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Evidence for involvement of non-classical pathways in the protection from UV-induced DNA damage by vitamin D-related compounds

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ABSTRACT

The vitamin D hormone, 1,25dihydroxyvitamin D_3 ($1,25(OH)_2D_3$), and related compounds derived from vitamin D_3 or lumisterol as a result of metabolism via the enzyme CYP11A1, have been shown, when applied 24 hours before or immediately after UV irradiation, to protect human skin cells and skin from DNA damage due to UV exposure, by reducing both cyclobutane pyrimidine dimers (CPD) and oxidative damage in the form of 8-oxo-7,8-dihydro-2'- deoxyguanosine (8-OHdG). We now report that knockdown of either the vitamin D receptor or the endoplasmic reticulum protein ERp57 by siRNA abolished the reductions in UV-induced DNA damage with 20-hydroxyvitamin D_3 or 24-hydroxylumisterol$_3$, as previously shown for $1,25(OH)_2D_3$. Treatment with $1,25(OH)_2D_3$ reduced oxygen consumption rates in UV-exposed and sham-exposed human keratinocytes and reduced phosphorylation of CREB (cyclic AMP response binding element protein). Both these actions have been shown to inhibit skin carcinogenesis after chronic UV exposure, consistent with the anticarcinogenic activity of $1,25(OH)_2D_3$. The requirement for a vitamin D receptor for the photoprotective actions of $1,25(OH)_2D_3$ and of naturally occurring CYP11A1-derived vitamin D related compounds may explain why mice lacking the vitamin D receptor in skin are more susceptible to UV-induced skin cancers, whereas mice lacking the 1α-hydroxylase and thus unable to make $1,25(OH)_2D_3$ are not more susceptible.

KEYWORDS: 1α,25-dihydroxyvitamin D_3; lumisterol; UV-induced DNA damage; vitamin D receptor; ERp57; CREB phosphorylation; oxidative phosphorylation.

Introduction

Vitamin D3 is primarily made in skin through the absorption of UVB photons by 7-dehydrocholesterol, opening the B-ring of the sterol to form pre-vitamin D3. At body temperature, pre-vitamin D3 isomerizes to vitamin D3 (1). However, the same sunlight exposure which produces vitamin D3 also causes several types of DNA damage in skin cells (2, 3). Some DNA bases directly absorb photons. Upon UV absorbance, adjacent pyrimidine bases of the same DNA strand form dimeric photolesions (4, 5). The most prevalent UV-induced lesions are the cis-syn cyclobutane pyrimidine dimers (CPD), mostly formed between the 5-6 bonds of adjacent thymine and cytosine pyrimidines (6). The commonest forms of CPD are thymine dimers, which are present in numbers proportional to total CPD of all types (7). Although thymine dimers (even if not properly repaired) are not in theory mutagenic, they have been shown to cause mutations in practice (8, 9). Thymine-cytosine dimers and cytosine-cytosine dimers are highly mutagenic if not correctly repaired by nucleotide excision repair (9).

Indirect DNA damage or oxidative damage to purine bases was shown to contribute to mutagenesis, cancer, aging and other pathological conditions (10). The main endogenous agents which cause this damage are free radicals such as reactive oxygen species (ROS) (11) and reactive nitrogen species (RNS) (12). Oxidative DNA damage, with free radicals targeting guanine, produces the main photolesion, 8-oxo-7,8-dihydro-2'- deoxyguanosine (8-oxoguanine/8-OHdG) (13). 8-OHdG is used as a biomarker for DNA damage by oxidative stress. Oxidative stress may cause non-canonical base

pairing and incorrect pairing by DNA polymerase, leading to DNA damage (8, 9), mutations (9, 14) and contributing to photocarcinogenesis (15).

Pre-vitamin D3 and vitamin D3 are not the only vitamin D compounds made in skin. Vitamin D3 is metabolized in skin cells through 25-hydroxyvitamin D (25(OH)D) to the active hormone, 1,25-dihydroxyvitamin D3 ($1,25(OH)_2D_3$) (16, 17). Vitamin D3 in skin can also be metabolized by the enzyme CYP11A1 (18, 19), which increases in expression after exposure to UV (20), to produce 20-hydroxyvitamin D_3 ($20(OH)D_3$) and other compounds (Figure 1) (21-23). Furthermore, continued absorption of UV by pre-vitamin D3 produces so called, "overirradiation products" including the major product, lumisterol (24). Lumisterol can also be metabolised by CYP11A1 to produce several hydroxylated metabolites including 24-hydroxylumisterol$_3$ ($24(OH)L_3$) (Figure 1) (25, 26).

Sunlight produces both vitamin D3 and DNA damage. While DNA damage occurs relatively quickly, from immediately to a few hours (27, 28), production of vitamin D3 takes many hours (1). This gives a window of opportunity to test whether vitamin D compounds added topically, either before, or in most cases immediately after UV, could reduce UV-induced DNA damage. Many studies, including studies carried out with Tony Norman, reported that the vitamin D hormone, $1,25(OH)_2D_3$, reduced UV-induced DNA damage in skin cells, skin explants and in human subjects (28-38). Vitamin-D like compounds such as $20(OH)D_3$ and the synthetic compound 1,25-dihydroxylumisterol$_3$, synthesized by Bill Okamura in Tony Norman's group (39), also have been shown to reduce UV-induced CPD in separate studies (33, 34, 38, 40-43). It seems likely then, that vitamin D compounds, synthesized in skin as a result of sunlight exposure contribute to protection of DNA in skin cells from the next exposure to UV. This skin adaptation is similar in timing to the development of a thickened stratum corneum (44), the outer layer of skin which attenuates UV

penetration (45), and pigmentation (tan), both of which contribute to reduced UV damage and photocarcinogenesis (46, 47).

Indeed, in mice, topical application of $1,25(OH)_2D_3$ immediately after UV exposure over 10 weeks, significantly reduced skin tumour development over the subsequent 30 weeks (34). $1,25(OH)_2D_3$ normally requires the vitamin D receptor (VDR), a member of the steroid hormone receptor superfamily, to effect changes in cellular function (48, 49). In support of the hypothesis that $1,25(OH)_2D_3$ contributes to skin adaptation to sunlight exposure, mice with a non-functional VDR developed more skin tumours than wild-type mice after UV exposure (50) or after oral administration of a chemical carcinogen (51). Somewhat surprisingly, mice with ablation of CYP27B1, the 1α-hydroxylase that converts 25-hydroxyvitamin D to $1,25(OH)_2D_3$, are not more susceptible to UV-induced skin tumours (52). This raises the likelihood that other vitamin D compounds made in skin (Figure 1) may also contribute to protection of DNA from UV exposure. There is some evidence to support this proposal (38, 41-43, 53), but more is needed.

$1,25(OH)_2D_3$ binds to the VDR, which acts principally as a modifier of transcription in the nucleus (54). Tony Norman's group was the first to demonstrate a non-classical pathway of $1,25(OH)_2D_3$ response: a rapid intestinal calcium uptake response to $1,25(OH)_2D_3$ was shown to be via plasma membrane-associated VDR and/or endoplasmic reticulum protein, ERp57, a member of the protein disulphide isomerase family (PDIA3) (55-59). The receptor has been called 1,25D3-MARRS (Membrane Associated Rapid Response Steroid binding) (60-62). This non-classical pathway was also shown to be present in bone cells (59, 63, 64).

In collaboration with Tony Norman, we published evidence that, at least in part, the protective effect of $1,25(OH)_2D_3$ to reduce UV-induced CPD depends on both VDR and ERp57 (65). These earlier studies in skin cells from patients with Hereditary Vitamin D Resistant Rickets type 1, due to anomalies in the VDR (66-69), showed that $1,25(OH)_2D_3$ did not reduce CPD in fibroblasts from a patient with an early stop codon in the *VDR* gene, but was effective in reducing CPD in fibroblasts from patients with mutations in either the DNA binding domain or the ligand binding domain (65). Treatment of skin fibroblasts with a neutralizing antibody (which would not be expected to pass across the cell membrane) to ERp57 or siRNA to ERp57 also abolished the protective effect of $1,25(OH)_2D_3$ on CPD after UV (65). Immunoprecipitation experiments on non-nuclear fractions of these skin cells revealed a VDR-ERp57 complex. Furthermore, the use of DIDS (4,40-diisothiocyanatostilbene-2,20-disulfonic acid), a chloride channel blocker shown to prevent $1,25(OH)_2D_3$-induced chloride currents in osteoblasts (70), also blocked the reduction in post-UV CPD by $1,25(OH)_2D_3$ (37).

But there are many unknowns. While $20(OH)D_3$ and its metabolites derived from vitamin D3 via CYP11A1 reduce both CPD and oxidative DNA damage in the form of 8-OHdG (38, 42, 71), there is a shortage of similar information on the CYP11A1 derivatives of pre-vitamin D, such as $24(OH)L_3$. Recent studies, however, have demonstrated a photoprotective role for CYP11A1-derived hydroxylumisterol compounds in human keratinocytes (71, 72). In addition, while a non-functional VDR or knockdown of ERp57 reduced UV-induced CPD in skin fibroblasts, it is not known whether knockdown of VDR or ERp57 abolishes the protection against UV-induced CPD by $1,25(OH)_2D_3$ in keratinocytes, the main epidermal cell type. Nor is there data on whether VDR or ERp57 are also required for protection by $1,25(OH)_2D_3$ against oxidative DNA damage or whether any protection against DNA damage by CYP11A1-derived metabolites of vitamin D3, like $20(OH)D_3$ or of pre-vitamin D3, like $24(OH)L_3$, requires VDR and/or ERp57. Furthermore, evidence has accumulated for

vitamin D hydroxyderivatives acting on alternative nuclear receptors including retinoic acid orphan receptor (ROR)α and γ (73), aryl hydrocarbon receptor (AhR) (74) and liver X receptors (LXR) (75). These questions and the possibility that $1,25(OH)_2D_3$ affects some keratinocyte functions known to play a role in photocarcinogensis were tested in the current study.

Methods

Vitamin D compounds: $24(OH)L_3$ and $20(OH)D_3$ were enzymatically synthesised using recombinant CYP11A1, procedures described previously (21, 26). Other compounds were purchased; $1,25(OH)_2D_3$ (Sapphire Bioscience Pty Ltd, Sydney, Australia). All compounds were dissolved in spectroscopic grade ethanol (Merck, Darmstadt, Germany) and stored under argon gas at -80 °C. The absorbance of these compounds was determined by Nanodrop ND-1000 Spectrophotometer and concentration was calculated using Beer-Lambert law. The final concentration of ethanol vehicle used in experiments was 0.1% v/v.

Solar simulated irradiation: Cells were exposed to solar simulated UV radiation (ssUV), provided by Oriel Sol1A^{TM} 94042A 450W solar simulator (Newport Corporation, California, USA) with an inbuilt attenuation filter to eliminate UVC. UVA and UVB output was calibrated using a OL756 spectroradiometer (Optronic Laboratories, Gooch & Housego, Florida, USA) as previously described (37). The UV dose was determined from previous studies to cause reasonable levels of DNA damage in human keratinocytes, but was not high enough to result in a large increases in apoptosis (36, 76). Irradiation was carried out as previously described (37). All treatments with vitamin D compounds were added immediately after exposure to UV.

Keratinocyte culture: Legal parents or guardians gave written informed consent for collection of skin from circumcision under a protocol approved by the University of Sydney Human Ethics Committee (Reference number 2015/063). Keratinocytes, (passages 1–5) from at least two independent donors were used in all experiments. Keratinocytes were cultured from skin samples and used for studies as previously described (37). Keratinocytes at passage 2-5 were used for experiments.

Small interfering RNA (siRNA) transfection: Opti-MEM™ Reduced Serum Medium powder (22600134) (Thermofisher Scientific, Massachusetts, USA), small interfering RNA (siRNA) transfection reagent, non-directed siRNA sequence (siCTRL RNA) and small interfering RNA targeted to VDR (siVDR), small interfering RNA targeted to ERp57 (siERp57), were all from Santa Cruz Biotechnologies, Texas, USA. Cell culture media was replaced with 100 µl Opti-MEM™ serum free media (Thermofisher Scientific, Massachusetts, USA) and incubated at 37 °C for 5-10 minutes. For VDR, ERp57 knockdown, cells were transfected with 50 mM siVDR, siERp57 or siControl RNA diluted in siRNA transfection medium/ Opti-MEM™ serum free media (Santa Cruz Biotechnologies, CA, USA) in the presence of the recommended concentration of lipid-based siRNA transfection reagent (Santa Cruz Biotechnologies) according to the manufacturer's instructions, and as previously described (65).

Western blots used protein lysates from keratinocytes seeded in 6-well plates at a density of 500,000 cells/well as previously described (78). Additional antibodies used for protein detection were anti-ERp57 (mouse monoclonal, Santa Cruz Biotechnologies), anti-VDR (mouse monoclonal, Santa Cruz Biotechnologies), anti-phospho-CREB-Ser^{133} (mouse monoclonal, Cell Signaling Technology, MA, USA) and anti-tubulin at 1µg/ml (mouse monoclonal, Santa Cruz Biotechnologies).

Immunochemistry. Immunohistochemistry: Cells were incubated for 3 hours at 37°C, based on previous studies (34, 37), followed by fixation with ice-cold 100% methanol at -20°C for 5 minutes and washed 3 times with MiliQ water (Mili-Q integral water purification system®, Millipore SAS, Molsheim, France). Cells were left to air-dry overnight before staining for DNA damage in the form of thymine dimers as an index of cyclobutane pyrimidine dimers (CPDs) (79) or 8-hydroxydeoxyguanosine (8-OHdG), as previously described (27). Briefly, cells were first treated with 1% H2O2 (v/v in PBS) for 5 minutes to block endogenous peroxidase activity, while covered in foil, followed by 3 washes with MiliQ water. Antigen retrieval involved nuclear DNA denaturation with 70mM NaOH diluted in 70% ethanol, followed by aspiration and proteolytic digestion with Proteinase K (Final concentration: 1µg/ml in 0.1mM CaCl2) at room temperature for 5 minutes for CPD or 10 minutes at 37°C for 8-OHdG. The wells were gently washed with MiliQ water twice. Non-specific staining was blocked with 50% horse serum (v/v in PBS) (Sigma-Aldrich, Missouri, USA). Immunohistochemistry was performed using the anti-thymine dimer antibody (mouse monoclonal clone H3, Sigma-Aldrich, Missouri, USA) at 5 µg/ml or the 8-OHdG antibody (mouse monoclonal lgG2b, clone 15A3, Santa Cruz, California, USA) at 2 µg/ml overnight at 4°C, followed by incubation at room temperature with goat anti-mouse IgG F(ab')2 secondary antibody, biotin

conjugate at 1:500 (Thermo Fisher Scientific, Massachusetts, USA) for 20 minutes at room temperature, and HRP-Streptavidin conjugate at 1:150 (Invitrogen, California, USA) for 15 minutes at room temperature. Isotype control was performed with mouse lgG instead of primary antibody. All steps were separated by washing stesp with Tween-PBS (PBST) for 3 times. HRP substrate diaminobenzidine (DAB) (Enhanced Liquid substrate System for Immunohistochemistry, Sigma--Aldrich, Missouri, USA) was applied for 5 minutes to visualize the staining of DNA damage. Coverslips were rinsed with MiliQ water and mounted with Entellin (Merck KGaA, Darmstadt, Germany) onto glass slides after being air-dried.

Mouse monoclonal IgG1 ERp57 antibody and mouse monoclonal IgG2a VDR antibody (both from Santa Cruz Biotechnologies, California, USA) were used for immunohistochemistry of the VDR and ERp57. Cells were fixed with 10% (v/v) formaldehyde in PBS for 5 minutes at room temperature and permeabilized with 0.1% (v/v) Triton-X in PBS for 3 minutes at room temperature. Endogenous peroxidase activity was blocked by 6% (v/v) H2O2 in PBS for 10 minutes. Nonspecific antibody binding was inhibited by 10% (v/v) horse serum (blocker) in PBS for 1 hour. VDR and ERp57 antibodies in 10% horse serum at 2.5 μg/ml concentration were incubated with the cells overnight at 4 °C in blocker. Isotype control antibodies were used under similar experimental concentrations and conditions. Cells were rinsed with PBS five times and incubated with anti-mouse IgG HRP-linked antibody in blocker for 1 hour at room temperature. Cells were rinsed with PBS, incubated with DAB and rinsed with Mili-Q water and mounted as described above for analysis.

Brightfield images were taken on the Stereo Investigator Scope (MBF Bioscience, Vermont, USA) or Zeiss-Axioscan light microscope (Zeiss, Oberkochen, Germany) at the Bosch Advanced Microscopy Facility at The University of Sydney. The image was thresholded in ImageJ imaging software (National Institute of Health, Maryland, USA) against the isotype control which showed low staining as previously reported (Dixon,2008). Thus, the threshold was set based on the non-irradiated sample (SHAM) to pick up minimum mean gray value. Mean gray value is the average gray value within the selected area, calculated as the sum of the gray values of all the pixels divided by the number of pixels. The set threshold was applied to all the images. The average of the mean gray value for each coverslip was obtained from 10 fixed-area regions, where cells were well attached, as previously reported (Rybchyn et al.,2018).

These immunohistochemical methods for determining CPD and 8-OHdG after UV have been validated with COMET assays, which use specific endonucleases to detect CPD or 8-OHdG followed by single cell electrophoresis to detect DNA strand breaks (27, 37).

Seahorse energetics: The cellular oxygen consumption rates (OCR) were determined in an XF96 Extracellular Flux Analyzer (Seahorse Bioscience, Agilent, CA, USA) immediately following irradiation or sham irradiation of cells. $1,25(OH)_2D_3$ at 10 nM (or vehicle) was injected immediately after irradiation (earliest possible); N=22-24 per treatment. Data analysed using Wave 2.3 (Seahorse Bioscience) and Prism (GraphPad Software, CA, USA) (36)

Reactive oxygen species (ROS) detection: ROS levels were measured using the ROS-Glo™ H_2O_2 assay (Promega) as previously described (36).

Tunicamycin studies: Tunicamycin (Sigma-Aldrich, Missouri, USA), an N-linked glycosylation inhibitor, was added into the medium at 1 μg/ml, based on previous optimization experiments, and incubated with cells for 48 h. This was followed by ssUV irradiation and processing for CPD or 8-OHdG at 3 h, as described above.

Statistical analysis: Keratinocyte experiments were performed in triplicate or as otherwise indicated and experiments were repeated at least twice with different tissue donors. Data were graphed and analysed with use of GraphPad Prism software (GraphPad Software, California, USA). Unless stated otherwise, results are presented as mean + standard error of the mean (SEM) and significance between treatment groups was analysed with one-way ANOVA with Tukey post-test (36).

Results:

Like $1,25(OH)_2D_3$ and $20(OH)D_3$ (38, 41), $24(OH)L_3$ reduced UV-induced CPD and 8OHdG in the nuclei of human primary keratinocytes in a concentration-dependent manner (Figure 2).

To test whether the VDR or ERp57 was required for the reduction in CPD or oxidative DNA damage by $1,25(OH)_2D_3$ or $24(OH)L_3$, cells were transfected with small interfering RNA (siRNA) targeted to VDR mRNA (siVDR) or with siRNA to ERp57 (siERp57) or with a non-directed siRNA

sequence (siCTRL). VDR or ER-p57 knockdown by siRNA was verified by western blot (Figure 3A for VDR or 3C for ERp57) and, as an additional control, by immunohistochemistry and image analysis for VDR or ERp57 (Figures 3B, D). Significant reductions in VDR protein expression were observed in cells transfected with siVDR compared to siCTRL (***p<0.001) (Figure 3B), similar to western blot results (Figure 3A), but there were no significant differences observed in ERp57 expression between either siVDR or siCTRL samples. Likewise, significant reductions in ERp57 protein expression were observed in cells transfected with siERp57 compared to siCTRL (***p<0.001) (Figure 3D), similar to western blot results (Figure 3C), but there were no significant differences observed in VDR expression between either siERp57 or siCTRL samples.

Keratinocytes were exposed to the solar simulator 48 hours after siRNA transfection and immediately treated with $1,25(OH)_2D_3$, $20(OH)D_3$ or $24(OH)L_3$ at 10^{-9} M. After 3 hours, significant reductions in UV-induced CPD (Figure 4A) or 8-OHdG (Figure 4B) resulted from these treatments in cells treated with siCTRL (***p<0.001). Reductions of either UV-induced CPD or 8-OHdG with any of these compounds were abolished in the presence of siVDR or siERp57 (Figure 4A, B).

In a separate set of experiments, keratinocytes were treated with tunicamycin, an N-glycosylation inhibitor (79), at 1μg/mL for 48 h. The aim of those experiments was to abolish cell surface expression of the Calcium Sensing Receptor (CaSR) (80), which was achieved, in order to test modulators of CaSR. In the experiments, the vitamin D hormone, $1,25(OH)_2D_3$, was used as a positive control for photoprotection. Rather surprisingly, treatment of keratinocytes with tunicamycin abolished the $1,25(OH)_2D_3$-induced reductions in UV-induced CPD and 8OHdG (Figure 5).

UV exposure causes mitochondrial damage (36, 81), which reduces oxygen consumption rates as measured by SeaHorse analysis (Figure 6A, B). In contrast, compounds which reduce oxygen consumption rates without causing mitochondrial damage have been characterized as anti-carcinogenic (82). Oxygen consumption rates (OCR), a measure of mitochondrial oxidative phosphorylation, were lower in keratinocytes exposed to $1,25(OH)_2D_3$ compared with vehicle (Figure 6A). At 90 minutes after UV, OCR were significantly lower in keratinocytes treated with $1,25(OH)_2D_3$ (from time shown in arrow), whether SHAM irradiated or exposed to UV (Figures 6A, B). Of interest is the observation that treatment with oligomycin, which inhibits oxidative phosphorylation, did not affect the reduction in CPD (Figure 6C) or 8OHdG (not shown) caused by

the addition of $1,25(OH)_2D_3$. Production of reactive oxygen species (ROS) was significantly increased by UV exposure and significantly reduced in either SHAM or UV exposed keratinocytes, in the presence of $1,25(OH)_2D_3$.

Increased phosphorylation of CREB (cyclic AMP response element binding protein) is a recently identified marker of (photo)carcinogenic agents (83). As shown in Figure 7, CREB phosphorylation is increased by UV exposure in human keratinocytes, but decreased in UV-exposed keratinocytes by $1,25(OH)_2D_3$.

Discussion:

Several studies reported the photoprotective effect of $1,25(OH)_2D_3$ in reducing UV-induced DNA damage from *in vitro*, *in vivo* and *ex vivo* studies (reviewed in (84-86). Compounds derived from vitamin D or the "over-irradiation" product lumisterol have also been reported to reduce UV-induced CPD in keratinocytes (32, 33, 71, 72) and in mice (34, 38). We show here for the first time that the CYP11A1 derivative of lumisterol $24(OH)L_3$, reduces oxidative damage as well as CPD, provide a concentration response curve for $24(OH)L_3$ in comparison with $1,25(OH)_2D_3$ and also show for the first time that this compound and $20(OH)D_3$, like $1,25(OH)_2D_3$ reduces DNA damage in human keratinocytes when added immediately after UV exposure. Previous studies in collaboration with Tony Norman reported that both VDR and the endoplasmic reticulum protein, ERp57 are required for reduction of UV-induced CPD by$1,25(OH)_2D_3$ (65), but the receptors through which $1,25(OH)_2D_3$ reduces oxidative DNA damage and the receptors required by vitamin-like compounds to reduce UV-induced photo lesions were not known. The current study showed for the first time that both VDR and ERp57 are indeed required for reductions in oxidative DNA damage by $1,25(OH)_2D_3$ and for the reduction in both types of UV-induced DNA damage by the CYP11A1 metabolites, $20(OH)D_3$ and $24(OH)L_3$. The absolute requirement for VDR for the reduction in DNA damage, together with the apparent ability of these vitamin D-like compounds derived from vitamin D or lumisterol to reduce UV-induced DNA damage, go some way towards explaining why knock-down of the VDR leads to increased susceptibility to photocarcinogenesis (50). In contrast, lack of the 1α-hydroxylase does not apparently increase susceptibility to UV-induced skin tumors (52, 87).

Agents such as 1,25-dihydroxylumisterol$_3$, 20(OH)D$_3$ and other vitamin D compounds made in skin (18, 19, 21, 38, 88), which have been shown to reduce UV-induced CPD (32, 38, 41, 42), generally have reduced binding to the G-pocket of the VDR in comparison to 1,25(OH)$_2$D$_3$ (58, 89). They interact with the VDR, however, and the addition of OH at C1α modifies such interaction (90-92). In this context, it is worth noting that some VDR, but not necessarily a fully functional VDR, seems to be required to enable 1,25(OH)$_2$D$_3$ to reduce CPD after UV, since this protective effect was shown with VDR which had a mutated ligand-binding domain or a mutation in the DNA binding domain (65).

In that study, we demonstrated the involvement of the endoplasmic reticulum stress protein, ERp57 (also known as PDIA3 or MARRS (93)) in the photoprotective response to 1,25(OH)$_2$D$_3$ (65). As well as siRNA to the protein, a neutralizing antibody to ERp57, which would not enter the cell, abolished the response, supporting the idea of a membrane position for this protein for this function (94). It is likely that the VDR was also present in the cell membrane as shown by the Norman group (57). Co-IP studies of non-nuclear fractions of human primary fibroblasts showed that ERp57 co-immunoprecipitated with VDR and VDR with ERp57 (65).

The global importance of ERp57 was demonstrated in ERp57 -/- embryos where ERp57 deficiency was lethal at embryonic day 13.5, while embryos at embryonic day 12.5 were reported to be much smaller than the wild type embryos (95). In the current study, in the presence of siERp57, which significantly reduced ERp57 expression, the reduction of UV-induced CPD and 8-OHdG by 1,25(OH)$_2$D$_3$, 20(OH)D$_3$ and 24(OH)L$_3$ was also significantly reduced. Further studies of the role of ERp57 in photoprotection will require the use of animals which have a conditional knock-out of this protein in the epidermis.

The current study also showed that when keratinocytes had been incubated with tunicamycin, a known inhibitor of glycosylation (79), the protection by 1,25(OH)$_2$D$_3$ against UV-induced CPD was abolished and that against 8-OHdG impaired. The explanation for this finding remains unclear and warrants further investigation. In that set of experiments, the tunicamycin was actually used to inhibit glycosylation of the calcium-sensing receptor in order to prevent its movement to the cell membrane. The 1,25(OH)$_2$D$_3$ was included as a positive control for DNA damage reduction. Glycosylation is not apparently a feature of post-translational modification of VDR, though slightly higher molecular weight bands have been reported in some tissues (96), which could be a result of post-translational modification (97). These authors reported hyperglycemia-induced enzyme mediated glycosylation

(OGlcNAcylation) of VDR (97). Tunicamycin competes with N-acetylglucosamine phosphotransferase to inhibit N-glycoslyation (79) and would not be expected to inhibit OGlcNAcylation. A search of the literature revealed no evidence of glycosylation of ERp57. Nevertheless, it is possible that tunicamycin interferes with some chaperone protein important for translocating either VDR or ERp57 to the plasma membrane.Testing this proposal was beyond the scope of the current study, but the observations support, at least in part, the hypothesis that membrane associated VDR and/or ERp57 is important for some aspects of photoprotection. Tunicamycin also triggers endoplasmic reticulum stress (98) and is thus is likely have an effect on a typical stress marker, such as ERp57 (99).

As noted above, $1,25(OH)_2D_3$ reduced UV-induced CPD in fibroblasts with DNA binding domain mutations (65). $1\alpha,25(OH)_2$-lumisterol$_3$ has little ability to modulate gene transcription, but was shown to initiate non-genomic action with a similar potency to $1,25(OH)_2D_3$ (58, 100). $1\alpha,25(OH)_2$-lumisterol$_3$, similar to $1,25(OH)_2D_3$, reduced UV-induced CPD in human primary fibroblasts and keratinocytes (29, 32) and reduced photocarcinogenesis in Skh:hr1 mice, though not to the extent seen with $1,25(OH)_2D_3$ (34). All these data and the involvement of ERp57 support the proposal that photoprotection by $1,25(OH)_2D_3$ and other vitamin D-like compounds at concentrations similar to $1,25(OH)_2D_3$ signal, at least in part, by non-genomic pathways. The chloride channel inhibitor, DIDS (4,40-diisothiocyanatostilbene-2,20-disulfonic acid), inhibited UV-induced CPD reduction by $1,25(OH)_2D_3$ and $20(OH)D_3$, further supporting involvement of a non-classical vitamin D pathway in the reduction of UV-induced DNA lesions by these vitamin-D like compounds (37, 101).

Some of the actions of $1,25(OH)_2D_3$ shown here that could contribute to protection from UV damage and subsequent photocarcinogenesis seem unlikely to be mediated by changes in gene transcription. These include suppression by $1,25(OH)_2D_3$ of otherwise upregulated phosphorylation of AKT (acutely transforming retrovirus AKT8 in rodent T-cell lymphoma) and ERK1/2 (extracellular regulated kinase-1/2), after UV (36). $1,25(OH)_2D_3$ also increased PTEN expression after UV (102). UVB normally downregulates PTEN in cells and whole skin in an AKT and ERK1/2-dependent manner (103). In turn, PTEN downregulation impairs global genomic nucleotide excision repair, which is needed to remove UV-induced DNA lesions such as CPDs (104). The $1,25(OH)_2D_3$-induced reduction in reactive oxygen species as soon as 15 min after UV probably contributes to reduced oxidative DNA lesions and is likely to result in less damage to repair enzymes and thus to enhance repair (36). Likewise, after UV, the $1,25(OH)_2D_3$-induced reduction in reactive nitrogen species and

damaging nitrosylation of proteins, including DNA repair enzymes, is likely to facilitate DNA repair (37).

In the current study, we observed a reduction in oxygen consumption rate, indicative of a reduction in oxidative phosphorylation, in both sham and UV-exposed keratinocytes. We had previously reported that energy from glycolysis was required for DNA repair (36) and it is clear from the results presented here that even treatment with a well known inhibitor of oxidative phosphorylation, oligomycin, had no effect of the ability of $1,25(OH)_2D_3$ to reduce DNA lesions. On the other hand, increased activity of the electron transport chain in later stages of oxidative phosphorylation, has been shown to be critically important for induction of skin cancers in mice by UV (82). If this process is blocked, actinic tumors do not develop (82). Furthermore, agents which interrupt the mitochondrial respiratory chain, such as metformin (82), and it seems from the current data, $1,25(OH)_2D_3$, reduce tumorigenesis (34, 105).

As observed in this study, increased phosphorylation of CREB at Serine ^{133} occurs after UV exposure. This increased CREB phosphorylationseems to be important in the early stages of skin tumor development in mice (83). There is some evidence that the increased CREB phosphorylation after UV is at least, in part, downstream of increased ERK1/2 phosphorylation (106). So the reduction in ERK1/2 phosphorylation after UV with$1,25(OH)_2D_3$, reported earlier (36) may partly explain the reduced CREB-phosphorylation after UV noted here. In turn, reduced phosphorylation of CREB-Ser^{133} even when the reduction is relatively modest as seen here seen here with $1,25(OH)_2D_3$ and as described for the plant-derived flavonol, kaempferol, are enough to significantly suppress UV-induced skin tumor development (34, 107).

Conclusion: Although Professor Anthony Norman did not live to see the extension of his work in photoprotection, the accumulating data further supports the ideas he promulgated for mechanisms of action of the vitamin D hormone, including a role for membrane VDR and non-classical effects on various cell signaling pathways, as well as the classical steroid hormone modulation of gene transcription.

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Figure legends:

Figure 1. Production and metabolism of vitamin D3 and related compounds in the skin. Pre-vitamin D3 is synthesized when the B-ring of 7-dehydrocholesterol is broken on the absorption of a photon of UVB. At body temperature, pre-vitamin D3 is converted to vitamin D3. Continued absorption of UV photons by pre-vitamin D or vitamin D3 results in conversion to over-irradiation products such as lumisterol3, tachysterol3, or suprasterols, or 5,6transvitamin D3.Vitamin D3 is converted in the skin to 25-hydroxyvitamin D3 by CYP2R1 (or possibly CYP27A1) and then to 1,25-dihydroxyvitamin D3 by CYP27B1. The cholesterol side-chain cleavage enzyme CYP11A1 is also expressed in the skin and upregulated by UV. It can convert vitamin D3 into 20-hydroxyvitamin D3 and at least 10 other products. CYP11A1 can also convert lumisterol3 into 24-hydroxylumisterol3 and several other lumisterol derivatives. (Adapted from (87).

Figure 2. Concentration-dependent reduction in CPD or 8-OHdG by $24(OH)L_3$ or $1,25(OH)_2D_3$ in human primary keratinocytes. Representative photomicrographs of immunohistochemical staining of UV-induced CPD (A) or 8-OHdG (C), 3 hours after treatment with vehicle [0.1% (v/v) ethanol], for 5 concentrations of $1,25(OH)_2D_3$ or $24(OH)L_3$. Dark brown staining, as shown by arrows, indicates the presence of CPD positive nuclei (scale bar = 100 μm). (B) Image analysis of IHC images presented as CPD positive nuclei (B) or 8-OHdG positive nuclei (D) as a percentage of total area. Solid grey bars show $1,25(OH)_2D_3$ while stippled bars show $24(OH)L_3$. Results are from a single experiment performed in triplicate, representative of 3 separate experiments with similar results, Mean + SEM; $^{**}p<0.01$, $^{***}p<0.001$, $^{****}p<0.0001$, when compared to UV vehicle, NS-not significant between data sets.

Figure 3. **VDR or ERp57 knockdown by siRNA reduced VDR or ERp57 protein expression respectively, but did not affect the expression of the non-knocked down protein.** Representative western blots showing VDR (A) or ERp57 and VDR (D) in human primary keratinocytes 48 h following transfection with small interfering RNA targeted to VDR mRNA (siVDR) (A lanes 4 - 6), siRNA targeted to ERp57 (siERp57) (D lanes 4 - 6) or or a non-directed siRNA sequence (siCTRL) (lanes 1 – 3). β-actin is shown as the loading control. Representative photomicrographs of keratinocytes stained for VDR or ERp57 following transfection with siVDR (B) or siERp57 (E) or siCTRL. Image analysis of IHC images presented as positive VDR (C) or ERp57 (F) protein expression as a percentage of total cellular area 48 h after transfection with siVDR, siERp57 or siCTRL. Results were from a single experiment performed in triplicate, representative of two separate experiments with similar results. Mean + SEM; ***p<0.001, when compared to siCTRL vehicle, NS- not significant between data sets.

Figure 4. **Reductions in UV-induced CPD or 8-OHdG by 1,25(OH)$_2$D$_3$, 20(OH)D$_3$ or 24(OH)L$_3$ were abolished by either VDR or ERp57 knockdown in human primary keratinocytes**. Image analysis of IHC images of CPD (A) or 8-OHdG (B). Results are each from a single experiment performed in triplicate, representative of 3 separate experiments with similar results. Data are presented as positive nuclei as a percentage of total area. Mean + SEM; ****p<0.0001***p<0.001, when compared to UV vehicle, NS not significant between data sets.

Figure 5. **Tunicamycin pre-treatment abolished the protective effect of 1,25(OH)$_2$D$_3$ on UV-induced CPD and 8-OHdG.** Keratinocytes were treated with 1 ug/ml tunicamycin or vehicle (DMSO) for 48 hours followed by exposure to UV and treatment with vehicle or 1,25(OH)$_2$D$_3$ (10^{-9}

M). **a) CPD** or **b) 8-OHdG** (y-axis) are shown as optical density relative to SHAM. Open white bars show vehicle without UVR; solid black bars show vehicle with UVR; light grey bars show $1,25(OH)_2D_3$ with UVR. Means +SEM. Results are from 3 independent experiments each in triplicate (n=9). ****p<0.0001, **p<0.01, *p<0.05, and n.s. not significant compared with UV+Vehicle (non tunicamycin treated) by Mixed model analysis and Sidak's multiple comparison post-test.

Figure 6. **$1,25(OH)_2D_3$ reduced oxygen consumption rate and ROS production under basal conditions and after UV exposure but oxidative phosphorylation was not needed for protection from DNA damage after UV.** Cells subjected to Seahorse XF after UVirradiation ("UVR") or nonirradiation ("SHAM") ± $1,25(OH)_2D_3$ ("1,25D") (mean ± SEM, n = 22). (A) Oxygen consumption rate (OCR) following 1,25D/vehicle at injection point (arrow). (B) Graph showing OCR percentage 90 min after injection; Bars indicate significant differences between groups ****p<0.0001, *p<0.05. (C) Graph of CPD in keratinocytes measured by image analysis showing the effect of oligomycin treatment (n=9). (D) Reactive oxygen species (ROS) in arbitrary luminescence units ± UV and or 1,25D (n=9). Bars indicate significant differences between groups *p<0.05; **p<0.01; ***p<0.001; ****p < 0.0001.

Figure 7. **$1,25(OH)_2D_3$ reduced UV upregulated p-CREB**. Human keratinocytes cultured in 96 well plates were irradiated with 400 mJ/cm^2 UVB followed by treatment with vehicle or 10 nM $1,25(OH)_2D_3$ for 90 mins. A) Blot was incubated with anti-p-CREB antibody and tubulin which is shown as a loading control b) Densitometry of triplicate blots for p-CREB expression (Mean + SD) was normalized to UV+Vehicle and shown as relative expression. ****p<0.001 when compared with vehicle-treated cells after UV.

Figure 1.

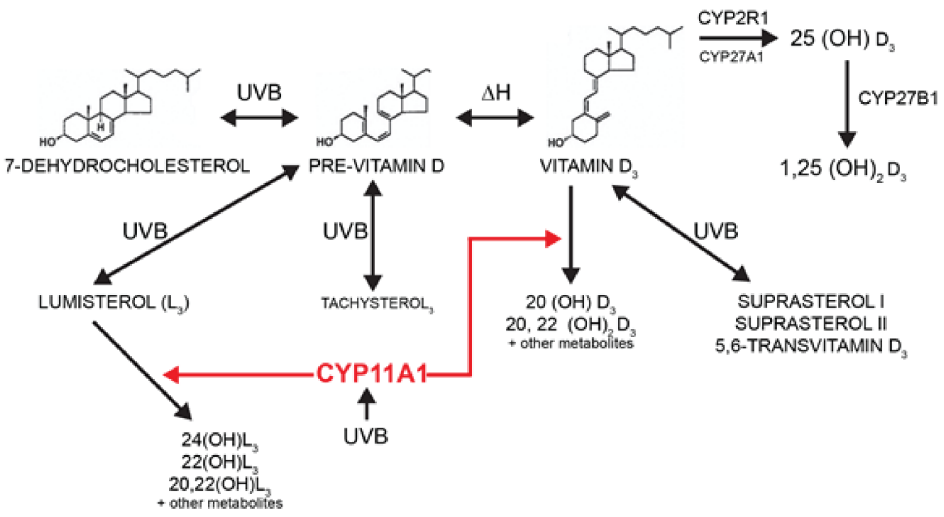
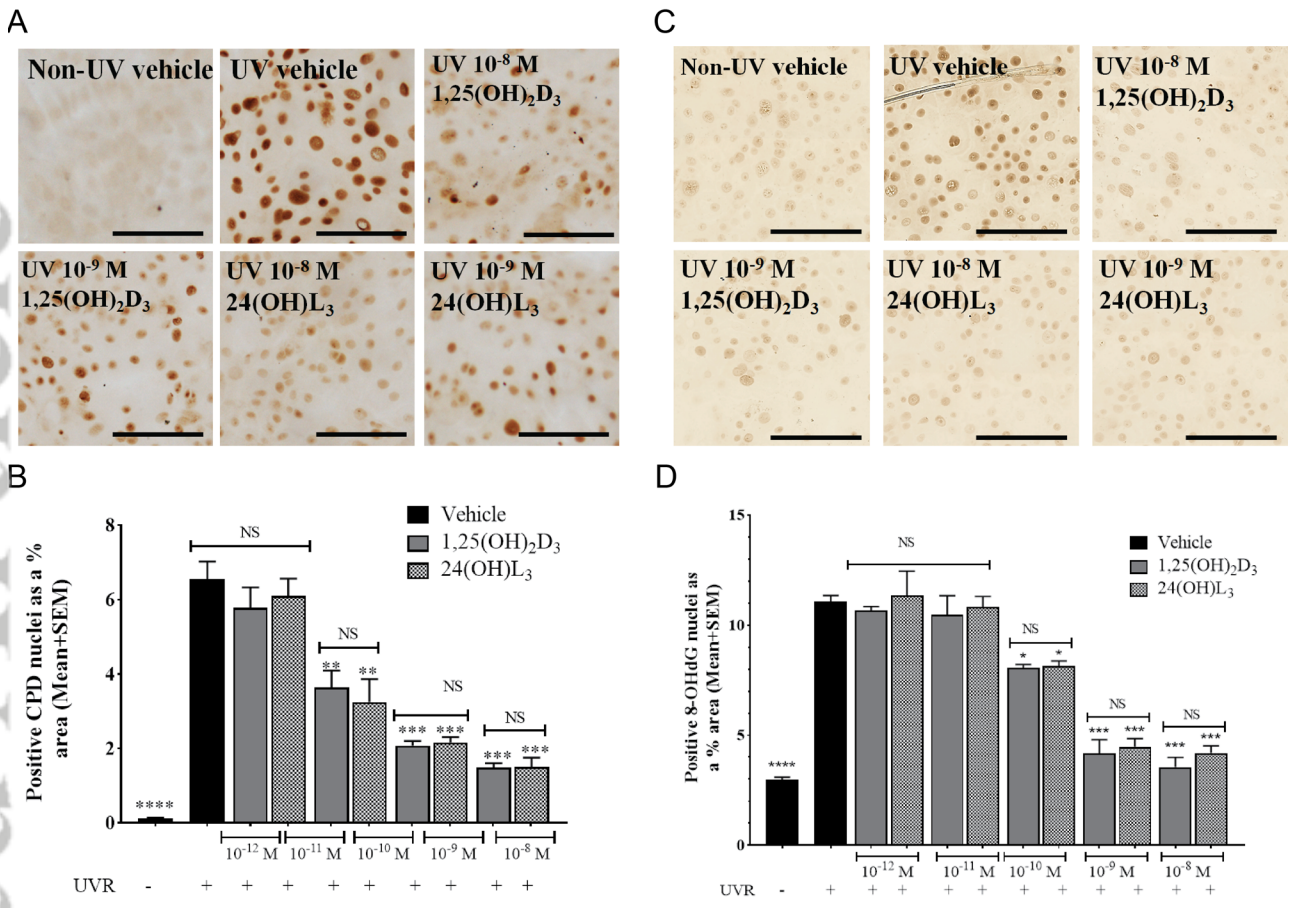


Figure 2.



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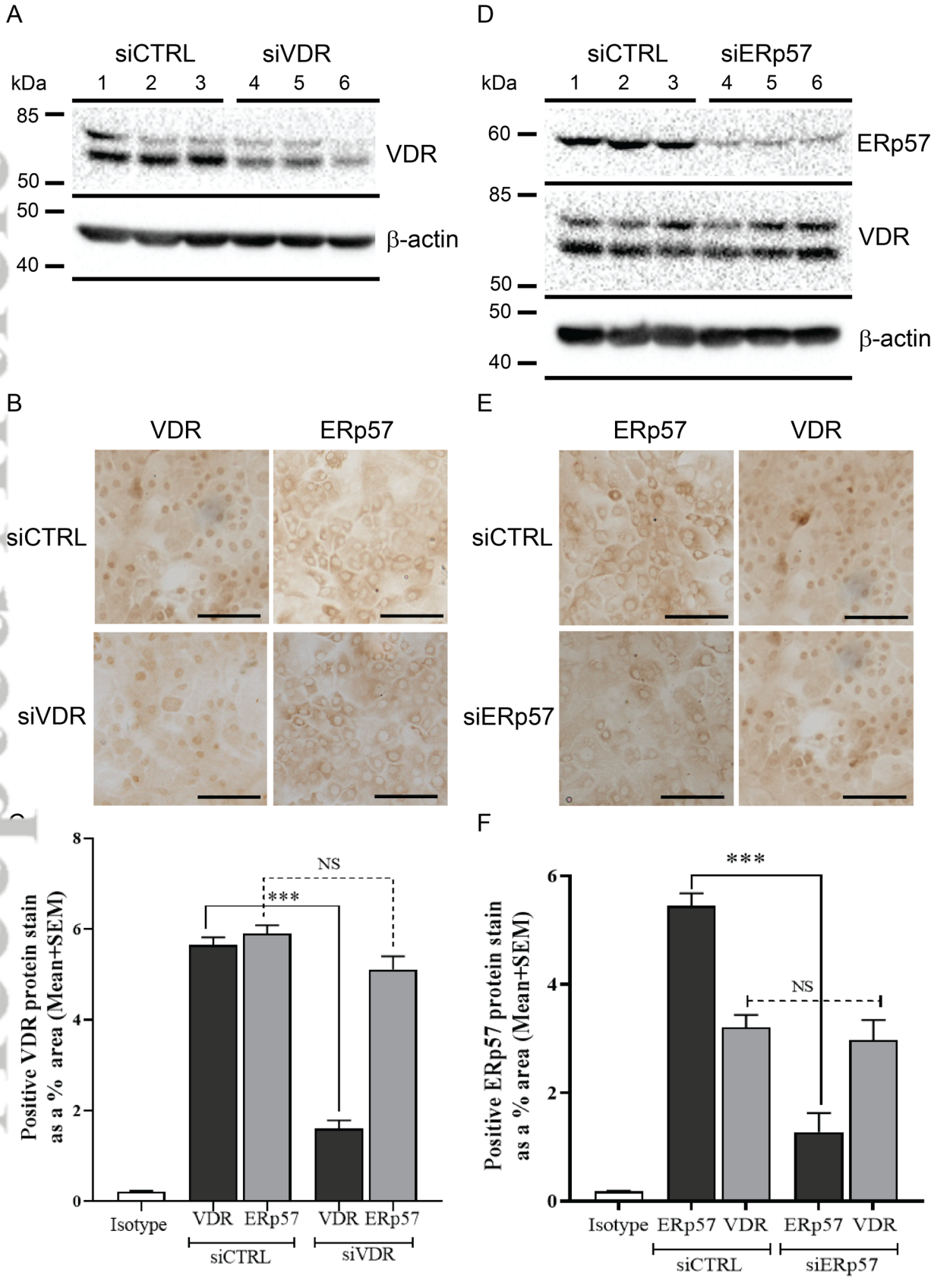

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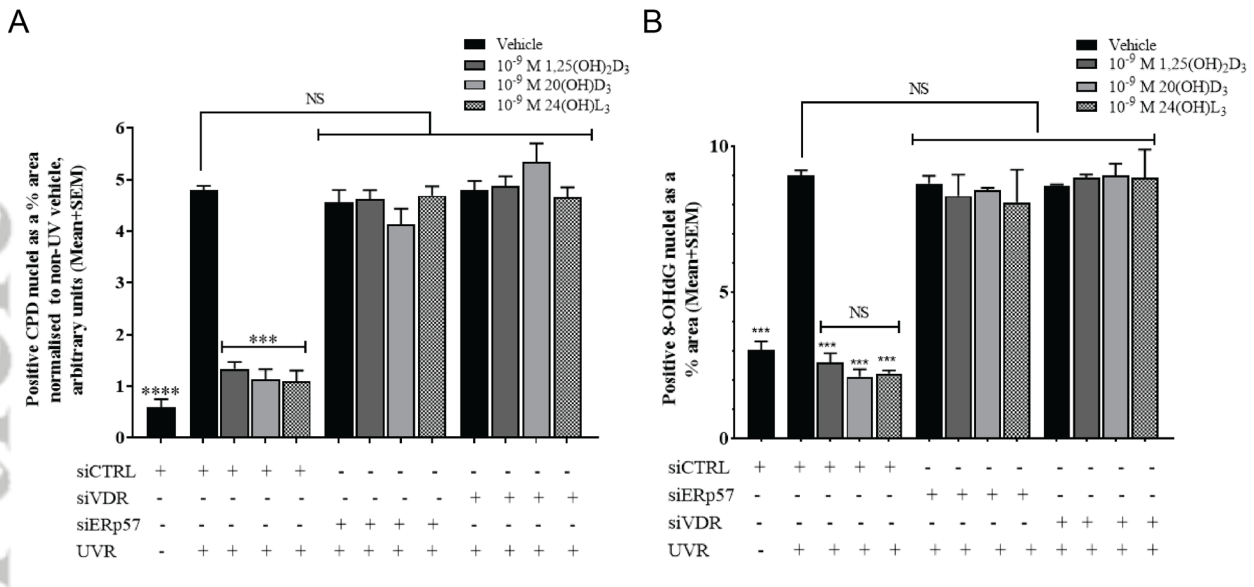

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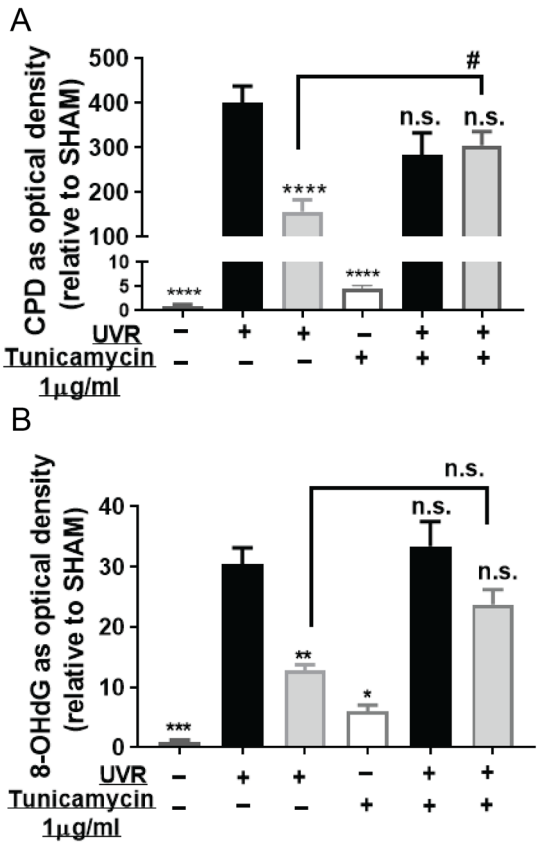

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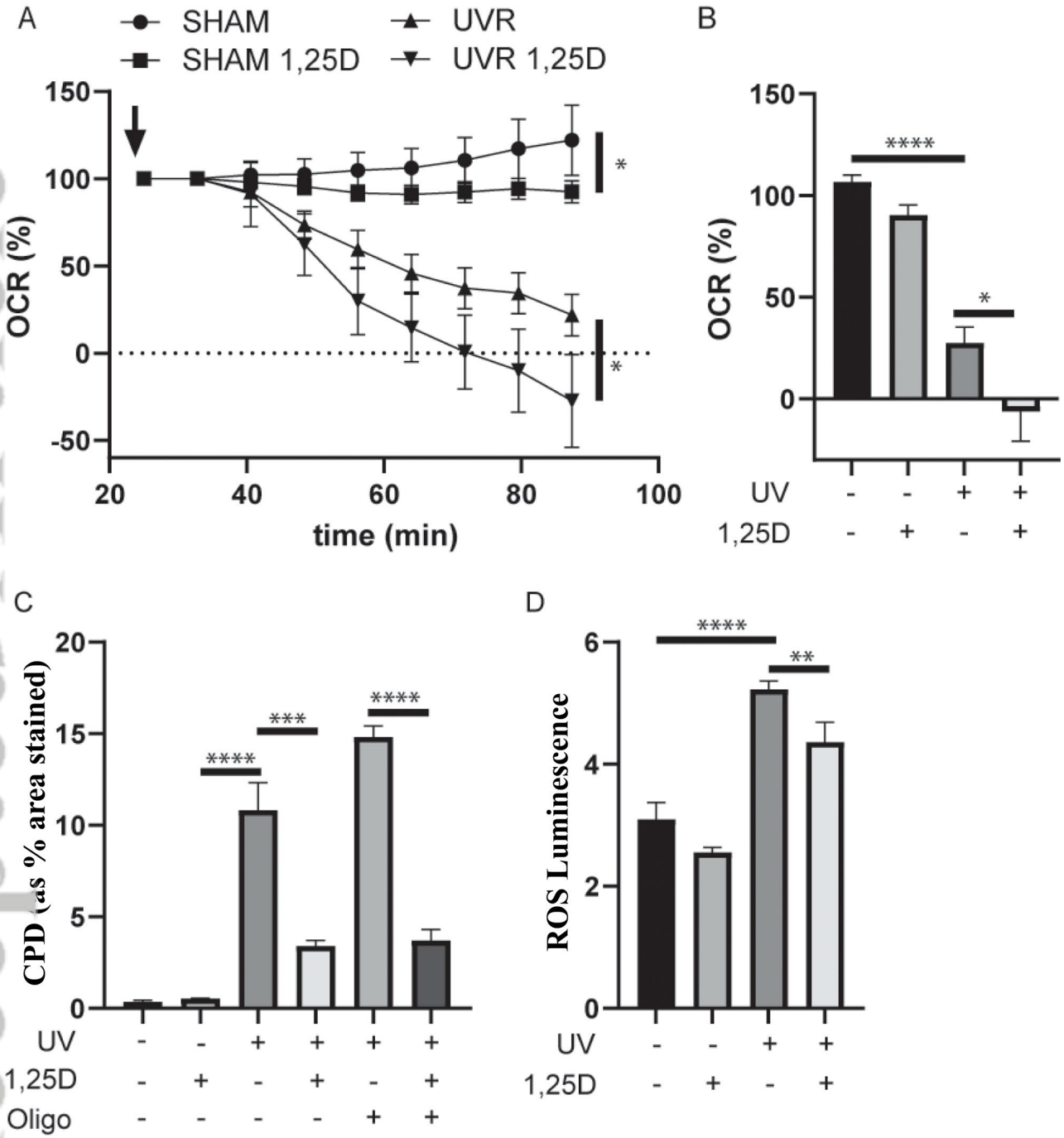

Figure 7.