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PAPER

1α,25 Dihydroxyvitamin D_3 enhances cellular defences against UV-induced oxidative and other forms of DNA damage in skin†

Clare Gordon-Thomson,[a] Ritu Gupta,[a] Wannit Tongkao-on,[a] Anthony Ryan,[a] Gary M. Halliday[b] and Rebecca S. Mason*[a]

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DNA damage induced by ultraviolet radiation is the key initiator for skin carcinogenesis since mutations may arise from the photoproducts and it also contributes to photoimmune suppression. The active vitamin D hormone, 1α,25 dihydroxyvitamin D_3 (1,25$(OH)_2D_3$) reduces thymine dimers, the major photoproduct found in human skin after UV exposure, and suppresses the accumulation of nitric oxide derivatives that lead to more toxic reactive nitrogen species (RNS). We examined whether other forms of DNA damage are reduced by 1,25$(OH)_2D_3$, and hypothesized that photoprotection by 1,25$(OH)_2D_3$ is, in part, due to the suppression of various forms of promutagenic DNA damage, including thymine dimers, through a reduction of genotoxic RNS. Different forms of UV-induced DNA damage were investigated in irradiated skin cells treated with or without 1,25$(OH)_2D_3$, or inhibitors of metabolism and inducible nitric oxide synthase. Keratinocytes were also treated with nitric oxide donors in the absence of UV light. DNA damage was assessed by comet assay incorporating site specific DNA repair endonucleases, and by immunohistochemistry using antibodies to thymine dimers or 8-oxo-7,8-dihydro-2′-deoxyguanosine, and quantified by image analysis. Strand breaks in T4 endonuclease V, endonuclease IV and human 8-oxoguanine DNA glycosylase digests increased more than 2-fold in UV irradiated human keratinocytes, and were reduced by 1,25$(OH)_2D_3$ treatment after UV exposure, and also by low temperature, sodium azide and an inhibitor of inducible nitric oxide synthase. Conversely, nitric oxide donors induced all three types of DNA damage in the absence of UV. We present data to show that 1,25$(OH)_2D_3$ protects skin cells from at least three forms of UV-induced DNA damage, and provide further evidence to support the proposal that a reduction in RNS by 1,25$(OH)_2D_3$ is a likely mechanism for its photoprotective effect against oxidative and nitrative DNA damage, as well as cyclobutane pyrimidine dimers.

Introduction

Ultraviolet (UV) radiation can alter the molecular structure of DNA by direct UV absorption and also indirectly by metabolic interactions with free radicals generated by UV. Reactive oxygen species (ROS) are formed by UV activation of numerous photoreceptors in skin cells. UV radiation also increases the gaseous free radical nitric oxide (NO) in skin by upregulating the expression of nitric oxide synthases (NOS),[1–3] as well as by UVA photodecomposition of stable NO derivatives.[4,5] Increased levels of NO can combine with the oxidative stress-induced superoxide anion radical (O_2^-) to form peroxynitrite ($OONO^-$), an unstable intermediate of the NO pathway. Peroxynitrite is a powerful oxidative and nitrative agent that causes oxidative and nitrative modifications to DNA bases and the sugar–phosphate backbone.[6]

The principal form of UV-induced DNA damage produced in human skin is the thymine–thymine dimer, one type of *cys–syn* cyclobutane pyrimidine dimer (CPD).[7–9] CPDs are known to be produced photochemically by direct absorption of high energy photons of UVC and UVB that breaks the 5–6 double bonds in adjacent pyrimidines. The abnormal covalent bonding that follows joins the two pyrimidines by a stable ring configuration forming a bipyrimidine product that distorts the DNA helix.[10,11] There are now also numerous reports of CPD production by the less energetic but more predominant wavelengths of UVA in sunlight.[9,12–16] Chemical production of CPDs in isolated DNA has also been demonstrated, proposed to occur *via* a triplet energy transfer mechanism.[17,18]

Other forms of DNA damage such as base modifications are produced by the interactions of free radicals, including highly toxic derivatives of NO such as peroxynitrite, a potent nitrative and oxidative agent. Oxidation of the primary amine on the base guanine produces 8-oxo-7,8-dihydro-2′-deoxyguanosine (8-oxodG);[10,19] the most abundant form of oxidatively-induced

[a]*Department of Physiology, The Bosch Institute, The University of Sydney, NSW 2006, Australia. E-mail: rebeccam@physiol.usyd.edu.au; Fax: +61 2 93512510; Tel: +61 2 93512561*
[b]*Department of Dermatology, The Bosch Institute, The University of Sydney, NSW 2006, Australia*
† Contribution to the Vitamin D Update collected papers.

DNA damage found in human skin.[9,20,21] Nitration of the base guanine forms 8-nitroguanine,[6,22] a residue with a short half-life that undergoes rapid depurination within a few hours resulting in a promutagenic abasic site.[23,24] DNA strand breaks are also produced by nitrosation of primary amines by another derivative of NO, nitrous anhydride (N_2O_3).[25] Overproduction of NO can also inactivate DNA repair enzymes by nitrosylation;[26] and has been shown to preferentially inhibit the excision and ligation steps of nuclear excision repair (NER) of CPDs.[27]

UV-induced DNA damage is considered to be one of the key initiating factors for photocarcinogenesis as it can lead to gene mutations and photoimmune suppression.[28–30] The active vitamin D hormone 1α,25 dihydroxyvitamin D_3 (1,25$(OH)_2D_3$), which is synthesized in skin,[31–33] has been shown to protect skin cells from the damaging effects of UV.[34] 1,25$(OH)_2D_3$ and some low calcaemic analogs reduced thymine dimers in irradiated skin cells in culture and in human and mouse skin.[35–42] This was demonstrated by immunohistochemistry using a monoclonal antibody to thymine dimers,[43] and quantified by image analysis. Furthermore, 1,25$(OH)_2D_3$ and the analog 1α,25 dihydroxylumisterol$_3$ (JN) also reduced UV-induced inflammation, photoimmune suppression and photocarcinogenesis in Skh:hr1 hairless mice.[44,45]

An unexplained observation in these studies was that thymine dimers increased up to 6 h post UV in irradiated keratinocytes.[39] Others have reported similar increases in CPDs after UV irradiation.[12,46–50] These investigators however, have either not commented on this or have speculated that it might be due to the presence of an unspecified cytokine,[12] or due to the DNA becoming more accessible to the anti-thymine dimer antibody during antigen retrieval steps, although no data was provided to support this proposal.[12] A reduction in thymine dimers by 1,25-$(OH)_2D_3$ occurred within 30 minutes post UV,[39] which is fast for a DNA repair process.[51] An alternative possibility is that CPD production is not only due to direct absorption of UV by DNA, but also involves an as yet unknown metabolic process. 1,25-$(OH)_2D_3$ may thus protect UV irradiated cells, not only by enhancing repair, but by suppressing the metabolic production of CPDs. Metabolites of the nitric oxide (NO) pathway may be implicated in the metabolic production of CPDs, as thymine dimers were reduced in irradiated skin cells by inhibitors of NOS,[39,42] and 1,25$(OH)_2D_3$ reduced UV-induced accumulation of the peroxynitrite degradation product, nitrite,[39] and also nitrotyrosine, another index of RNS.[44]

Here we investigate whether 1,25$(OH)_2D_3$ protects cells from other predominant forms of UV-induced DNA damage as well as thymine dimers, and whether RNS may be involved in their production. DNA damage in UV irradiated skin cells was assessed after treatment with or without 1,25$(OH)_2D_3$, metabolic inhibitors and inhibitors of nitric oxide synthase. DNA damage was also examined in keratinocytes treated with NO donors in the absence of UV. DNA damage was detected and quantified at the single cell level by comet assay[52] using site-specific endonucleases (T4 endonuclease V for CPD and abasic sites, endonuclease IV for abasic sites, and human 8-oxoguanine-DNA glycosylase for 8-oxodG. DNA damage was also examined by immunohistochemistry using monoclonal antibodies to thymine dimers and 8-oxo-7,8-dihydro-2′-deoxyguanosine, and quantified by image analysis.

Methods

Cell culture

The human studies were approved by the University of Sydney Human Research Ethics Committee. Skin cells were isolated from human neonatal foreskins under informed consent using methods previously described with some modifications.[53,54] Keratinocyte growth medium (KGM) consisted of Dulbecco's Modified Eagle's Medium-DMEM without calcium purchased from Invitrogen (Gibco, CA, USA), 2.2 g L^{-1} $NaHCO_3$, 293 mg L^{-1} glutamine, 10 mM HEPES buffer, and supplemented with 5% v/v heat-inactivated foetal bovine serum (FBS), 10 ng mL^{-1} epidermal growth factor, 1 pM cholera toxin, and 0.5 mg L^{-1} hydrocortisone. All other reagents were purchased from Sigma-Aldrich (Australia) unless otherwise indicated. Cells were used at passage 1–6 and were plated onto poly-L-lysine-coated glass coverslips (Menzel-Glaser, Braunschweig, Germany) in 96-well plates for immunohistochemistry, or in 60 mm tissue culture dishes (BD Biosciences, CA, USA) for comet assays. Experiments were carried out at approximately 60–80% confluence. Growth media was changed to 5% FBS in culture medium with no other supplementation for at least 24 h before treatment to allow cell signal pathways to normalize.

Irradiation

The solar simulated UV source for the *in vitro* studies was a fluorescent FS20T12 UVB lamp and a FL20SBL UVA lamp (Phillips, Amsterdam, The Netherlands) filtered through 0.125 mm cellulose tri-acetate (Eastman Chemical Products, Kingsport, TN) to remove wavelengths below 290 nm. The output spectra for these lamps have been previously published.[39] UV doses were 200 mJ cm^{-2} UVB and 1200 mJ cm^{-2} UVA for immunohistochemistry, or 840 mJ cm^{-2} UVB and 2300 mJ cm^{-2} UVA for comet assays. Cells were irradiated under Martinez buffer solution containing 145 mM NaCl, 5.5 mM KCl, 1.2 mM $MgCl_2(H_2O)_6$, 1.2 mM $NaH_2PO_4(H_2O)_2$, 7.5 mM NaHEPES, 7.5 mM HEPES, 10 mM D-glucose and 1 mM $CaCl_2(H_2O)_2$. Sham-irradiated cells were subjected to similar procedures but were shielded during the irradiation.

Treatments

After irradiation, cells were incubated with 1 nM 1α,25 dihydroxyvitamin D_3 (purchased from Sigma-Aldrich or Cayman Chemical Co., MI, USA) or 0.1% ethanol (vehicle controls) in KGM with 5% FBS at 37 °C. In the inhibition studies, irradiated cells were incubated in KGM with 5% FBS for 3 h post UV with either 20 nM 1400W (*N*-[[3-(aminomethyl)phenyl]methyl]-ethanimide dihydrochloride; Cayman Chemical Co.); or 2.5 mM sodium azide (Ajax Chemicals, Australia); or at low temperature (4 °C) in a refrigerator, or at 37 °C (controls). Unirradiated cells were incubated in KGM with 5% FBS in the absence of any light at 37 °C for 3 h with nitric oxide donors, 2 mM Sin 1 chloride (SIN; 5-amino-3-(4-morpholinyl)-1,2,3-oxadiazolium/3-morpholinosydonimine; Cayman Chemical Co.) and 2 mM sodium nitroprusside (SNP). Optimum concentrations for all treatments were established empirically.

Murine studies

Animal studies were performed in compliance with the guidelines of the National Health and Medical Research Council of Australia and approved by the Animal Ethics Committee of the University of Sydney. Mice were maintained as previously described.[44] The solar simulated UV source consisted of a bank of one fluorescent UVB tube (Phillips TL40W 12R/S, Eindhoven, The Netherlands) flanked by 6 UVA tubes (Hitachi 40W F40T 10/BL, Tokyo, Japan) and filtered through 0.125 mm thick cellulose tri-acetate to eliminate wavelengths below 290 nm. Skh:hr1 female hairless mice were divided into treatment groups of three and subjected to a single exposure of 3.32 kJ m^{-2} UVB and 53.17 kJ m^{-2} UVA (equivalent to approximately three times the minimal erythemal dose). Treatment was by topical application to the dorsum of irradiated and sham irradiated mice with 23 pmol cm^{-2} $1,25(OH)_2D_3$ in a base lotion of 1,2-propandiol in water (1 : 1). Control mice were painted with vehicle (ethanol) in base lotion. Skin samples were taken at 24 h post UV, fixed in Histochoice (Amresco, Ohio, USA) and histologically processed into wax.

Immunocytochemistry and image analysis

Cell cultures were fixed at −20 °C with 100% methanol after 3 h treatment unless otherwise indicated and processed for immunocytochemistry and image analysis as described previously.[39] A mouse monoclonal IgG_1 lambda anti-thymine dimer antibody and a labelled streptavidin biotin secondary antibody (LSAB Plus kit) and diaminobenzidine (DAB) kit (purchased from Dako, Denmark) were used for thymine dimer detection. Stained cells were examined and analysed as described previously.[39] The amount of positive nuclei is represented as a proportion of total nuclei. Sectioned mouse skin was processed for immunohistochemistry using a monoclonal antibody to 8-oxoguanine, clone 2E2 (Trevigen Inc., MD, USA) at a concentration of 2.5 μg mL^{-1}, together with the Animal Research Kit (Dako), as previously described in mouse studies for thymine dimers.[39] To control for specificity of the primary antibodies an isotype control at the same protein concentration was used, which resulted in no staining. Images were captured on a Nikon E800 microscope, Japan, equipped with a digital camera supported by Leica image analysis software. Oxidative DNA damage was quantified using ImageJ and the amount of positive nuclei represented as a proportion of the area of epidermis.

Comet assays

DNA damage was quantified by comet assay as described previously[55] using a FLARE assay kit (Trevigen Inc.). Briefly, single cell suspensions (5000 cells per mL per sample) were embedded in agarose on FLARE slides (R&D Systems, MN, USA). After cell lysis, DNA was digested with either T4 endonuclease V/T4-pyrimidine dimer glycosylase (R&D Systems), human 8-oxoguanine DNA glycosylase (R&D Systems), or endonuclease IV (New England Biolabs, MA, USA) diluted 1 : 1000 in reaction buffer at 37 °C for 30 min. Negative controls were incubated in buffer only without enzyme. DNA breaks were exposed by unwinding in alkali solution (30 mM NaCl; 1 mM EDTA, pH 12.1) and separated by electrophoresis at 1 V cm^{-1} for 20 min. Digital images of SYBR Green 1 stained comets were captured by epifluorescence microscopy and image analysis performed with Tritek CometScore Freeware version 1.5 (www.AutoComet.com). DNA damage was quantified using the metric 'tail moment' that accounts for the extent of migration of strand breaks (tail length) and the relative amount of DNA in the tail compared to intact DNA in the nucleus (% fluorescent intensity in tail). The mean tail moment of one hundred comets per slide of enzyme treated samples were normalised by subtraction of the mean tail moment measured in replicate slides not treated with enzyme (no enzyme negative control) in order to correct for other strand breaks and extraneous DNA damage produced during processing.

Statistical analysis

All experiments shown were repeated 2–6 times with similar results. Data are presented either as representative single experiments or as normalized results from several pooled experiments. For each immunocytochemistry experiment, values were based on 3 to 5 separate wells. In each comet assay, values were based on 2 replicate slides each prepared from 2 separate cell cultures of the same donor/treatment. Results are expressed as means ± standard deviation (SD) or standard error of mean (SEM). Significant differences between means were determined by one-way analysis of variance (ANOVA) with the Tukey–Kramer multiple comparisons test using the GraphPad Instat program (GraphPad Software Inc., San Diego, CA) unless stated otherwise.

Results

$1,25(OH)_2D_3$ reduces T4 endonuclease V- and endonuclease IV-sensitive sites in UV irradiated keratinocytes

Strand breaks generated by T4 endonuclease V (T4N5) yielded comet-like figures with extensive comet tails in UV irradiated vehicle-treated keratinocytes (Fig. 1a, left). Comet tails produced in irradiated keratinocytes treated with $1,25(OH)_2D_3$ were notably less extensive than that in the UV irradiated vehicle-treated samples (Fig. 1a, middle); while sham irradiated keratinocytes showed little or no migration of damaged DNA from nuclei (Fig. 1a, right).

In time course studies over 24 h, strand breaks generated by T4N5 digestion (T4N5-sensitive sites) increased significantly in the UV irradiated keratinocytes post UV ($^{\gg}p < 0.001$; Fig. 1b). A two-fold increase in T4N5-sensitive sites was seen at 6 h compared to 0.5 h, which is consistent with earlier studies using an antibody to thymine dimers and image analysis in keratinocytes.[39] At 24 h post UV the T4N5-sensitive sites were reduced in UV irradiated keratinocytes to a level that was not significantly different from the unirradiated samples at each time point (0 h shown only). Treatment with 1 nM $1,25(OH)_2D_3$ immediately after UV exposure significantly reduced T4N5-sensitive sites at 0.5 h and onwards ($^{***}p < 0.001$; Fig. 1b). T4N5-sensitive sites in unirradiated keratinocytes treated with $1,25(OH)_2D_3$ at each time point were not significantly different from unirradiated keratinocytes at 0 h (therefore not shown).

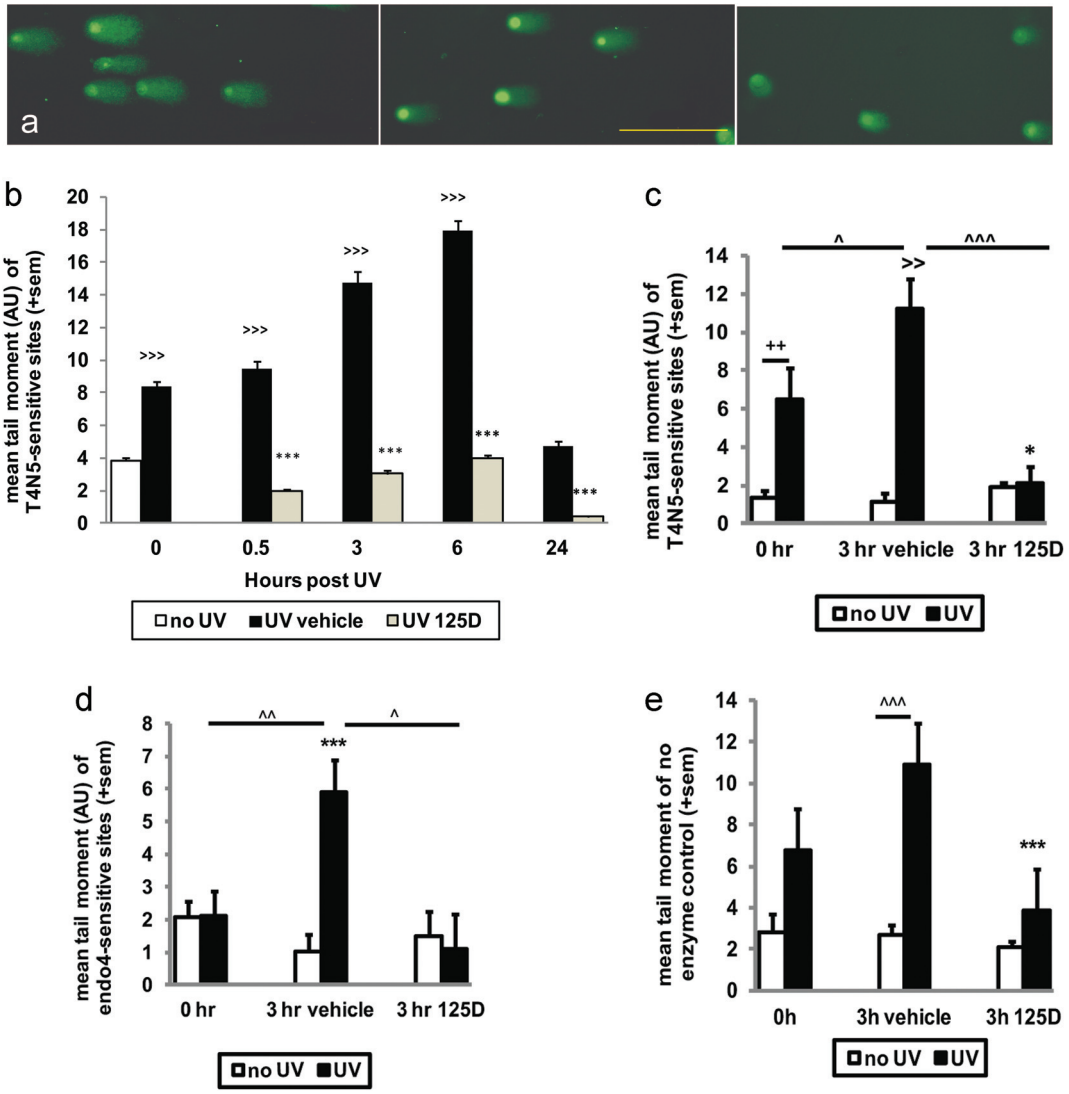

Fig. 1 $1,25(OH)_2D_3$ reduces UV-induced T4N5- and endo4-sensitive sites. (a) In comet assays, broken strands of DNA migrate from the nucleus to form a comet following digestion with endonucleases and single cell electrophoresis. DNA damage is measured as tail moment (tail length × percentage fluorescence in tail) using Cometscore analysis. At 3 h after UV irradiation comets with extensive comet tails were observed (left), which were reduced in the presence of 1 nM $1,25(OH)_2D_3$ (middle). No UV (right panel). Scale bar = 200 μm. (b) In time-dependent comet assays, T4N5-sensitive sites (CPD and abasic sites) increase in UV irradiated vehicle-treated keratinocytes from 0.5 to 6 h post UV and were significantly different from unirradiated vehicle-treated keratinocytes at each time point ($^{\gg}p < 0.001$). Only data from no UV at 0 h is shown as all unirradiated treatment groups were not statistically different from 0 h. T4N5-sensitive sites were significantly reduced in UV irradiated keratinocytes treated post UV with 1 nM $1,25(OH)_2D_3$ compared with UV irradiated vehicle-treated cells at each time point ($^{***}p < 0.001$). Each point represents data pooled from 2 identical experiments. The mean tail moment was calculated from >100 comets/sample ($n = 4$) and adjusted for net enzyme-sensitive sites (mean tail moment of enzyme-treated sample minus mean tail moment of no enzyme controls, ± SEM). (c) In separate experiments to (b), T4N5-sensitive sites (CPD and abasic sites) in UV irradiated cells at 0 h were significantly higher than in unirradiated cells (no UV) at the same time point ($^{++}p < 0.01$) and were significantly increased in UV irradiated vehicle-treated keratinocytes at 3 h, compared to UV irradiated vehicle-treated cells at 0 h ($^{\wedge}p < 0.05$) and at 3 h ($^{\gg}p < 0.01$). T4N5-sensitive sites were significantly reduced in UV irradiated cells in the presence of $1,25(OH)_2D_3$ at 3 h, compared to UV irradiated cells at 0 h ($^{*}p < 0.05$) and at 3 h ($^{\wedge\wedge\wedge}p < 0.001$). Each point represents data pooled from 3 identical experiments. The mean tail moment was calculated after subtraction of the no enzyme controls, as in Fig. 1b ($n = 6$). (d) Endo4-sensitive sites (abasic sites) in UV irradiated cells at 0 h were not significantly different from the unirradiated cells (no UV), but were significantly increased in UV irradiated vehicle-treated keratinocytes at 3 h post UV compared with 0 h ($^{\wedge\wedge}p < 0.01$) and with unirradiated cells (no UV) at 3 h ($^{***}p < 0.001$). Endo4-sensitive sites were significantly reduced in UV irradiated cells in the presence of $1,25(OH)_2D_3$ at 3 h, compared with UV irradiated vehicle-treated cells at 3 h ($^{\wedge}p < 0.05$). Each point represents data pooled from 3 identical experiments. The mean tail moment was calculated after subtraction of the no enzyme controls, as in Fig. 1b ($n = 6$). (e) The mean tail moment of the no enzyme controls (unknown photoproducts, see text) at 0 h was higher in the UV irradiated keratinocytes than in the unirradiated cells (no UV), but this was not statistically significant. The mean tail moment of the no enzyme controls in UV irradiated vehicle-treated keratinocytes was significantly increased at 3 h post UV, compared to no UV at the same time point ($^{\wedge\wedge\wedge}p < 0.001$), but was significantly reduced in UV irradiated cells in the presence of $1,25(OH)_2D_3$ at 3 h, compared to UV irradiated cells treated with vehicle at 3 h ($^{***}p < 0.001$). Each point represents data pooled from 7 similar experiments. The mean tail moment was calculated from >100 comets/sample, ± SEM.

T4 endonuclease V (T4N5) once considered the gold standard for CPD detection, also has apurinic/apyrimidinic (AP-lyase) activity and therefore cleaves abasic as well as bipyrimidine sites, although with less efficiency. To discriminate between the CPD and abasic fractions in the T4N5 digests, we employed endonuclease IV (endo4), which cleaves the phosphodiester bond at abasic sites only.[15] Tail moments were measured at 2 time points, immediately after UV exposure at 0 h, and at 3 h post UV, the later being the optimal time point used for treatments in our experiments. The putative CPD fraction was determined by subtracting the mean tail moment of the endo4 digests from the mean tail moment of replicate samples digested with T4N5.

Immediately after irradiation (0 h), the mean tail moment of T4N5-sensitive sites (CPD and abasic sites) in UV irradiated vehicle-treated keratinocytes was significantly higher than in unirradiated (no UV) T4N5 digests at the same time point ($^{++}p < 0.01$: Fig. 1c). As seen in the previous set of experiments, T4N5-sensitive sites were significantly increased in UV irradiated vehicle-treated keratinocytes at 3 h post UV, compared to that at 0 h ($^{\wedge}p < 0.05$). Treatment with 1 nM $1,25(OH)_2D_3$ for 3 h post UV significantly reduced T4N5-sensitive sites compared to UV irradiated vehicle-treated keratinocytes at 0 h and at 3 h ($^{*}p < 0.05$ and $^{\wedge\wedge\wedge}p < 0.001$ respectively; Fig. 1c).

At 0 h, the endo4-sensitive (abasic) sites in UV irradiated keratinocytes were not significantly different from the non-irradiated cells (Fig. 1d). Endo4-sensitive sites were significantly increased in UV irradiated vehicle-treated cells at 3 h ($^{\wedge\wedge}p < 0.01$; Fig. 1d), but this was significantly lower (1.9-fold) than in replicate samples digested with T4N5 ($p < 0.01$). There was a significant reduction in endo4-sensitive sites in UV irradiated keratinocytes treated with 1 nM $1,25(OH)_2D_3$ for 3 h post UV, compared to irradiated vehicle-treated keratinocytes at the same time point ($^{\wedge}p < 0.05$).

The putative CPD fraction calculated by performing digestions with T4N5 and with endo4 in replicated samples and subtracting one from the other as described (Jiang, Rabbi *et al.* 2009), was 1.2-fold higher at 3 h in UV irradiated keratinocytes compared to 0 h (mean tail moment at 0 h = 4.4 ± SEM 1.4 arbitrary units (AU); mean tail moment at 3 h = 5.36 ± SEM 1.3 AU), but this was not statistically significant. However, treatment with $1,25(OH)_2D_3$ significantly reduced the CPD fraction by 5.4 fold at 3 h post UV (mean tail moment for $1,25(OH)_2D_3$ treated cells = 0.99 ± SEM 0.98 AU); compared to vehicle at 3 h post UV ($p = 0.0374$ by unpaired t test; $n = 6$, pooled data from 3 identical experiments).

Data from undigested samples (no enzyme controls) from the comet assays ($n = 7$) was pooled and analysed for variations in the treatment groups. The tail moment of the no enzyme control group represents both spurious and genuine non-specific single and double strand breaks generated by the treatments and during the assay. At 0 h, the mean tail moment of the no enzyme controls of UV irradiated vehicle-treated keratinocytes was higher than in unirradiated keratinocytes, but this was not statistically significant. At 3 h post UV, the mean tail moment in UV irradiated vehicle-treated keratinocytes was significantly higher than the unirradiated cells at the same time point ($^{\wedge\wedge\wedge}p < 0.001$; Fig. 1e). There was a significant reduction in the mean tail moment of the no enzyme controls of UV irradiated keratinocytes treated with $1,25(OH)_2D_3$ at 3 h post UV, compared to UV irradiated vehicle-treated keratinocytes at the same time point ($^{***}p < 0.001$; Fig. 1e).

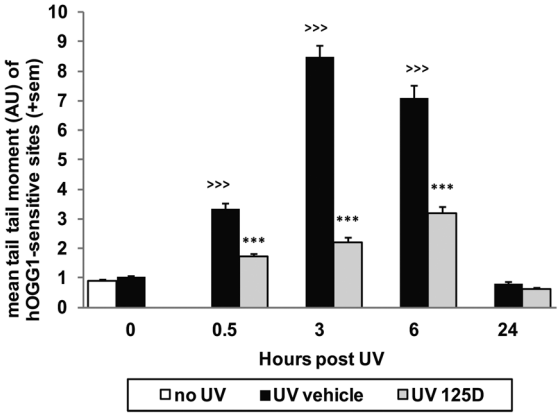

Fig. 2 $1,25(OH)_2D_3$ reduces UV-induced hOGG-sensitive sites. In time-dependent comet assays, hOGG-sensitive sites (8-oxodG) increased significantly in UV irradiated vehicle-treated keratinocytes from 0.5 to 3 h post UV compared with unirradiated vehicle-treated cells (no UV) at each time point: ($^{\gg}p < 0.001$). Only data from no UV at 0 h is shown as all unirradiated treatment groups were not statistically different from 0 h. hOGG1-sensitive sites were reduced in UV irradiated keratinocytes treated with 1 nM $1,25(OH)_2D_3$ compared with vehicle ($^{***}p < 0.001$). Each point represents data pooled from 2 identical experiments. The mean tail moment was calculated as in Fig. 1b ($n = 4$).

$1,25(OH)_2D_3$ reduces UV-induced oxidative DNA damage

Time course studies with comet assays incorporating digestion with the hOGG repair enzyme that specifically cleaves 8-oxodG showed no increase in hOGG-sensitive sites in UV irradiated vehicle-treated keratinocytes at 0 h compared to unirradiated keratinocytes immediately after irradiation at 0 h (Fig. 2). A more than 3-fold significant increase in hOGG-sensitive sites was seen within 30 minutes post UV, which continued to increase to more than 8-fold by 3 h post UV ($^{\gg}p < 0.001$; Fig. 2). At 24 h post UV, the hOGG-sensitive sites were reduced in irradiated keratinocytes to a level that was not significantly different from the unirradiated samples at each time point (0 h shown only; Fig. 2). Treatment with 1 nM $1,25(OH)_2D_3$ immediately after UV exposure significantly reduced hOGG-sensitive sites in irradiated keratinocytes from 0.5 h onwards ($^{***}p < 0.001$; Fig. 2). The hOGG-sensitive sites in unirradiated keratinocytes treated with $1,25(OH)_2D_3$ at each time point were not significantly different from unirradiated keratinocytes at 0 h.

Positive nuclear staining with a monoclonal antibody to 8-oxodG, quantified by image analysis, was significantly reduced in UV irradiated Skh:hr1 mouse skin treated topically immediately after UV exposure with $1,25(OH)_2D_3$ at a concentration of 23 pmol cm^{-2} ($p = 0.0017$ by two-tailed t test), compared to UV irradiated vehicle-treated skin (Fig. 3a–e).

UV-induced DNA damage is suppressed by inhibitors of metabolism and iNOS

To test the role played by metabolism and RNS in the production of UV-induced DNA damage, keratinocytes were incubated immediately after UV for 3 h at low temperature (4 °C) to slow metabolism, or with 2.5 mM sodium azide (an inhibitor of oxidative phosphorylation). The NOS inhibitor (1400W) was used

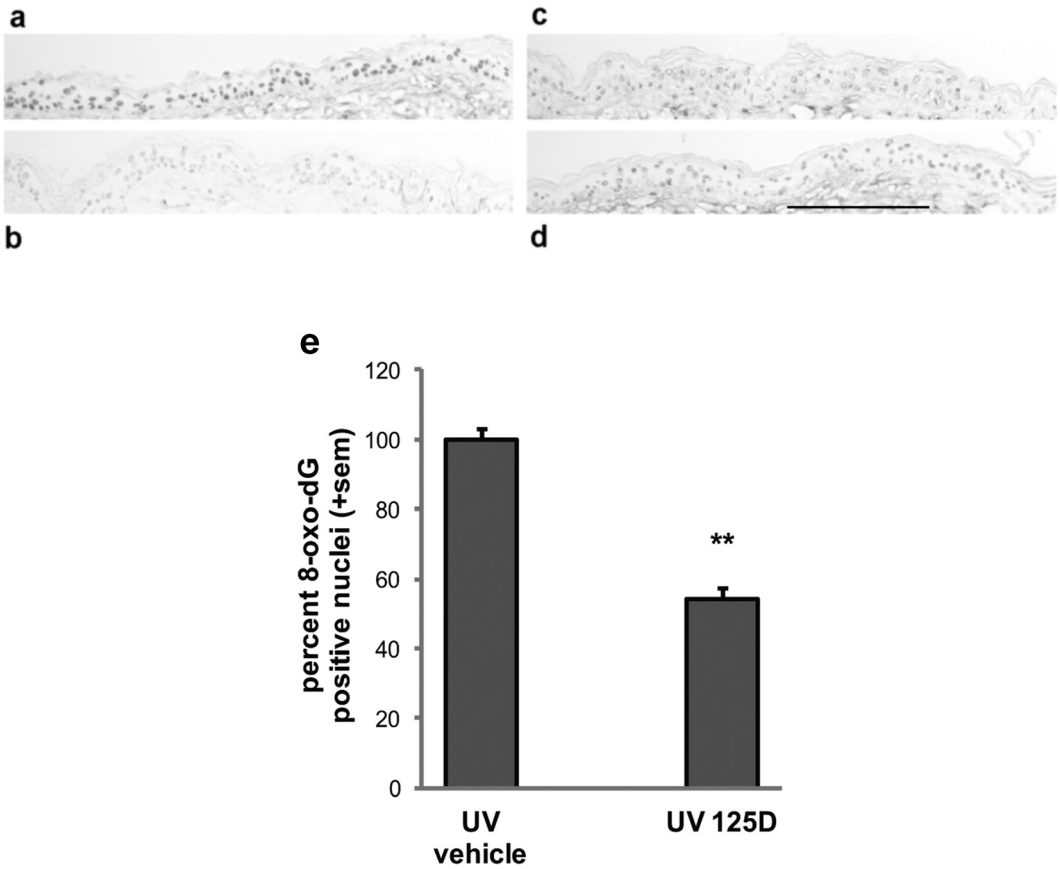

Fig. 3 $1,25(OH)_2D_3$ reduces 8-oxodG in UV irradiated mouse skin. Topical application of $1,25(OH)_2D_3$ reduced positive nuclear staining to an antibody to 8-oxodG in nuclei in the Skh:hr1 mouse epidermis 24 h post UV. (a) UV vehicle; (b) UV 23 pmol cm^{-2} $1,25(OH)_2D_3$; (c), (d) unirradiated skin treated with vehicle or $1,25(OH)_2D_3$ respectively. (Scale bar = 50 μM). (e) Positive nuclear staining in mouse skin with the 8-oxodG antibody was significantly reduced by $1,25(OH)_2D_3$ at 24 h post UV(**$p = 0.0017$ by unpaired t test; $n = 9$).

at 20 nM concentration to selectively inhibit the inducible isoform.[56]

The mean tail moments for T4N5-, endo4- and hOGG-sensitive sites were significantly higher in irradiated keratinocytes incubated at 37 °C for 3 h post UV, compared to unirradiated keratinocytes and keratinocytes incubated with the inhibitors ($^{\gg}$, ***, and $^{\wedge\wedge\wedge}p < 0.001$; for T4N5- endo4- and hOGG-sensitive sites respectively; Fig. 4a). T4N5-, endo4- and hOGG-sensitive sites in irradiated keratinocytes treated with the inhibitors were not significantly higher than in the unirradiated cells (no UV). The T4N5-sensitive sites in irradiated samples incubated at 37 °C were also significantly higher than the endo4-sensitive sites in replicate samples ($^{++}p < 0.01$; Fig. 4a).

The CPD fraction (T4N5 minus endo4) when calculated was significantly increased at 3 h in UV irradiated vehicle-treated keratinocytes incubated at 37 °C, compared to the unirradiated cells ($^{\gg}p < 0.001$; Fig. 4b). The CPD fraction was significantly reduced in the presence of all three inhibitors, compared to CPDs in UV irradiated keratinocytes at 37 °C (*$p = 0.026$ for 4 °C incubation; *$p = 0.035$ for sodium azide treatment, and *$p = 0.021$ for incubation with the iNOS inhibitor, 1400W, by unpaired t test; Fig. 4b).

Positive nuclear staining with the antibody to thymine dimers, quantified by image analysis, was significantly reduced in irradiated keratinocytes incubated at 4 °C, compared to keratinocytes incubated at 37 °C. The average percentage of nuclei containing thymine dimers per total nuclei at 4 °C was 42% ± 11 (SEM), relative to thymine dimers produced in UV irradiated keratinocytes incubated at 37 °C: (*$p = 0.018$; Fig. 4c).

Nitric oxide donors induce DNA damage in the absence of UV

Human keratinocytes were treated with the nitric oxide donors SIN 1 chloride (SIN) or sodium nitroprusside (SNP) in the dark to test the proposal that derivatives of the NO pathway contribute to DNA damage, including CPDs. SIN decomposes spontaneously in physiological solution to NO and superoxide that combine to form peroxynitrite. The mean tail moment of T4N5-, endo4- and hOGG-sensitive sites significantly increased in unirradiated keratinocytes treated with the peroxynitrite donor (SIN), when compared to keratinocytes incubated without SIN for 3 h at 37 °C ($^{\gg}$, **$p < 0.01$ and $^{\wedge}p < 0.05$ for T4N5-, endo4- and hOGG-sensitive sites respectively; Fig. 5a). T4N5-, endo4- and hOGG-sensitive sites produced in UV irradiated keratinocytes at 3 h post UV were significantly higher than in unirradiated keratinocytes incubated for 3 h ($^{\gg}$, ***$p < 0.001$ for T4N5, and endo4-sensitive sites respectively, and $^{\wedge\wedge}p < 0.01$ for

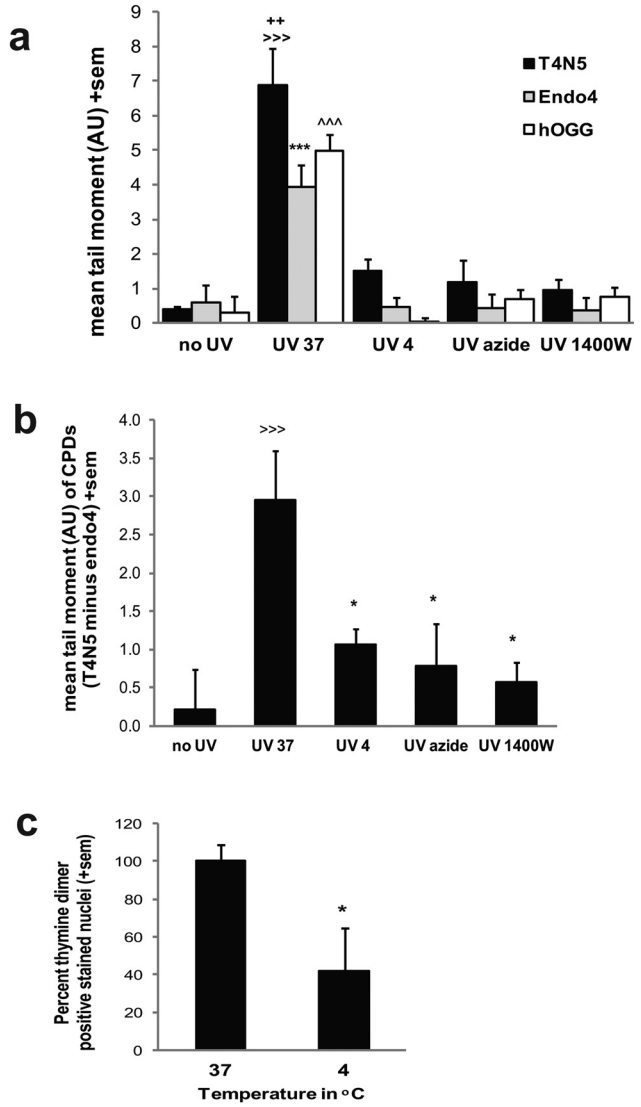

Fig. 4 UV-induced DNA damage is reduced by inhibitors of metabolism (4 °C and sodium azide) and with an iNOS inhibitor (1400W). (a) At 3 h, T4N5-, endo4- and hOGG1-sensitive sites were significantly higher in UV irradiated vehicle-treated keratinocytes incubated at 37 °C for 3 h compared with unirradiated cells (no UV; $^{\gg}$ and *** and $^{\wedge\wedge\wedge}p <$ 0.001 for T4N5-, endo4- and hOGG-sensitive sites respectively). T4N5-sensitive sites in UV irradiated cells at 37 °C were significantly higher than endo4-sensitive sites incubated at the same temperature ($^{++}p <$ 0.01). Each point represents data pooled from 2 identical experiments. The mean tail moment was calculated as in Fig. 1b ($n = 4$). (b) The CPD fraction (T4N5 minus endo4) when calculated was significantly increased in UV irradiated keratinocytes incubated at 37 °C, relative to unirradiated cells (no UV; $^{\gg}p < 0.001$). CPDs were significantly reduced in keratinocytes incubated with the inhibitors compared with those in UV irradiated keratinocytes incubated at 37 °C ($^{*}p < 0.05$, by unpaired t test). (c) Positive nuclear staining with the thymine dimer antibody in UV irradiated keratinocytes incubated at 37 °C for 3 h was significantly reduced by low temperature (4 °C) ($^{*}p = 0.018$ by unpaired t test). Data was normalized to data from the control incubation temperature of 37 °C. Graph represents the pooled data from 6 experiments ±SEM.

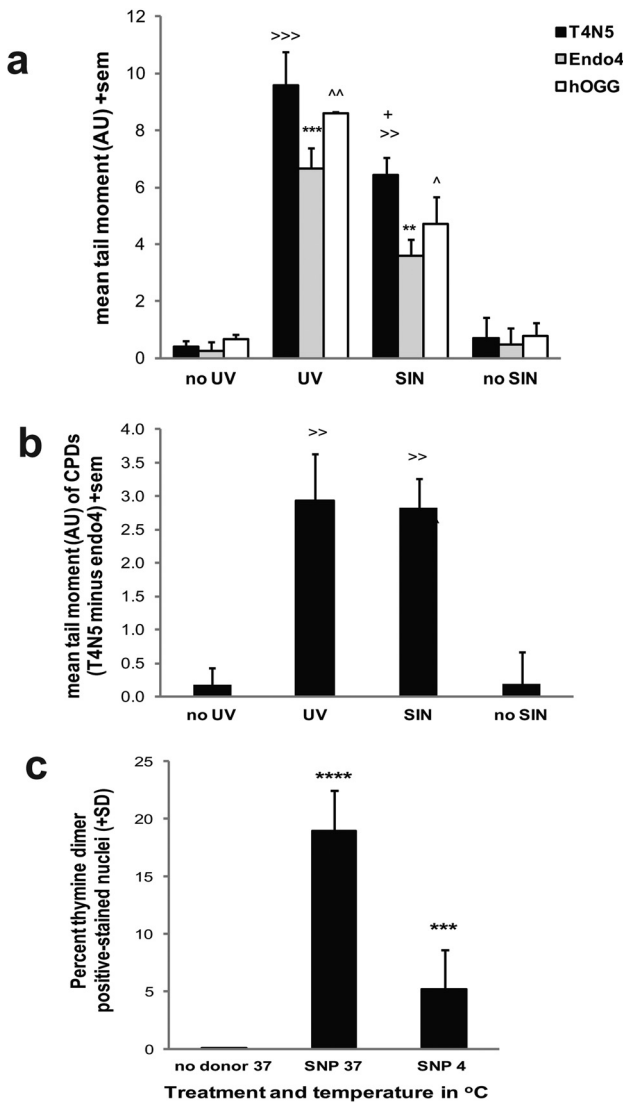

Fig. 5 Nitric oxide donors induce DNA damage in keratinocytes in the absence of UV (a) At 3 h, T4N5-, endo4- and hOGG1-sensitive sites were significantly higher in UV irradiated keratinocytes compared with unirradiated keratinocytes (no UV; $^{\gg}$, $^{***}p < 0.001$ for T4N5- and endo4-sensitive sites respectively, and $^{\wedge\wedge}p < 0.05$ for hOGG-sensitive sites). T4N5-, endo4- and hOGG1-sensitive sites were also significantly increased in keratinocytes treated with 2 nM of the NO donor, Sin 1 chloride (SIN) for 3 h in the absence of UV compared with untreated cells (no SIN; $^{\gg}$ and $^{**}p < 0.01$ for T4N5- and endo4-sensitive sites respectively; and $^{\wedge}p < 0.05$ for hOGG-sensitive sites). Each point represents data pooled from 2 identical experiments. The mean tail moment was calculated as in Fig. 1b ($n = 4$). (b) The CPD fraction (T4N4 minus endo4) when calculated was significantly increased in keratinocytes incubated with 2 mM SIN; significantly different from keratinocytes incubated without the NO donor (no SIN; $^{\gg}p < 0.01$), and was not significantly different from the CPD fraction in UV irradiated keratinocytes. The CPD fraction in the UV irradiated keratinocytes was significantly higher than in unirradiated keratinocytes (no UV; $^{\gg}p < 0.01$). (c) Positive nuclear staining with the thymine dimer antibody increased in unirradiated keratinocytes treated with SNP (2 mM) for 3 h: ($^{****}p <$ 0.0001 by student's t-test). Thymine dimers were reduced but not abolished in SNP-treated cells incubated at 4 °C ($^{***}p < 0.001$). Each point represents the mean of quadruplicate wells ± SD. Similar results were seen in at least 2 other experiments.

hOGG-sensitive sites; Fig. 5a), but were not significantly higher than in keratinocytes treated with SIN (Fig. 5a). The T4N5-sensitive sites in keratinocytes treated for 3 h with SIN were also significantly higher than replicates digested with endo4 (^{+}p = 0.0103; Fig. 5a).

The CPD fraction (T4N5 minus endo4) when calculated was significantly higher in keratinocytes incubated with SIN in the absence of UV than without SIN ($^{\gg}p$ = 0.006; Fig. 5b). The CPD fraction produced in the UV irradiated keratinocytes was significantly higher than in unirradiated cells ($^{\gg}p < 0.01$), but not significantly different from the CPD fraction induced by SIN (Fig. 5b).

Thymine dimers were generated in the absence of UV in the presence of SNP incubated at 37 °C as assessed by image analysis after immunohistochemistry with a thymine dimer antibody (Fig. 5c). Thymine dimers were reduced but not abolished in cells incubated with SNP at 4 °C (***p<0.001; Fig. 5c).

Discussion

In earlier studies we demonstrated that $1,25(OH)_2D_3$ reduced thymine dimers, the major form of pyrimidine dimer, which were quantified in skin cells by image analysis following immunohistochemical staining with a monoclonal antibody.[34] Here we provide further evidence that $1,25(OH)_2D_3$ protects skin cells from UV-induced CPD and also other DNA photoproducts, using a different approach, by comet assay incorporating digestion with DNA repair enzymes, as well as by immunohistochemistry.

The T4N5-sensitive sites (*i.e.* CPD plus abasic sites) were significantly higher immediately after irradiation (time zero) when compared to the non-irradiated keratinocytes as expected ($p < 0.01$), but unexpectedly they continued to increase significantly up to 6 h after irradiation ($p < 0.001$). The addition of $1,25(OH)_2D_3$ to the cultures immediately after irradiation (time zero) significantly reduced T4N5-sensitive sites within 30 minutes ($p < 0.001$), consistent with earlier measurements by thymine dimer antibody and image analysis.[39] The CPD fraction when calculated by subtracting the data of the endo4 digests from replicate T4N5 digests revealed a fold increase between 0 h (immediately after UV) and 3 h post UV, but this was not statistically significant in one set of experiments (refer to Fig. 1 and associated text), but was significantly higher in a second set of experiments (Fig. 4b). Additional investigations with a more direct measure for CPDs such as high performance liquid chromatography associated with tandem mass spectrometry would be useful to validate these results. Important, however, is the confirmation, using an entirely different method to antibody detection, that the addition of $1,25(OH)_2D_3$ immediately after irradiation significantly reduced the CPD fraction ($p < 0.05$), as well as other forms of DNA damage (abasic sites and 8-oxodG) within a very short time frame.

The increase in pyrimidine dimers with time after UV suggests that CPDs may be produced metabolically as well as photochemically, but this has raised questions as the general consensus for CPD production is that UV absorption is required. However, metabolic production of CPDs cannot be excluded, as CPDs were induced in unirradiated keratinocytes after treatment with nitric oxide donors, and CPDs were reduced in the presence of the metabolic inhibitors, low temperature and sodium azide. These findings are consistent with others who reported the chemical production of CPDs in isolated and bacterial DNA, and it was proposed that this was due to a triplet–triplet energy transfer mechanism by excited state species (*e.g.* *N*-phenylphthalimidine).[17,18,57]

A potential mechanism for the metabolic production of CPDs in skin would be the increased production of NO by the upregulation of NOS,[1–3] and by enzyme-independent decomposition of *S*-nitrosothiols and nitrite.[4,5,22] NO on its own can have beneficial properties and is known to prevent oxidative damage and lipid peroxidation,[58] but when NO combines with ROS, which is also increased by UV, it is converted to more highly reactive NO derivatives, such as peroxynitrite that is known to interact with DNA and induce modifications to its structure by oxidation and nitration.[6,22,59] Supporting this proposal, we have demonstrated CPD induction in unirradiated keratinocytes with the peroxynitrite donor, SIN. It has been speculated that peroxynitrite can form excited state species during dissociation reactions which may contribute to pyrimidine dimer induction. It is interesting that thymine dimers were also induced in unirradiated keratinocytes by SNP, a ferricyanide complex that spontaneously releases NO, nitroxyl anions (NO^{-}),[60] and free iron that can lead to the formation of highly reactive hydroxyl radicals by the Fenton reaction;[61] and therefore a possibility for more highly toxic intermediates of the NO pathway being involved. For more details on the biochemistry and reactions of peroxynitrite and other free radicals and oxidants refer to reviews by others.[62,63]

Since $1,25(OH)_2D_3$ reduces the accumulation of stable products of the NO pathway,[39,44] this may well contribute to a reduction in other more genotoxic RNS. Blockade of inducible NOS with 1400W reduced abasic sites, 8-oxodG, and also CPDs in harmony with earlier studies showing a reduction in thymine dimers by the NOS inhibitor, aminoguanidine[42] and *N*-methyl-L-arginine (L-NMMA).[39] These results are consistent with the proposal that a mechanism for photoprotection against DNA damage by $1,25(OH)_2D_3$ is by reducing RNS, which we have previously demonstrated by showing a reduction in the photoproducts, nitrite and nitrotyrosine.[39,44] A reduction in NO products by $1,25(OH)_2D_3$ could be mediated by increased p53 accumulation, since p53 represses inducible NOS gene expression.[64] It is interesting that while $1,25(OH)_2D_3$ is not a known antioxidant, it reduced UV-induced 8-oxodG by 48 ± 4% (SEM) within 30 minutes in this study. An antioxidant system, metallothionein, has been shown to be upregulated by $1,25(OH)_2D_3$.[65] However, the reduction in DNA damage by $1,25(OH)_2D_3$ within the relatively short time frame of 30 minutes is not fully compatible with the time for transcriptional activities for NOS and metallothionein upregulation in normal skin.[3,66]

The present study shows for the first time the photoprotective effect of $1,25(OH)_2D_3$ against various other types of DNA damage induced indirectly by UV in skin cells, besides CPDs. In comet assays, abasic sites (endo4-sensitive sites) were not evident immediately after irradiation, unlike CPDs, but were significantly increased by 3 h post UV ($p < 0.01$). The abasic sites, in part, represent the difficult to detect nitrated bases such as 8-nitroguanine that undergo rapid apurination,[23,67] as well as residues produced during base excision repair. The addition of

$1,25(OH)_2D_3$ at time zero reduced abasic sites in irradiated keratinocytes measured at 3 h post UV, when compared to irradiated keratinocytes incubated with vehicle for 3 h ($p < 0.05$). Photoprotection against 8-oxodG, the predominant oxidatively-induced DNA photoproduct[21] is also afforded by $1,25(OH)_2D_3$. Similar to the endo4 digests there was no measurable hOGG-sensitive sites at time zero, but these increased significantly up to 3 h post UV, and were significantly reduced by $1,25(OH)_2D_3$ within 30 minutes of treatment ($p < 0.001$). Furthermore, a significant reduction in positive nuclear staining with an antibody to 8-oxodG was measured by image analysis in irradiated skin of the Skh:hr1 hairless mouse after topical application of $1,25(OH)_2D_3$ ($p < 0.01$). DNA strand breaks observed in the negative controls that were not treated with a repair enzyme in the comet assays were also reduced by $1,25(OH)_2D_3$, suggesting that $1,25(OH)_2D_3$ may also protect cells from other unidentified photoproducts, including double strand breaks.

The pathway for photoprotection by $1,25(OH)_2D_3$ has not been established. We proposed that photoprotection against DNA damage by $1,25(OH)_2D_3$ is mediated by the steroid non-genomic pathway that is fast-acting and apparent within 0.5 h after treatment. Previous studies with synthetic vitamin D analogs that have fixed conformations support this proposal. For example, the 6-s-*cis* locked rapid-acting agonist 1,25-dihydroxylumisterol$_3$ that has no gene transactivating activity protected skin cells from UV-induced thymine dimers; whereas photoprotection against thymine dimers was not afforded by a non-genomic antagonist 1β, 25 dihydroxyvitamin D_3.[38,42,68] Furthermore, there was no suppression of the photoprotective effect of $1,25(OH)_2D_3$ against thymine dimers in skin cell cultures from patients with hereditary vitamin D resistant rickets that express a vitamin D receptor (VDR) with a defective DNA binding domain that fails to evoke a genomic response.[69] The activation of outwardly rectifying chloride currents by $1,25(OH)_2D_3$ is also required for photoprotection, as thymine dimers were not reduced by $1,25(OH)_2D_3$ in normal irradiated skin cells after treatment with a chloride channel blocker (DIDS).[70]

Although some of the reduced DNA damage after UV in the presence of $1,25(OH)_2D_3$ may be the result of reduced generation of DNA lesions, it is also likely that $1,25(OH)_2D_3$ enhances DNA repair processes. The rapid reduction of CPDs within 30 minutes by $1,25(OH)_2D_3$ cannot be explained entirely by enhanced DNA repair, as the NER pathway for CPD is relatively slow with a half-life of more than 7 h.[51,71–75] We previously proposed that the increased accumulation of nuclear p53 induced by $1,25(OH)_2D_3$ in UV irradiated keratinocytes, promoted an early and accelerated DNA damage response that upregulates DNA repair genes.[39] Whether nuclear upregulation of p53 is critical for these processes is unclear,[70] but keratinocytes from mice lacking a functional vitamin D receptor show markedly reduced clearance of CPDs and 6–4 photoproducts.[76] While we did not observe after UV or $1,25(OH)_2D_3$ treatment in human keratinocytes an increase in expression of XPG (xeroderma pigmentosum, complementation group G), an enzyme involved in NER,[41] it is a complex multistep process involving many different proteins that have not as yet been tested. A microarray study of $1,25(OH)_2D_3$-treated keratinocytes reported upregulation of two other key genes involved – XPC and XPE.[77] Abasic sites and 8-oxodG photoproducts are repaired by base excision repair, which has not been examined in relation to regulation by $1,25(OH)_2D_3$. As noted earlier, $1,25(OH)_2D_3$ reduces nitric oxide products,[39,44] which could otherwise inhibit DNA repair.[27]

Since DNA damage has been shown to be a mediator for the post UV events of mutagenesis and immune suppression that promote the development of skin cancer; and photoprotection by $1,25(OH)_2D_3$ against UV-induced immune suppression and experimental photocarcinogenesis has been demonstrated in Skh:hr1 hairless mice,[44] this work identifies a novel potential therapeutic target for decreasing DNA photoproducts using vitamin D compounds, which could be important for skin cancer prevention.

Abbreviations

CPD	cyclobutane pyrimidine dimer
Endo4	endonuclease IV
$1,25(OH)_2D_3$	1α,25 dihydroxyvitamin D_3
8-oxodG	8-oxo-7,8-dihydro-2′-deoxyguanosine
hOGG	human 8-oxoguanine DNA glycosylase
NO	nitric oxide
NOS	nitric oxide synthase
NER	nucleotide excision repair
RNS	reactive nitrogen species
ROS	reactive oxygen species
T4N5	T4 endonuclease V
SIN	Sin 1 chloride
SNP	sodium nitroprusside

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