, cholesta-5,7-dien-3β-ol)
VDR	vitamin D receptor
PDIA3	Protein disulfide isomerase associated 3

References

Barragan M, Good M, Kolls JK. Regulation of dendritic cell function by vitamin D. Nutrients. 2015; 7:8127–8151. [PubMed: 26402698]

Bikle DD. Vitamin D regulated keratinocyte differentiation. J. Cell. Biochem. 2004; 92:436–444. [PubMed: 15156556]

Bikle DD. Vitamin D receptor, UVR, and skin cancer: a potential protective mechanism. J. Invest. Dermatol. U. S. 2008:2357–2361.

Bikle DD. Vitamin D metabolism and function in the skin. Mol. Cell. Endocrinol. 2011a; 347:80–89. [PubMed: 21664236]

Bikle DD. Vitamin D: an ancient hormone. Exp. Dermatol. 2011b; 20:7–13. [PubMed: 21197695]

Bikle DD. Vitamin D regulation of immune function. Vitam. Horm. 2011c; 86:1–21. [PubMed: 21419265]

Bikle DD. Vitamin D and the skin: physiology and pathophysiology. Rev. Endocr. Metab. Disord. 2012; 13:3–19. [PubMed: 21845365]

Bikle DD, Chang S, Crumrine D, Elalieh H, Man MQ, Choi EH, Dardenne O, Xie Z, Arnaud RS, Feingold K, Elias PM. 25 Hydroxyvitamin D 1 alpha-hydroxylase is required for optimal epidermal differentiation and permeability barrier homeostasis. J. Invest. Dermatol. 2004a; 122:984–992. [PubMed: 15102089]

Bikle DD, Oda Y, Xie Z. Calcium and 1,25(OH)2D: interacting drivers of epidermal differentiation. J. Steroid Biochem. Mol. Biol. 2004b; 89–90:355–360.

Bohm M, Grassel S. Role of proopiomelanocortin-derived peptides and their receptors in the osteoarticular system: from basic to translational research. Endocr. Rev. 2012; 33:623–651. [PubMed: 22736674]

Bonventre JV. Antifibrotic vitamin D analogs. J. Clin. Invest. 2013; 123:4570–4573. [PubMed: 24162671]

Cannell JJ, Grant WB, Holick MF. Vitamin D and inflammation. Dermatoendocrinol. 2014; 6:e983401. [PubMed: 26413186]

Chakraborty AK, Funasaka Y, Slominski A, Bolognia J, Sodi S, Ichihashi M, Pawelek JM. UV light and MSH receptors. Ann. N. Y. Acad. Sci. 1999; 885:100–116. [PubMed: 10816644]

Chen J, Wang J, Kim TK, Tieu EW, Tang EK, Lin Z, Kovacic D, Miller DD, Postlethwaite A, Tuckey RC, Slominski AT, Li W. Novel vitamin D analogs as potential therapeutics: metabolism, toxicity profiling, and antiproliferative activity. Anticancer Res. 2014; 34:2153–2163. [PubMed: 24778017]

Cheng CY, Slominski AT, Tuckey RC. Hydroxylation of 20-hydroxyvitamin D3 by human CYP3A4. J. Steroid Biochem. Mol. Biol. 2016; 159:131–141. [PubMed: 26970587]

Chretien M, Seidah NG. Precursor polyproteins in endocrine and neuroendocrine systems. Int. J. Pept. Protein Res. 1984; 23:335–341. [PubMed: 6144640]

Cirillo N, Prime SS. Keratinocytes synthesize and activate cortisol. J. Cell. Biochem. 2011; 112:1499–1505. [PubMed: 21344493]

De Haes P, Garmyn M, Verstuyf A, De Clercq P, Vandewalle M, Degreef H, Vantieghem K, Bouillon R, Segaert S. 1,25-Dihydroxyvitamin D3 and analogues protect primary human keratinocytes

against UVB-induced DNA damage. J. Photochem Photobiol. B. 2005; 78:141–148. [PubMed: 15664501]

Ebeling PR. Vitamin D and bone health: epidemiologic studies. Bonekey Rep. 2014; 3:511. [PubMed: 24818003]

Eckert RL, Rorke EA. Molecular biology of keratinocyte differentiation. Environ. Health Perspect. 1989; 80:109–116. [PubMed: 2466639]

Eckhart L, Lippens S, Tschachler E, Declercq W. Cell death by cornification. Biochim. Biophys. Acta. 2013; 1833:3471–3480. [PubMed: 23792051]

Elias PM. Structure and function of the stratum corneum extracellular matrix. J. Invest. Dermatol. 2012; 132:2131–2133. [PubMed: 22895445]

Elias PM, Eichenfield LF, Fowler JF, Horowitz P, McLeod RP. Update on the structure and function of the skin barrier: atopic dermatitis as an exemplar of clinical implications. Semin. Cutan. Med. Surg. 2013; 32:S21–S24. [PubMed: 24156154]

Feldman D, Krishnan AV, Swami S, Giovannucci E, Feldman BJ. The role of vitamin D in reducing cancer risk and progression. Nat. Rev. Cancer. 2014; 14:342–357. [PubMed: 24705652]

Gordon-Thomson C, Gupta R, Tongkao-on W, Ryan A, Halliday GM, Mason RS. 1α,25 dihydroxyvitamin D3 enhances cellular defences against UV-induced oxidative and other forms of DNA damage in skin. Photochem Photobiol. Sci. 2012; 11:1837–1847. [PubMed: 23069805]

Hannen RF, Michael AE, Jaulim A, Bhogal R, Burrin JM, Philpott MP. Steroid synthesis by primary human keratinocytes; implications for skin disease. Biochem. Biophys. Res. Commun. 2011; 404:62–67. [PubMed: 21094146]

Harmer SC, Bicknell AB. Role of gamma-MSH peptides in the regulation of adrenal steroidogenesis. Peptides. 2005; 26:1944–1951. [PubMed: 15985307]

Haussler MR, Jurutka PW, Mizwicki M, Norman AW. Vitamin D receptor (VDR)-mediated actions of 1α,25(OH)$_2$ vitamin D$_3$: genomic and non-genomic mechanisms. Best. Pract. Res. Clin. Endocrinol. Metab. 2011; 25:543–559. [PubMed: 21872797]

Holick MF. Vitamin D and bone health. J. Nutr. 1996; 126:1159s–1164s. [PubMed: 8642450]

Holick MF. Vitamin D: a millenium perspective. J. Cell. Biochem. 2003; 88:296–307. [PubMed: 12520530]

Holick MF. Vitamin D: evolutionary, physiological and health perspectives. Curr. Drug Targets. 2011; 12:4–18. [PubMed: 20795941]

Holick MF. Sunlight, ultraviolet radiation, vitamin D and skin cancer: how much sunlight do we need? Adv. Exp. Med. Biol. 2014; 810:1–16. [PubMed: 25207357]

Holick MF, Frommer JE, McNeill SC, Richtand NM, Henley JW, Potts JT Jr. Photometabolism of 7-dehydrocholesterol to previtamin D3 in skin. Biochem. Biophys. Res. Commun. 1977; 76:107–114. [PubMed: 194588]

Ito N, Ito T, Kromminga A, Bettermann A, Takigawa M, Kees F, Straub RH, Paus R. Human hair follicles display a functional equivalent of the hypothalamic-pituitary-adrenal axis and synthesize cortisol. FASEB J. 2005; 19:1332–1334. [PubMed: 15946990]

Kim TK, Wang J, Janjetovic Z, Chen J, Tuckey RC, Nguyen MN, Tang EK, Miller D, Li W, Slominski AT. Correlation between secosteroid-induced vitamin D receptor activity in melanoma cells and computer-modeled receptor binding strength. Mol. Cell. Endocrinol. 2012; 361:143–152. [PubMed: 22546549]

Oda Y, Ishikawa MH, Hawker NP, Yun QC, Bikle DD. Differential role of two VDR coactivators, DRIP205 and SRC-3, in keratinocyte proliferation and differentiation. J. Steroid Biochem. Mol. Biol. 2007; 103:776–780. [PubMed: 17223341]

Patel HB, Bombardieri M, Sampaio AL, D'Acquisto F, Gray M, Grieco P, Getting SJ, Pitzalis C, Perretti M. Anti-inflammatory and antiosteoclastogenesis properties of endogenous melanocortin receptor type 3 in experimental arthritis. FASEB J. 2010; 24:4835–4843. [PubMed: 20702773]

Paus R, Langan EA, Vidali S, Ramot Y, Andersen B. Neuroendocrinology of the hair follicle: principles and clinical perspectives. Trends Mol. Med. 2014; 20:559–570. [PubMed: 25066729]

Pawelek JM, Chakraborty AK, Osber MP, Orlow SJ, Min KK, Rosenzweig KE, Bolognia JL. Molecular cascades in UV-induced melanogenesis: a central role for melanotropins? Pigment. Cell. Res. 1992; 5:348–356. [PubMed: 1292019]

Reichrath J, Collins ED, Epple S, Kerber A, Norman AW, Bahmer FA. Immunohistochemical detection of 1,25-dihydroxyvitamin D3 receptors (VDR) in human skin. a comparison of five antibodies. Pathol. Res. Pract. 1996; 192:281–289. [PubMed: 8739475]

Rid R, Wagner M, Maier CJ, Hundsberger H, Hintner H, Bauer JW, Onder K. Deciphering the calcitriol-induced transcriptomic response in keratinocytes: presentation of novel target genes. J. Mol. Endocrinol. 2013; 50:131–149. [PubMed: 23256991]

Rousseau K, Kauser S, Pritchard LE, Warhurst A, Oliver RL, Slominski A, Wei ET, Thody AJ, Tobin DJ, White A. Proopiomelanocortin (POMC), the ACTH/melanocortin precursor, is secreted by human epidermal keratinocytes and melanocytes and stimulates melanogenesis. FASEB J. 2007; 21:1844–1856. [PubMed: 17317724]

Sevilla LM, Latorre V, Sanchis A, Perez P. Epidermal inactivation of the glucocorticoid receptor triggers skin barrier defects and cutaneous inflammation. J. Invest. Dermatol. 2012

Skobowiat C, Slominski AT. UVB activates hypothalamic-pituitary-adrenal Axis in C57BL/6 mice. J. Invest. Dermatol. 2015; 135:1638–1648. [PubMed: 25317845]

Skobowiat C, Slominski AT. Ultraviolet B stimulates proopiomelanocortin signalling in the arcuate nucleus of the hypothalamus in mice. Exp. Dermatol. 2016; 25:120–123. [PubMed: 26513428]

Skobowiat C, Dowdy JC, Sayre RM, Tuckey RC, Slominski A. Cutaneous hypothalamic-pituitary-adrenal axis homolog: regulation by ultraviolet radiation. Am. J. Physiol. Endocrinol. Metab. 2011; 301:E484–E493. [PubMed: 21673307]

Skobowiat C, Sayre RM, Dowdy JC, Slominski AT. Ultraviolet radiation regulates cortisol activity in a waveband-dependent manner in human skin ex vivo. Br. J. Dermatol. 2013a; 168:595–601. [PubMed: 23363016]

Skobowiat C, Nejati R, Lu L, Williams RW, Slominski AT. Genetic variation of the cutaneous HPA axis: an analysis of UVB-induced differential responses. Gene. 2013b; 530:1–7. [PubMed: 23962689]

Slominski AT. Ultraviolet radiation (UVR) activates central neuro-endocrine-immune system. Photodermatol. Photoimmunol. Photomed. 2015; 31:121–123. [PubMed: 25660238]

Slominski A, Baker J, Ermak G, Chakraborty A, Pawelek J. Ultraviolet B stimulates production of corticotropin releasing factor (CRF) by human melanocytes. FEBS Lett. 1996; 399:175–176. [PubMed: 8980146]

Slominski A, Ermak G, Mazurkiewicz JE, Baker J, Wortsman J. Characterization of corticotropin-releasing hormone (CRH) in human skin. J. Clin. Endocrinol. Metab. 1998; 83:1020–1024. [PubMed: 9506767]

Slominski A, Roloff B, Curry J, Dahiya M, Szczesniewski A, Wortsman J. The skin produces urocortin. J. Clin. Endocrinol. Metab. 2000; 85:815–823. [PubMed: 10690896]

Slominski A, Wortsman J, Pisarchik A, Zbytek B, Linton EA, Mazurkiewicz JE, Wei ET. Cutaneous expression of corticotropin-releasing hormone (CRH), urocortin, and CRH receptors. FASEB J. 2001; 15:1678–1693. [PubMed: 11481215]

Slominski A, Pisarchik A, Tobin DJ, Mazurkiewicz JE, Wortsman J. Differential expression of a cutaneous corticotropin-releasing hormone system. Endocrinology. 2004; 145:941–950. [PubMed: 14605004]

Slominski A, Zbytek B, Szczesniewski A, Semak I, Kaminski J, Sweatman T, Wortsman J. CRH stimulation of corticosteroids production in melanocytes is mediated by ACTH. Am. J. Physiol. Endocrinol. Metab. 2005a; 288:E701–E706. [PubMed: 15572653]

Slominski A, Zbytek B, Semak I, Sweatman T, Wortsman J. CRH stimulates POMC activity and corticosterone production in dermal fibroblasts. J. Neuroimmunol. 2005b; 162:97–102. [PubMed: 15833364]

Slominski A, Semak I, Zjawiony J, Wortsman J, Li W, Szczesniewski A, Tuckey RC. The cytochrome P450scc system opens an alternate pathway of vitamin D3 metabolism. FEBS J. 2005c; 272:4080–4090. [PubMed: 16098191]

Slominski A, Zbytek B, Szczesniewski A, Wortsman J. Cultured human dermal fibroblasts do produce cortisol. J. Invest. Dermatol. U. S. 2006a:1177–1178.

Slominski A, Zbytek B, Pisarchik A, Slominski RM, Zmijewski MA, Wortsman J. CRH functions as a growth factor/cytokine in the skin. J. Cell. Physiol. 2006b; 206:780–791. [PubMed: 16245303]

Slominski A, Zbytek B, Zmijewski M, Slominski RM, Kauser S, Wortsman J, Tobin DJ. Corticotropin releasing hormone and the skin. Front. Biosci. 2006c; 11:2230–2248. [PubMed: 16720310]

Slominski AT, Li W, Bhattacharya SK, Smith RA, Johnson PL, Chen J, Nelson KE, Tuckey RC, Miller D, Jiao Y, Gu W, Postlethwaite AE. Vitamin D analogs 17,20S(OH)2pD and 17,20R(OH)2pD are noncalcemic and exhibit antifibrotic activity. J. Invest. Dermatol. 2011; 131:1167–1169. [PubMed: 21228816]

Slominski AT, Zmijewski MA, Skobowiat C, Zbytek B, Slominski RM, Steketee JD. Sensing the environment: regulation of local and global homeostasis by the skin's neuroendocrine system. Adv. Anat. Embryol. Cell Biol. 2012; 212(v, vii):1–115.

Slominski AT, Zmijewski MA, Zbytek B, Tobin DJ, Theoharides TC, Rivier J. Key role of CRF in the skin stress response system. Endocr. Rev. 2013a; 34:827–884. [PubMed: 23939821]

Slominski A, Janjetovic Z, Tuckey RC, Nguyen MN, Bhattacharya KG, Wang J, Li W, Jiao Y, Gu W, Brown M, Postlethwaite AE. 20S-Hydroxyvitamin D3, noncalcemic product of CYP11A1 action on vitamin D3, exhibits potent antifibrogenic activity in vivo. J. Clin. Endocrinol. Metab. 2013b; 98:E298–E303. [PubMed: 23295467]

Slominski A, Kim TK, Zmijewski MA, Janjetovic Z, Li W, Chen J, Kusniatsova EI, Semak I, Postlethwaite A, Miller DD, Zjawiony JK, Tuckey RC. Novel vitamin D photoproducts and their precursors in the skin. Dermatoendocrinology. 2013c; 5:7–19.

Slominski A, Zbytek B, Nikolakis G, Manna PR, Skobowiat C, Zmijewski M, Li W, Janjetovic Z, Postlethwaite A, Zouboulis CC, Tuckey RC. Steroidogenesis in the skin: implications for local immune functions. J. Steroid Biochem. Mol. Biol. 2013d; 137:107–123. [PubMed: 23435015]

Slominski AT, Kim TK, Li W, Yi AK, Postlethwaite A, Tuckey RC. The role of CYP11A1 in the production of vitamin D metabolites and their role in the regulation of epidermal functions. J. Steroid Biochem. Mol. Biol. 2014; 144(Pt A):28–39. [PubMed: 24176765]

Slominski AT, Janjetovic Z, Kim TK, Wasilewski P, Rosas S, Hanna S, Sayre RM, Dowdy JC, Li W, Tuckey RC. Novel non-calcemic secosteroids that are produced by human epidermal keratinocytes protect against solar radiation. J. Steroid Biochem. Mol. Biol. 2015a; 148:52–63. [PubMed: 25617667]

Slominski AT, Li W, Kim TK, Semak I, Wang J, Zjawiony JK, Tuckey RC. Novel activities of CYP11A1 and their potential physiological significance. J. Steroid Biochem. Mol. Biol. 2015b; 151:25–37. [PubMed: 25448732]

Slominski AT, Manna PR, Tuckey RC. On the role of skin in the regulation of local and systemic steroidogenic activities. Steroids. 2015c; 103:72–88. [PubMed: 25988614]

Talabér G, Jondal M, Okret S. Extra-adrenal glucocorticoid synthesis: immune regulation and aspects on local organ homeostasis. Mol. Cell. Endocrinol. 2013; 380:89–98. [PubMed: 23707789]

Tang EK, Chen J, Janjetovic Z, Tieu EW, Slominski AT, Li W, Tuckey RC. Hydroxylation of CYP11A1-derived products of vitamin D3 metabolism by human and mouse CYP27B1. Drug Metab. Dispos. 2013; 41:1112–1124. [PubMed: 23454830]

Tieu EW, Tang EK, Chen J, Li W, Nguyen MN, Janjetovic Z, Slominski A, Tuckey RC. Rat CYP24A1 acts on 20-hydroxyvitamin D(3) producing hydroxylated products with increased biological activity. Biochem. Pharmacol. 2012; 84:1696–1704. [PubMed: 23041230]

Tongkao-On W, Carter S, Reeve VE, Dixon KM, Gordon-Thomson C, Halliday GM, Tuckey RC, Mason RS. CYP11A1 in skin: an alternative route to photoprotection by vitamin D compounds. J. Steroid Biochem. Mol. Biol. 2015; 148:72–78. [PubMed: 25448743]

Tuckey RC, Li W, Shehabi HZ, Janjetovic Z, Nguyen MN, Kim TK, Chen J, Howell DE, Benson HA, Sweatman T, Baldisseri DM, Slominski A. Production of 22-hydroxy metabolites of vitamin d3 by cytochrome p450scc (CYP11A1) and analysis of their biological activities on skin cells. Drug Metab. Dispos. 2011; 39:1577–1588. [PubMed: 21677063]

Wasiewicz T, Szyszka P, Cichorek M, Janjetovic Z, Tuckey RC, Slominski AT, Zmijewski MA. Antitumor effects of vitamin d analogs on hamster and mouse melanoma cell lines in relation to melanin pigmentation. Int. J. Mol. Sci. 2015; 16:6645–6667. [PubMed: 25811927]

Watt FM. Involucrin and other markers of keratinocyte terminal differentiation. J. Invest. Dermatol. 1983; 81:100s–103s. [PubMed: 6345687]

Wei R, Christakos S. Mechanisms underlying the regulation of innate and adaptive immunity by vitamin D. Nutrients. 2015; 7:8251–8260. [PubMed: 26404359]

Wierzbicka JM, Binek A, Ahrends T, Nowacka JD, Szydłowska A, Turczyk, Wąsiewicz T, Wierzbicki PM, Sądej R, Tuckey RC, Slominski AT, Chybicki J, Adrych K, Kmieć Z, Żmijewski MA. Differential antitumor effects of vitamin D analogues on colorectal carcinoma in culture. Int. J. Oncol. 2015; 47:1084–1096. [PubMed: 26260259]

Wierzbicka JM, Zmijewski MA, Antoniewicz J, Sobjanek M, Slominski AT. Differentiation of keratinocytes modulates expression of epidermal elements of the cutaneous analog of hypothalamus-pituitary-adrenal axis. J. Cell. Physiol. 2016

Zbytek B, Pfeffer LM, Slominski AT. Corticotropin-releasing hormone stimulates NF-kappaB in human epidermal keratinocytes. J. Endocrinol. 2004; 181:R1–R7. [PubMed: 15171702]

Zbytek B, Wortsman J, Slominski A. Characterization of a ultraviolet B-induced corticotropin-releasing hormone-proopiomelanocortin system in human melanocytes. Mol. Endocrinol. 2006a; 20:2539–2547. [PubMed: 16740657]

Zbytek B, Pfeffer LM, Slominski AT. CRH inhibits NF-kappa B signaling in human melanocytes. Peptides. 2006b; 27:3276–3283. [PubMed: 16959375]

Zbytek B, Janjetovic Z, Tuckey RC, Zmijewski MA, Sweatman TW, Jones E, Nguyen MN, Slominski AT. 20-hydroxyvitamin D3, a product of vitamin D3 hydroxylation by cytochrome P450scc, stimulates keratinocyte differentiation. J. Invest. Dermatol. 2008; 128:2271–2280. [PubMed: 18368131]

Zhu JG, Ochalek JT, Kaufmann M, Jones G, Deluca HF. CYP2R1 is a major, but not exclusive, contributor to 25-hydroxyvitamin D production in vivo. Proc. Natl. Acad. Sci. U. S. A. 2013; 110:15650–15655. [PubMed: 24019477]

Zmijewski MA, Slominski AT. CRF1 receptor splicing in epidermal keratinocytes: potential biological role and environmental regulations. J. Cell. Physiol. 2009; 218:593–602. [PubMed: 19006179]

Zmijewski MA, Slominski AT. Neuroendocrinology of the skin: an overview and selective analysis. Dermatoendocrinology. 2011; 3:3–10.

Zmijewski MA, Slominski AT. Is Mc1r an important regulator of non-pigmentary responses to UV radiation? Exp. Dermatol. 2013; 22:790–791. [PubMed: 24279915]

Zmijewski MA, Li W, Chen J, Kim TK, Zjawiony JK, Sweatman TW, Miller DD, Slominski AT. Synthesis and photochemical transformation of 3beta,21-dihydroxypregna-5,7-dien-20-one to novel secosteroids that show anti-melanoma activity. Steroids. 2011; 76:193–203. [PubMed: 21070794]

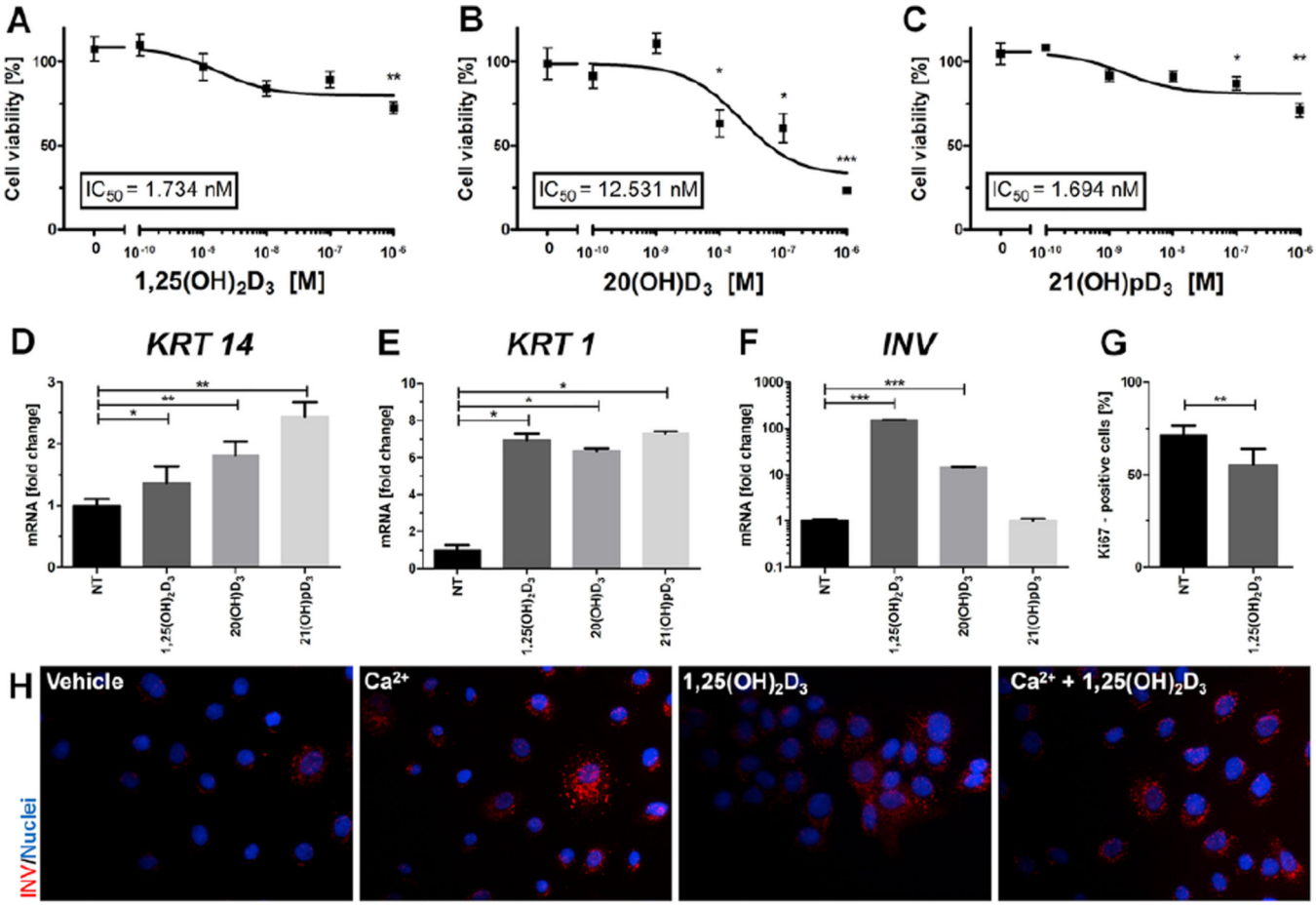

Fig. 1. $1,25(OH)_2D_3$ and it analogs inhibit cell proliferation and stimulate differentiation of primary human epidermal keratinocytes (HPEKp cell line)

Inhibition of the growth of primary human epidermal keratinocyte by $1,25(OH)_2D_3$ (A), $20(OH)D_3$ (B) and 21(OH)pD (C) was measured at 48 h using the SRB protein assay. Concentration-response curves were plotted and the IC_{50} value determined as the concentration of the secosteroid which caused a 50% decrease in cell proliferation, calculated using Graph Pad Prism 5. Real-time quantitative PCR analyses were carried out on the expression of key differentiation markers, cytokeratin 1 (*KRT1*, D), cytokeratin 14 (*KRT14*, E) and involucrin (*IVN*, F), in primary human epidermal keratinocytes stimulated with 0.1 μM $1,25(OH)_2D_3$, $20(OH)D_3$ or 21(OH)pD for 24 h. Immunofluorescence labeling for Ki67 in control and stimulated cells with $1,25(OH)_2D_3$ (0.1 μM) indicated a decrease in number of cells which entered cell cycle (G). Data are presented as means ± SD. Involucrin immunofluorescent-stained primary human epidermal keratinocytes (H) displayed increases in cell differentiation between control and stimulated cells with $1,25(OH)_2D_3$ (0.1 μM), Ca^{2+} (2.5 mM) or both. Magnification 400×. *P < 0.05, **P < 0.01, ***P < 0.001 compared to control.

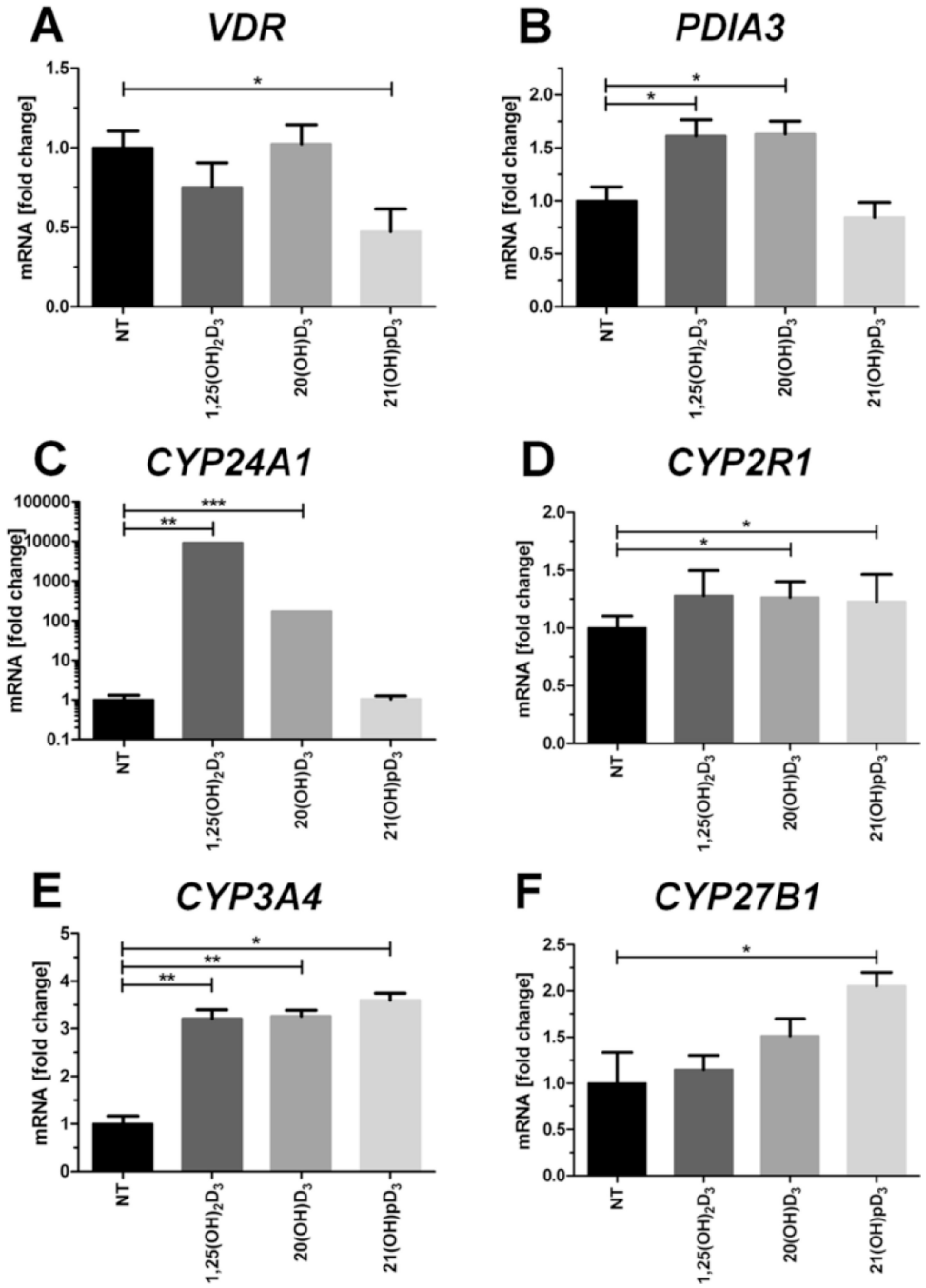

Fig. 2. $1,25(OH)_2D_3$ and it analogs modulate the expression of vitamin D-related genes in primary human epidermal keratinocytes (HPEKp cell line)

Real-time quantitative PCR analyses were carried out on the expression of key vitamin D-related genes: *VDR* (**A**), *PDIA3* (**B**), *CYP24A1* (**C**), *CYP2R1* (**D**), *CYP3A4* (**E**) and *CYP27B1* (**F**) in primary human epidermal keratinocytes stimulated with 0.1 μM $1,25(OH)_2D_3$, $20(OH)D_3$, or 21(OH)pD for 24 h. Data are presented as means ± SD. *P < 0.05, **P < 0.01, ***P < 0.001 compared to control.

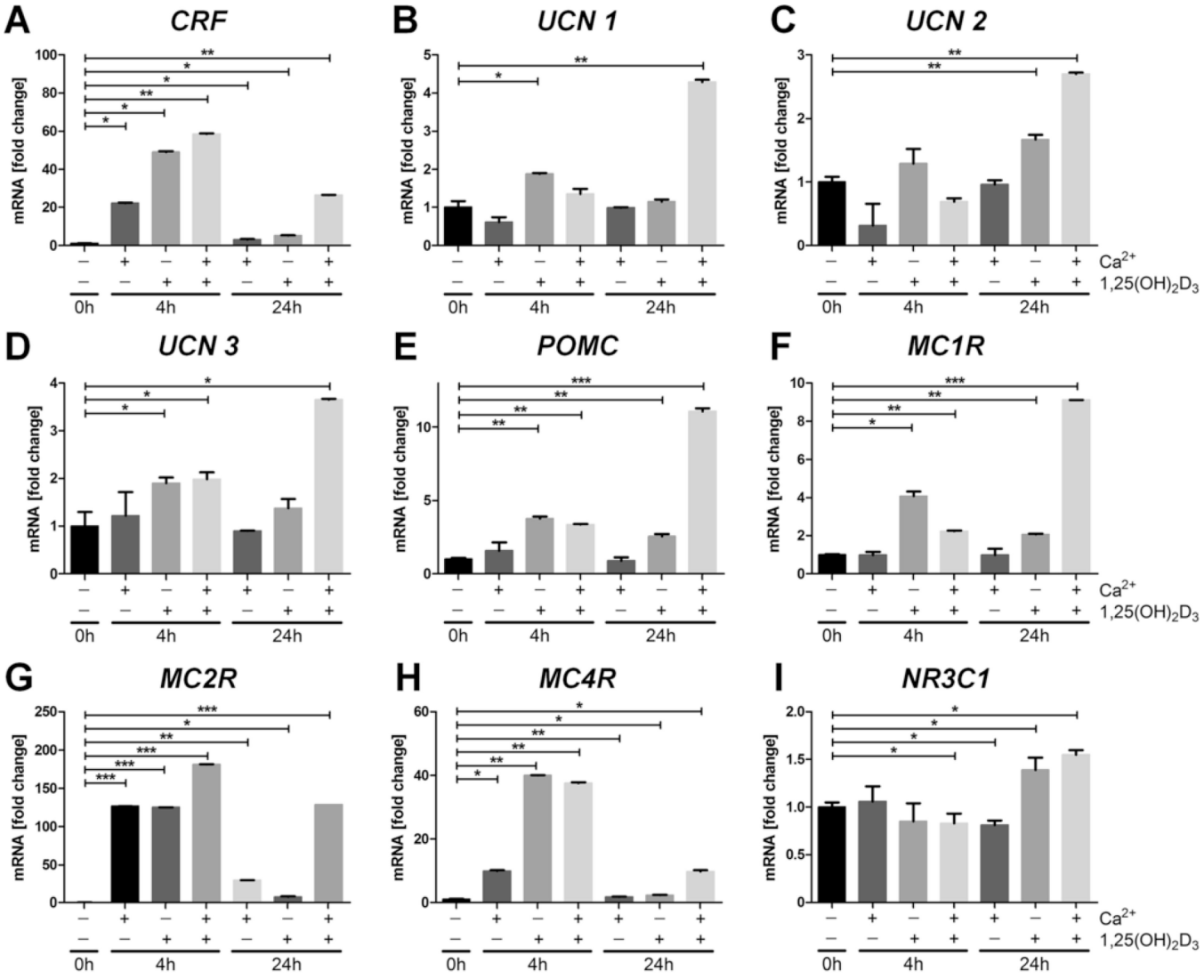

Fig. 3. $1,25(OH)_2D_3$ and calcium modulate the expression of neuropeptides in primary human epidermal keratinocytes (HPEKp cell line)

Real-time quantitative PCR analyses of neuropeptide gene expression were carried out on *CRF* (**A**), *UCN1* (**B**), *UCN2* (**C**), *UCN3* (**D**), *POMC* (**E**), *MC1R* (**F**), *MC2R* (**G**), *MC4R* (**H**) and *NR3C1* (**I**) in primary human epidermal keratinocytes stimulated with $1,25(OH)_2D_3$ (0.1 μM), Ca^{2+} (2.5 mM), or both, for 4 and 24 h. Data are presented as means ± SD. *P < 0.05, **P < 0.01 compared to control.

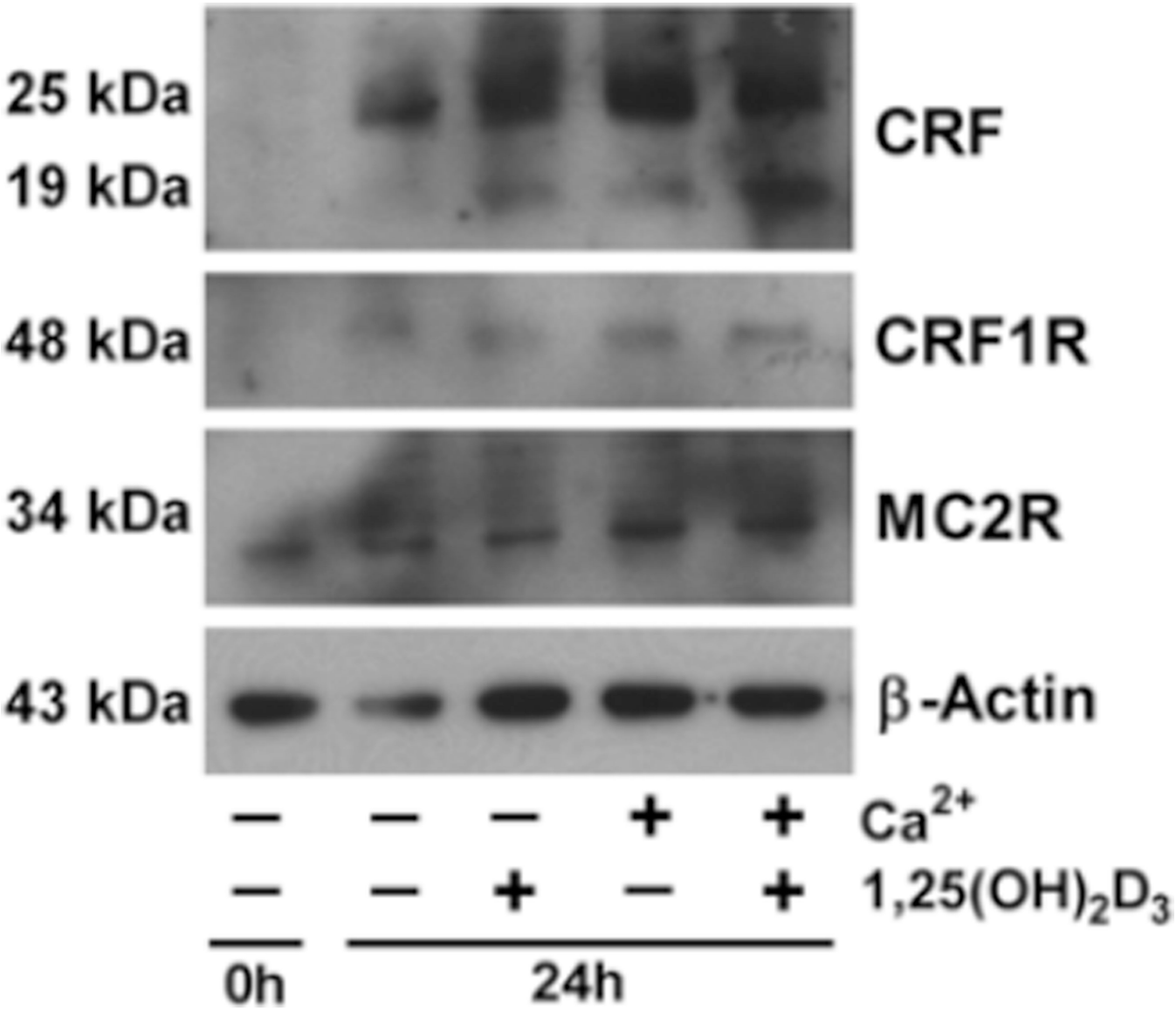

Fig. 4. Western Blot analyses of key proteins of the sHPA axis

CRF, CRFR1 and MC2R protein levels were measured in primary human epidermal keratinocytes stimulated with $1,25(OH)_2D_3$ (0.1 μM), Ca^{2+} (2.5 mM), or both, for 24 h. β-Actin levels were measured as a control.

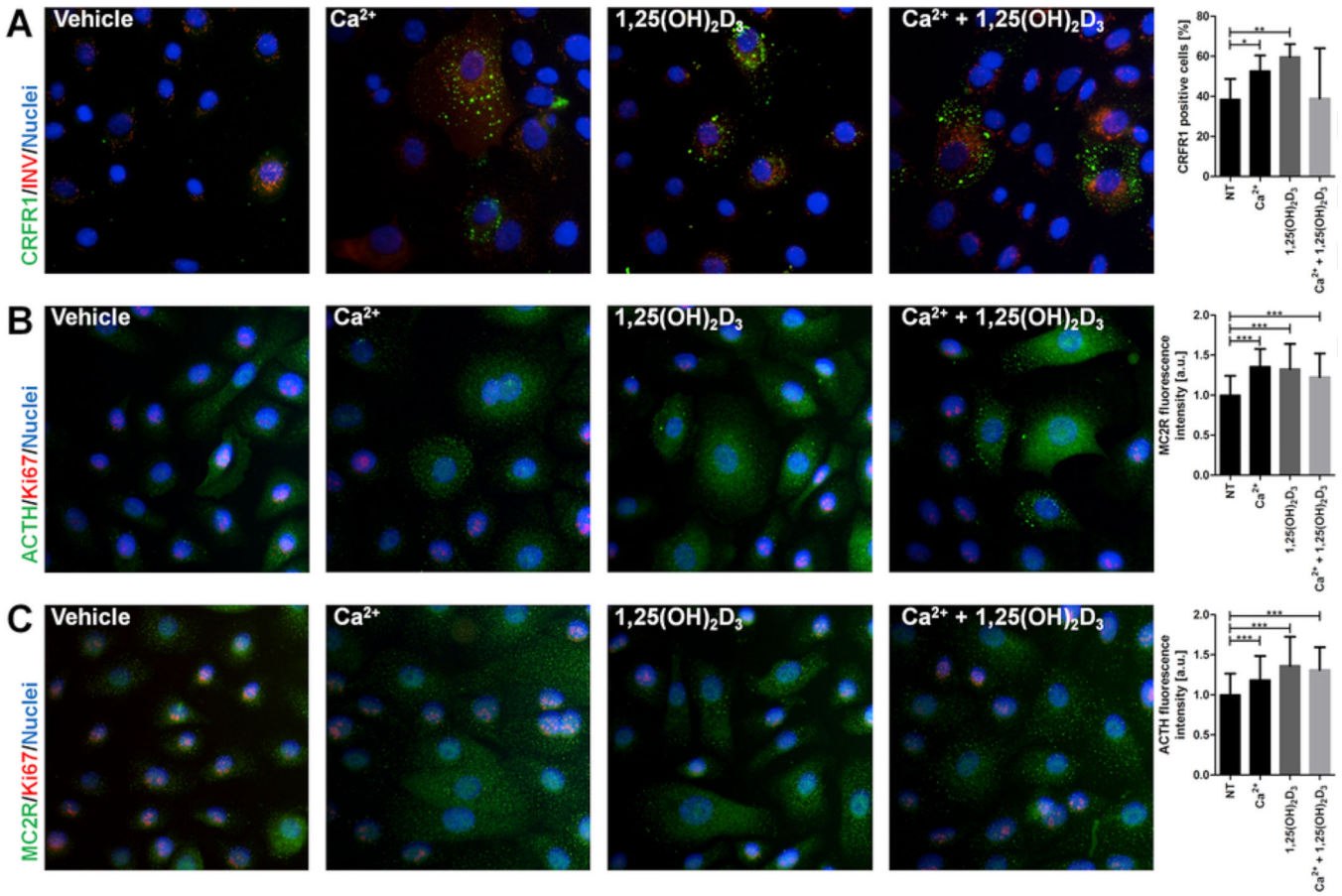

Fig. 5. 1,25(OH)$_2$D$_3$ and calcium modulate the protein level of neuropeptides and their receptors in primary human epidermal keratinocytes (HPEKp cell line)

Involucrin (red) and CRFR1 (green) immunofluorescent-stained primary human epidermal keratinocytes demonstrated increases in CRFR1 (**A**) in cells stimulated with 1,25(OH)$_2$D$_3$ (0.1 μM), Ca^{2+} (2.5 mM), or both, compared to the control. Ki67 (red) and ACTH (green) immunofluorescent stained primary human epidermal keratinocytes (**B**) demonstrated increases in ACTH in cells stimulated with 1,25(OH)$_2$D$_3$ (0.1 μM), Ca^{2+} (2.5 mM), or both, compared to control. Ki67 (red) and MC2R (green) immunofluorescent-stained primary human epidermal keratinocytes (**C**) demonstrated increases in MC1R in cells stimulated with 1,25(OH)$_2$D$_3$ (0.1 μM), Ca^{2+} (2.5 mM), or both, compared to control. Fluorescence intensity, was evaluated with the image analysis software, ImageJ (graphs on the right). Magnification 400×. Data are presented as means ± SD.*P < 0.05, **P < 0.01, ***P < 0.001 compared to control. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

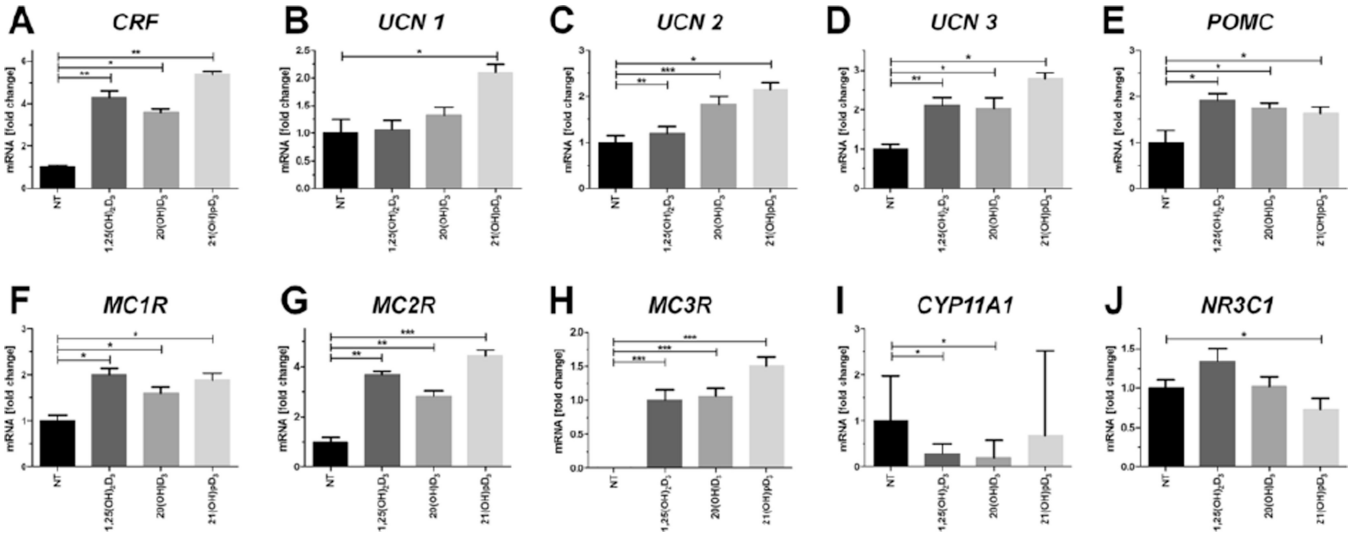

Fig. 6. 1,25(OH)$_2$D$_3$ and it analogs modulate the expression of sHPA axis elements in primary human epidermal keratinocytes (HPEKp cell line)

Real-time quantitative PCR analyses of the expression of key genes of the HPA axis were carried out for *CRF* (**A**), *UCN1* (**B**), *UCN2* (**C**), *UCN3* (**D**), *CRFR1* (**E**), *POMC* (**F**), *MC1R* (**G**), *MC2R* (**H**), *MC3R* (**I**) and *NR3C1* (**J**) in primary human epidermal keratinocytes stimulated with 0.1 μM 1,25(OH)$_2$D$_3$, 20(OH)D$_3$ or 21(OH)pD for 24 h. Data are presented as means ± SD. *P < 0.05, **P < 0.01, ***P < 0.001 compared to control.

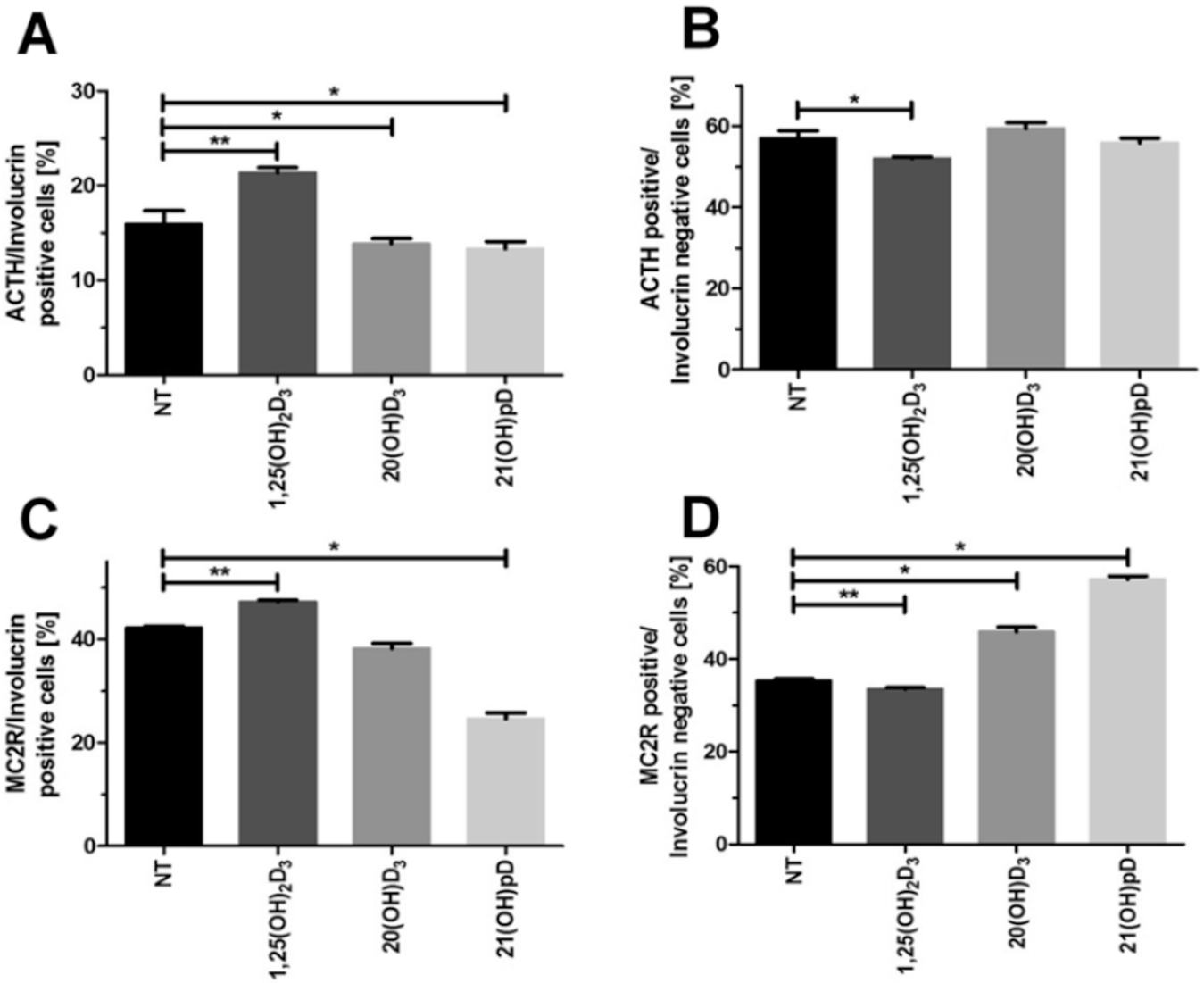

Fig. 7. $1,25(OH)_2D_3$ and it analogs selectively modulate the expression of ACTH and MC2R in differentiated and undifferentiated primary human epidermal keratinocytes (HPEKp cell line)

Flow cytometry analyses were performed on ACTH and MC2R in involucrin positive and negative primary human epidermal keratinocytes stimulated with 0.1 μM $1,25(OH)_2D_3$, $20(OH)D_3$ or 21(OH)pD for 24 h. Data are presented as means ± SD. *P < 0.05, **P < 0.01, ***P < 0.001 compared to control.

Table 1

The list of PCR primers used in the study.

Gene name	Forward primer	Reverse primer
RPL37	TTCTGATGGCGGACTTTACC	CACTTGCTCTTTCTGTGGCA
IVL	TGGGTATTGACTGGAGGAGG	CTGCCTCAGCCTTACTGTGA
KRT 1	TGACCAAGGTGGACCTTCAG	ATGATGCTGTCCAGGTCGAG
KRT 14	TCTGCAGAAGGACATTGGC	GGCCTGCTGAGATCAAAGAC
CRF	CACCCTCAGCCCTTGGATTTC	GCCCTGGCCATTTCCAAGAC
UCN 1	CAGGCGAGCGGCCGCG	CTTGCCCACCGAGTCGAAT
UCN 2	GTGTCGGCCACTGCTGAGCCTGAGAGA	ATCTGATATGACCTGCATGACAGTGGCT
UCN 3	TGCTGCTCCTGCTGCTGCTC	GTGTCCTGGCGTGGCTTTCCC
POMC	GAGGGCAAGCGCTCCTACTCC	GGGGCCCTCGTCCTTCTTCTC
MC1R	ACTCACCCATGTACTGCTTC	TACAGCACGGCCATGAGCAC
MC2R	GACTGTCCTCGTGTGGTTTTG	GGCTGCCCAGCATATCAGAT
NR3C1	GAGACCAGATGTAAGCTCTCCT	GCAATCATTCCTTCCAGCAC
VDR	CCAGTTCGTGTGAATGATGG	GTCGTCCATGGTGAAGGA
PDIA3	CTCCGACGTGCTAGAACTCA	CAGGTGTTAGTGTTGGCAGT
CYP24A1	GCAGCCTAGTGCAGATTT	ATTCACCCAGAACTGTTG
CYP2R1	AGAGACCCAGAAGTGTTCCAT	GTCTTTCAGCACAGATGAGGTA
CYP3A4	AAGGCACCACCCACCTATGATACT	TACTTTGGGTCACGGTGAAGAGCA
CYP27B1	TGTTTGCATTTGCTCAGA	CCGGGAGAGCTCATACAG

Table 2

The list of antibodies used in the study.

Primary antibodies				
Antigen	**Isotype**	**Dilution**	**Supplier**	**Cat. #**
Involucrin	Mouse	1:100	Novocastra	NCL-INV
Ki67	Mouse	1:100	Novocastra	NCL-Ki67-MM1
CRF	Goat	1:100	Santa Cruz Biotechnology	sc-21675
CRFR1	Goat	1:200	Abcam	ab77686
ACTH		1:500	Dr. Allen, USA	
MC2R	Goat	1:500	Abcam	ab77347
GR	Rabbit	1:100	Santa Cruz Biotechnology	sc-8992

Secondary antibodies					
Antigen	**Isotype**	**Dilution**	**Conjugate**	**Supplier**	**Cat. #**
Anti-Goat IgG	Donkey	1:500	Alexa Fluor® 488	Life Technologies	A11055
Anti-Rabbit IgG	Goat	1:500	Alexa Fluor® 488	Life Technologies	A11008
Anti-Mouse IgG	Donkey	1:500	Alexa Fluor® 594	Life Technologies	A21203