UV-induced DNA Damage in Skin is Reduced by CaSR Inhibition

Chen Yang1, Mark Stephen Rybchyn1,2, Warusavithana Gunawardena Manori De Silva1,3, Jim Matthews3,4, Andrew J. A. Holland5,6, Arthur David Conigrave5 and Rebecca Sara Mason1,5

1Department of Physiology and Bosch Institute, School of Medical Sciences, University of Sydney, NSW, Australia
2School of Chemical Engineering, University of New South Wales, Sydney, NSW, Australia
3Sydney Informatics Hub, University of Sydney, Sydney, NSW, Australia
4Douglas Cohen Department of Paediatric Surgery, Faculty of Medicine and Health, The Children’s Hospital at Westmead Clinical School, The University of Sydney School of Medicine, Sydney, NSW, Australia
5School of Life and Environmental Sciences, Charles Perkins Centre (D17), University of Sydney, Sydney, NSW, Australia

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ABSTRACT

The epidermis maintains a cellular calcium gradient that supports keratinocyte differentiation from its basal layers (low) to outer layers (high) leading to the development of the stratum corneum, which resists penetration of UV radiation. The calcium-sensing receptor (CaSR) expressed in keratinocytes responds to the calcium gradient with signals that promote differentiation. In this study, we investigated whether the CaSR is involved more directly in protection from UV damage in studies of human keratinocytes in primary culture and in mouse skin studied in vivo. siRNA-directed reductions in CaSR protein levels in human keratinocytes significantly reduced UV-induced direct cyclobutane pyrimidine dimers (CPD) by ~80% and oxidative DNA damage (8-OHdG) by ~65% compared with control transfected cells. Similarly, in untransfected cells, the CaSR negative modulator, NPS-2143 (500 nM), reduced UV-induced CPD and 8-OHdG by ~70%. NPS-2143 also enhanced DNA repair and reduced reactive oxygen species (ROS) by ~35% in UV-exposed keratinocytes, consistent with reduced DNA damage after UV exposure. Topical application of NPS-2143 also protected hairless Skh: hr1 mice from UV-induced CPD, oxidative DNA damage and inflammation, similar to the reductions observed in response to the well-known photoprotection agent 1,25(OH)2D3 (calcitriol). Thus, negative modulators of the CaSR offer a new approach to reducing UV-induced skin damage.

INTRODUCTION

DNA damage is one of the key biological processes that contributes to UV-induced skin carcinogenesis (1,2). UVB (280–315 nm) directly excites the nucleobases in DNA and results in the oxygen-independent formation of two dimeric photoproducts: cyclobutane pyrimidine dimers (CPDs) and (6-4) pyrimidine-pyrimidines. In keratinocytes, UV exposure also induces production of reactive oxygen species (ROS), including superoxide (O2·−), singlet oxygen (1O2), hydrogen peroxide (H2O2) and the hydroxyl free radical (·OH) (3,4). Oxidative modifications to DNA, proteins, lipids and other cellular components occur when ROS levels exceed the capacity of intrinsic antioxidant defense mechanisms (5). Guanine is particularly susceptible to oxidative stress, forming 8-hydroxy-2′-deoxyguanosine (8-OHdG), due to its lower redox potential compared with the other three bases in DNA (6–8). Keratinocytes, like other mammalian cells, are equipped with DNA repair systems including base excision repair (BER), mismatch repair, double-stranded break repair and nucleotide excision repair (NER) (9). Inadequate repair of UV-induced DNA damage leads to genetic mutations and immune suppression, and in some cases progression to skin carcinogenesis (1,10–12).

The epidermis maintains a cellular calcium gradient that increases from the inner basal and spinous layers to the outer stratum corneum (13,14). This gradient supports outwardly orientated keratinocyte differentiation and, thus, the development of the stratum corneum. The CaSR in keratinocytes reads and responds to the cellular gradient either intracellularly, in organelles such as the endoplasmic reticulum (ER) and/or Golgi (15,16), where calcium ions are stored in millimolar concentration, or via its expression at the cell surface (13). Raising [Ca2+]i above 0.1 mM, causes a transient increase in [Ca2+]i through activation of the phospholipase C pathway leading to

Abbreviations:

| Abbreviation | Definition |
|--------------|------------|
| CPD | cyclobutane pyrimidine dimer; |
| ER | endoplasmic reticulum; |
| ROS | reactive oxygen species; |
| siRNA | small interfering RNA; |
| ssUV | solar-simulated UV irradiation; |
| UDS | unscheduled DNA synthesis; |
| VDR | vitamin D receptor; |

*Corresponding author email: rebecca.mason@sydney.edu.au (Rebecca Sara Mason)
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increased local levels of inositol 1,4,5-tris-phosphate (IP3) and consequent release of Ca2+ from intracellular stores in the ER and Golgi (17,18). Increased differentiation depends on a sustained phase of Ca2+ mobilization induced by elevated [Ca2+]c, which in turn seems to be due to increased Ca2+ influx through Ca2+ release-activated Ca2+ entry mediated by the store-operated channels. These are activated by the depletion of intracellular Ca2+ stores and activation of the IP3 receptor (19). Although thickening of the stratum corneum in response to UV exposure, due to keratinocyte proliferation and differentiation, is a natural defense mechanism (20,21), little is known about the possible role of intracellular or plasma membrane CaSR and its ligands in photobiology.

In contrast, it is well established that 1,25-dihydroxyvitamin D3 [1,25(OH)2D3 or 1,25D], the hormonally active form of vitamin D, is photoprotective. 1,25(OH)2D3 reduces UV-induced CDPs in human keratinocytes, melanocytes and fibroblast cultures, as well as in human skin in vivo and ex vivo (22–26). 1,25(OH)2D3 also decreases oxidative damage and 8-OHdG formation (26,27). When the vitamin D receptor (VDR) is knocked out, mice are more susceptible to chemical or UV-induced skin tumors (28,29) and mice in which there is tissue-selective double knock-out of the VDR and the calcium-sensing receptor (CaSR) in the epidermis exhibit spontaneous development of squamous cell carcinomas (30,31).

To investigate the role of the CaSR in the photo-sensitivity of skin keratinocytes, we used siRNA knockdown or treatment with NPS-2143, a negative modulator (calcilytic) of the CaSR (32). Calcilytics are well-known systemically for inducing acute stimulation of parathyroid hormone (PTH) secretion, which is under tonic inhibitory control by the CaSR (32), but their effects on the skin, and on UV-induced damage in particular, are unknown. Thus, we investigated the effects of CaSR knock-down and treatment with NPS-2143, on UV-exposed keratinocytes to investigate the role of the CaSR in photo-induced damage and its potential value as a target in photo-protection.

MATERIALS AND METHODS

Keratinocytes were cultured as previously described (23) from skin samples removed at elective surgery, with written informed consent from subjects or their parents/guardians, under University of Sydney Human Research Ethics Committee protocol no. 2015/063. Keratinocyte culture media was changed to DMEM without additives such as EGF and cholera toxin for 24 h before experiments to allow cells to become quiescent (33). DNA damage in vitro. Keratinocytes were seeded at a density of 20,000 cells well−1 in 5 mm glass coverslips (Menzelgläser) in 96-well plates and irradiated with an Oriel 1000W xenon-arc lamp solar simulator (Newport Corporation). The spectral output of this lamp has been described as in human skin (34). Irradiation used for this study was 400 mJ cm−2 UVB and 3600 mJ cm−2 UVA (4000 mJ cm−2). 1,25(OH)2D3 also increased differentiation in keratinocytes, which is tissue selective double knock-out of the VDR and the calcium-sensing receptor (CaSR) in the epidermis exhibit spontaneous development of squamous cell carcinomas (30,31).

Small interfering RNA transfection of keratinocytes. Primary human keratinocytes were seeded as described above. For siRNA experiments, at 80% confluence, the culture medium was replaced with Opti-MEM™ serum-free medium (ThermoFisher Scientific), 20 min prior to transfection. Transfection of siRNA was carried out according to the manufacturer’s instructions and as previously described (35). Caspase 3/7 siRNA (Catalogue number: sc-4344373), a pool of three target-specific siRNAs (sequence in Table S1) and scrambled control siRNA (siCtrl: sc-47741) (Santa Cruz). Briefly, siRNA at 25 nM were transfected into keratinocytes in the presence of 10% (v/v) lipid-based transfection reagent (35).Cell Signaling Technology). As previously described (27,34), Staining and image analysis for CPD and OHdG were assayed by immunohistochemical staining and image analysis as previously described (27,34). Staining and image analysis for CPD and OHdG produced similar results to those obtained using endonuclease digestion of the lesion, followed by Comet assay (27,35). DNA damage in vivo. All studies were approved by the Animal Ethics Committee of the University of Sydney (project no. 2015/794). Housing conditions and the solar simulator used for irradiation of mice have been described previously (24). Mice were subjected to a single exposure of 3 minimal erythemal doses of UV (UVB value at 399 mJ cm−2) (24,36). Mice were treated topically over 7 cm2 on the irradiated dorsal skin with 100 μL of the vehicle only, ethanol:propylene glycol:water 2:1:1 as previously described (24) or with 1,25(OH)2D3 or NPS-2143 immediately after irradiation. The dose of NPS-2143 was chosen as equivalent to 20X (228 pmol cm−2) or 200X (2280 pmol cm−2) that of an effective dose of 1,25(OH)2D3 11.4 pmol cm−2. Biopsies (2 cm × 2 cm squared dorsal skin) were taken from UV-irradiated, treatment applied dorsal skin 3 h post-UV, paraffin-embedded and processed for CPD as previously described (24). Non-irradiated samples as SHAM control were obtained from the abdomen. Three areas of each section were analyzed (triplicates). Other mice, subjected to the same UV exposure and treatments had dorsal skin thickness measured with calipers as previously described (24) before (pre-irradiation day zero) and up to 7 days post-UV.

Unscheduled DNA synthesis. Incorporation of 5-ethynyl-2′-deoxyuridine (EdU) into nuclear DNA was detected using Click™ EdU Alexa Fluor 488 Imaging Kit (Catalogue number A0303) according to the manufacturer’s instructions. Incorporation of this thymidine analog in a punctate pattern, in keratinocytes cultured without growth factors, measures unscheduled DNA synthesis rather than DNA replication (38). Visualization of
nuclear EdU-incorporation was carried out by confocal laser microscopy (LSM 510 Meta; Zeiss, Oberkochen Germany). Densitometry was performed using Image J software (National Institute of Health, Maryland). Stained cells were manually counted from the images taken by Zeiss LSM 510 Meta confocal microscope. Fluorescence intensity was measured in cells that were above the detection threshold and considered “UDS positive.”

**ROS measurement.** ROS levels were determined using the ROS-Glo™ H₂O₂ Assay (Promega, WI) according to the manufacturer’s instructions, as described previously (34).

**Statistical analyses.** The results are based on triplicates of each treatment with keratinocytes from different donors in each experiment, unless otherwise stated. Data were normalized to SHAM of each experiment and pooled from three or more experiments, expressed as mean ± SEM, unless otherwise indicated. All statistical analyses were performed using the GraphPad Prism version 8.0 statistical program (GraphPad Software Inc.). Unless otherwise stated, the analysis of comparisons between treatment groups was made using Mixed Effects Model analysis with Sidak’s post-comparison test.

### RESULTS

**siRNA-directed knockdown of CaSR reduced UV-induced DNA damage in primary human keratinocytes**

Human primary keratinocytes were transfected with small interfering RNA (siRNA) targeting CaSR (siCaSR) or non-specific siRNA (siCtrl). siCaSR transfection reduced CaSR protein to ~40% (P < 0.0001) of the control transfected cells (Fig. 1a). In the

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**Figure 1.** siRNA-mediated reduction in CaSR protein levels reduced UV-induced DNA damage in primary human keratinocytes. (a) Western blot image and densitometry of CaSR densities 48 h post siRNA transfection, at the time of UVR exposure. Expression was normalized to siCtrl to pool data from three independent experiments. α-Tubulin was a loading control. ****P < 0.001 when compared with CaSR protein level under siCtrl condition. DNA damage (b) CPD or (c) 8-OHdG (y-axis) by immunohistochemistry and image analysis in either siCtrl or siCaSR transfected keratinocytes at 3 h post-UV with or without 1,25(OH)₂D₃ (1,25D). Data were shown as mean ± SEM (n = 9) relative to SHAM (non-irradiated). ***P < 0.001, **P < 0.01 and *P < 0.05, when compared with siCtrl UV + vehicle; n.s., not significant among treatments. ####P < 0.0001 significantly different from UV + vehicle. Photomicrographs of (d) CPD and (e) 8-OHdG in the presence of siCtrl or siCaSR. Black arrows point to the dark brown staining in nuclei indicating the presence of CPD or 8-OHdG. Scale bar = 100 μm.
presence of siCtrl, UV induced substantial increases in CPD levels by ~500-fold relative to non-irradiated cells (siCtrl SHAM) (Fig. 1b,d) and a well-recognized photoprotective agent, 1 nM 1,25(OH)2D3 (22,23,26) added immediately after UV exposure, suppressed UV-induced CPD levels by ~75% (P < 0.01) (Fig. 1b, d). In siCaSR-transfected keratinocytes exposed to UV, CPD levels were significantly reduced in both the absence and presence of 1,25(OH)2D3 to levels observed in non-irradiated cells (siCaSR SHAM) (P < 0.01, Fig. 1b,d). A similar pattern of reduction was observed in UV-induced oxidative DNA damage as measured by 8-OHdG in response to the siCaSR condition (Fig. 1c,e).

**NPS-2143 protected keratinocytes against UV-induced DNA damage**

We then tested the effects of various concentrations of NPS-2143 on UV-induced CPD levels in keratinocytes. NPS-2143 was used in the range 5 pM to 500 nM. This concentration range was chosen based on previous results from studies in HEK-293 cells, which showed an antagonistic NPS-2143 response (32). In our primary keratinocytes, NPS-2143 had no effect in the range 5 pM–0.5 nM but suppressed UV-induced CPD by ~50% at all concentrations tested in the range 5 nM to 500 nM (P < 0.001, Fig. 2). From these results, a fixed NPS-2143 concentration of 500 nM was chosen for subsequent experiments.

We next tested the effect of extracellular calcium (Ca2+o) concentration on UV-induced DNA damage and its sensitivity to NPS-2143. Raising Ca2+o from its baseline level of 0.16 mM to 1.0 mM or 2.0 mM had no effect on the UV-induced increase in CPD or 8-OHdG levels in human keratinocytes (Fig. 3). NPS-2143 (500 nM) significantly reduced UV-induced CPD (P < 0.01) and 8-OHdG (P < 0.001) levels in the presence of all three Ca2+o concentrations tested (Fig. 3).

**NPS-2143 enhanced DNA repair after UV**

We investigated whether NPS-2143 promoted DNA repair in the context of UV-induced damage using the unscheduled DNA synthesis (UDS) assay (39), which measures incorporation of the fluorescent thymidine analog 5-ethynyl-20-deoxyuridine (EdU) into DNA. In these quiescent cells, a punctate pattern of staining was observed, indicative of DNA repair (38). Ninety minutes after UV exposure, staining remained low in vehicle-treated keratinocytes. Treatment with either 1,25(OH)2D3 (1 nM) or NPS-2143 (500 nM) increased the proportion of UDS positive cells (P < 0.05 for both) (Fig. 4a,b) and the average intensity of fluorescence per positive cell (P < 0.05 for both) (Figure S1) at this time after UV exposure.

**NPS-2143 reduced UV-induced oxidative stress**

ROS were then measured in keratinocytes after UV challenge, at a dose which induces oxidative DNA damage (4). Test treatments were again added immediately after UV exposure, as in previous experiments. At 15 min post-UV irradiation, ROS levels were ~35% lower in cells treated with 1 nM 1,25(OH)2D3 (P < 0.05) or with 500 nM NPS-2143 (P < 0.05) when compared with vehicle-treated cells (Fig. 4c). At 90 min post-irradiation, however, whereas 1,25(OH)2D3 continued to significantly suppress ROS levels by 25%, ROS levels in cells treated with NPS-2143 were not significantly different from either cells exposed to

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**Figure 2.** Suppression of UV-induced CPD by NPS-2143 in human keratinocytes. (a) CPD in vitro by immunohistochemistry and image analysis at 3 h post UV with treatments as indicated. Media contained 1.0 mM Ca2+o. Data were shown as mean + SEM (n = 9). ****P < 0.0001, ***P < 0.001, n.s. not significant, when compared with UV+vehicle. n.s. (with bar) not significantly different from one another. ####, P < 0.0001 significantly different from UV+vehicle. b) Photomicrographs of CPD in vitro b). Black arrows point to the dark brown staining in nuclei indicating the presence of CPD. Scale bar = 100 µm.
the vehicle control or to 1,25(OH)₂D₃ at that time-point
(Fig. 4c).

NPS-2143 protected mouse skin against UV-induced DNA damage and edema

Although keratinocytes in primary cell culture provide a powerful approach for studying epidermal biology, they imperfectly model the various cell types and three-dimensional topology of the epidermis (40). NPS-2143, painted topically on Skh:hr1 mice immediately after exposure to 3 minimal erythema doses (3MED) of ssUV at two doses (228 pmol cm⁻² and 2280 pmol cm⁻²), caused significant reductions in the levels of both CPD and 8-OHdG (P < 0.001), in a manner similar to 1,25(OH)₂D₃ (Fig. 5a–d). Skin edema is an inflammatory response to UV irradiation. We observed that skinfold thickness increased daily reaching a maximum at day 4 post-ssUV, at approximately twice the normal skinfold thickness. It then decreased gradually towards baseline. As shown in Fig. 5e, on day 4 after UV exposure, dorsal skin thickness was significantly reduced by treatment with NPS-2143 (P < 0.01) or 1,25(OH)₂D₃ (P < 0.01).

DISCUSSION

Although the CaSR is required for keratinocyte differentiation and barrier formation in skin (13,17,41), its significance for the modulation of DNA damage and repair after UV exposure is unknown. The results presented in this study provide evidence that the CaSR protein itself modulates UV-induced DNA damage and repair in skin. Thus keratinocytes in which (1) the level of CaSR protein has been reduced prior to UV exposure; or (2) treated with its negative modulator NPS-2143 shortly after UV exposure, exhibited significantly attenuated UV-induced increases in CPD and 8-OHdG. The results demonstrate that NPS-2143
was maximally effective in reducing UV-induced DNA damage at a concentration of 50–500 nM, consistent with its stimulatory impact on PTH in other cell types (32).

What might contribute to reduced DNA damage in the presence of NPS-2143?

CPD generation predominantly arises from direct absorption of UV by thymidine bases in DNA (42). 8-OHdG generation arises from oxidative damage to deoxyguanosines in DNA from excess ROS released by UV-damaged mitochondria (4,43). Increased repair of both CPD and 8-OHdG is critical (44,45). Under the conditions of this experiment, where the keratinocytes are not proliferating in the absence of growth factors, unscheduled DNA synthesis can be used to quantify DNA repair (39). NPS-2143, like 1,25(OH)2D3 (34), enhanced DNA repair, evidenced by increased incorporation of fluorescence-labeled thymidine analog, 5-ethynyl-2′-deoxyuridine (Fig. 4, Figure S1). NPS-2143 has been reported to inhibit epidermal wound healing in mice (46), consistent with an inhibitory effect on keratinocyte proliferation. Reduced rates of proliferation associated with arrest in G2, provide increased time for DNA repair (47). NPS-2143 has also been reported to suppress the proliferation of other cell types, such as colon cancer cells (48).

NPS-2143 may reduce UV-induced DNA damage via suppression of ROS production. In this study, keratinocytes treated immediately after exposure to UV with either 1,25(OH)2D3 or NPS-2143 exhibited significantly lower levels of ROS (Fig. 4c). Excess intracellular ROS, arising from UV-exposed mitochondria (4,49), directly promotes the formation of 8-OHdG (50). Excess ROS has also been reported to impair DNA repair by damaging DNA repair proteins (51). We were unable to detect a change in the level of the key DNA repair protein, xeroderma pigmentosum, complementation group C, also known as XPC, by western blotting in UV-exposed keratinocytes treated with NPS-2143 (data not shown). Recent evidence suggests that rates of dissociation of XPC from damaged DNA, rather than the total amount of XPC, determines the rate of DNA repair following UV exposure (52). NPS-2143 treatment or CaSR knockdown have also been reported to reduce ROS production stimulated by exposure to melamine in kidney tubular cells (53).

Significant reductions in UV-induced CPD and 8-OHdG were also observed in this study after treatment with topical NPS-2143 in mice, indicating that it is effective on UV-exposed native skin in vivo as well as on UV-exposed keratinocytes in vitro. The protection by NPS-2143 against UV-induced DNA damage in vivo was similar in magnitude to that produced by the well-described photoprotective agent, 1,25(OH)2D3 (22,23,26). In these mice, NPS-2143 also reduced UV-induced inflammation (Fig. 5e). A consistent inhibitory effect of NPS-2143 on inflammation in several other systems has been reported, with reported mechanisms that include reduced activation of the NLRP
inflammasome (54–56) and reductions in the pro-inflammatory cytokine IL-6 (57–60).

In mouse studies, which examined effects of CaSR knock-down (13,17,41), 90% knockdown of the CaSR protein in the epidermis eventually led to disruption of the epidermal permeability barrier. Treatment of immortalized keratinocytes with NPS-2143 has also been reported to inhibit some measures of cellular differentiation (61). In this study, siRNA knockdown of

Figure 5. NPS-2143 inhibits UV-induced DNA damage and skin edema in mice following acute UV irradiation. Mice were exposed to 3 MED of ssUV and immediately treated on the UV-irradiated dorsum with vehicle, 1,25(OH)2D3 or NPS-2143 as indicated. (a) CPD or (c) 8-OHdG by immunohistochemistry and image analysis in female SKH:hr1 mice, at 3 h post-UV exposure, three mice per group, with similar results obtained in a second set of mice. ****P < 0.0001, ***P < 0.001, **P < 0.01, *P < 0.05, compared with UV + vehicle, n.s. (with bar) not significantly different from one another. ####, P < 0.0001 significantly different from UV + vehicle. Photomicrographs of CPD (b) or 8-OHdG (d) in mouse skin. Black arrows point to the dark brown staining in nuclei indicating the presence of CPD or 8-OHdG. Scale bar = 100 μm. (e) Skin edema was measured as skinfold thickness on the 4th day after UVR exposure. Data were expressed as mean change in dorsal skinfold thickness ± SEM compared with non-irradiated skinfold thickness. **P < 0.01 and *P < 0.05 when compared with UV + vehicle group. n = 5 mice per group.
CaSR in keratinocytes was only 60%. The inhibition of the CaSR by treatment with NPS-2143 enhanced keratinocyte survival after UV, as measured by cell titler blue, in a similar manner to 1,25(OH)\textsubscript{2}D\textsubscript{3} (Figure S2). Furthermore, in vivo functional outcomes after treatment with NPS-2143 were similar to those seen with the known photoprotective agent, 1,25(OH)\textsubscript{2}D\textsubscript{3}. This indicates that even if some epithelial function was altered after application of topical NPS-2143 to mice, it did not prevent the significant reductions in UV-induced CPD or oxidative DNA damage or in skin edema, a marker of inflammation, observed with this agent.

At first sight, the findings of photoprotective effects of CaSR knockdown or pharmacologic inhibition in human keratinocytes are inconsistent with reports that mice with epidermal double knockout of the VDR and CaSR spontaneously develop squamous cell carcinomas (31,30). These tumors developed in the context of low calcium diets, however, and were not UV associated (31,30). Studies of UV-induced DNA damage in mice with single or double epidermal knockout of the VDR and/or CaSR may help to identify the mechanisms.

**Is intracellular CaSR involved?**

UV exposure results in increased keratinocyte proliferation and differentiation leading to increased production of the protective stratum corneum at the surface of the skin (62). However, it is not clear which components of the UV-induced molecular response including: (1) the production of CPD, ROS and 8-OHdG; (2) local activation of 1,25(OH)\textsubscript{2}D\textsubscript{3} synthesis and possibly; (3) local activation of the intracellular or plasma membrane CaSR are required for keratinocyte proliferation and differentiation.

The CaSR is synthesized in the ER and its N-terminus is delivered into the ER lumen where it is subjected to co-translational glycosylation and quality control checkpoints leading to its release and trafficking to the plasma membrane (63). Many activities of the CaSR are dependent on its expression at the plasma membrane, where it is activated by Ca\textsuperscript{2+} and other nutrients, including L-amino acids, and modulators including the polyamine spermine (64,65) and inhibited by polyvalent anions including inorganic phosphate (66). CaSR expression has also been demonstrated in pre-plasma membrane compartments in various cell types, including keratinocytes, revealing the existence of an intracellular reservoir of CaSR (18,48,67–70). Previously it was assumed that the intracellular CaSR is inactive and held in storage for transit to the plasma membrane. More recently, immature ER-localized CaSR has been reported to mediate a range of cellular actions (18,48,71) including in keratinocytes (18).

NPS-2143 is an uncharged aromatic compound that is lipophilic and thus expected to penetrate intracellularly (71,72) to impact the function of both plasma membrane and intracellularly located CaSR. In an extension of these studies, we used tunicamycin to prevent N-linked glycosylation of the CaSR in primary human keratinocytes and thus impair trafficking to the plasma membrane. Consistent with this idea, tunicamycin markedly suppressed CaSR expression in the plasma membrane (Figure S3). Interestingly, tunicamycin had no effect on NPS-2143 dependent suppression of UV-induced 8-OHdG but almost abolished NPS-2143-dependent reductions in UV-induced CPD (Figure S4a–d). These results suggest that UV-induced 8-OHdG protection is dependent on an intracellular pool of the CaSR, whereas UV-induced CPD protection requires CaSR expressed in the plasma membrane. Also consistent with this idea was the lack of effect of raised medium Ca\textsuperscript{2+} concentration (to 2 mM) on DNA damage in the either the absence or presence of NPS-2143.

In conclusion, CaSR knockdown or exposure to the CaSR inhibitor NPS-2143 protected human keratinocytes and native mouse skin against UV-induced DNA damage including the direct production of CPD and ROS-dependent production of 8-OHdG to a similar extent to that provided by 1,25(OH)\textsubscript{2}D\textsubscript{3}. The photoprotective activity of NPS-2143 arose at least in part from enhanced DNA repair and suppressed production of ROS. Regarding the mechanisms by which the CaSR modulates UV-induced skin damage, studies with tunicamycin support the proposal that both plasma membrane and intracellular pools of CaSR mediate the suppressive effects of its negative modulator NPS-2143. Further investigation is required to identify the specific signaling mechanisms that support these two distinct processes. Importantly, this study identifies the CaSR as a novel target in strategies aimed at protecting the skin against UV-induced DNA damage.

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**CONFLICTS OF INTEREST**

The authors state that they have no conflicts.

**SUPPORTING INFORMATION**

Additional supporting information may be found online in the Supporting Information section at the end of the article:

**Figure S1.** NPS-2143 promoted repair.

**Figure S2.** NPS-2143 and 1,25(OH)\textsubscript{2}D\textsubscript{3} enhanced cell survival after UV.

**Figure S3.** Tunicamycin reduced expression of CaSR at the plasma membrane.

**Figure S4.** Tunicamycin pre-treatment had no effect on NPS-2143 suppression of UV-induced 8-OHdG.

**Table S1.** siRNA duplex sequences obtained from Santa Cruz Biotechnology.

**REFERENCES**

1. Kripke, M. L., P. A. Cox, L. G. Alas and D. B. Yarosh (1992) Pyrimidine dimers in DNA initiate systemic immunosuppression in UV-irradiated mice. *Proc. Natl Acad. Sci.* 89, 7516–7520.

2. Nakagawa, A., N. Kobayashi, T. Muramatsu, Y. Yamashina, T. Shirai, M. W. Hashimoto, M. Ikenaga and T. Mori (1998) Three-dimensional visualization of ultraviolet-induced DNA damage and its repair in human cell nuclei. *J. Invest. Dermatol.* 110, 143–148.

3. Heck, D. E., A. M. Vetrano, T. M. Mariano and J. D. Laskin (2003) UVB light stimulates production of reactive oxygen species: Unexpected role for catalase. *J. Biol. Chem.* 278, 22342–22346.

4. Aitken, G. R., J. R. Henderson, S. C. Chang, C. J. McNeil and M. A. Birch-Machin (2007) Direct monitoring of UV-induced free
radical generation in HaCaT keratinocytes. *Clin Exp Dermatol* **32**, 722–727.

5. Pattison, D. I. and M. J. Davies (2006) Actions of ultraviolet light on cellular structures. *EXS* **131**, 131–157.

6. Peak, M. J., J. G. Peak and B. A. Carnes (1987) Induction of direct and indirect single-strand breaks in human cell DNA by far- and near-ultraviolet radiations: action spectrum and mechanisms. *Photochem. Photobiol.* **45**, 381–387.

7. Peak, M. J., J. G. Peak and C. A. Jones (1985) Different (direct and indirect) mechanisms for the induction of DNA-protein crosslinks in human cells by far- and near-ultraviolet radiations (290 and 405 nm). *Photochem. Photobiol.* **42**, 141–146.

8. Cadet, J., A. Grand and T. Douki (2014) Solar UV radiation-induced DNA-biarylmidine photoproducts: formation and mechanistic insights. In *Photoinduced Phenomena in Nucleic Acids II* (Edited by M. Bar-batti, Borin A. and Ullrich S.), Vol 356, pp. 249–275. Topics in Cur rent Chemistry. Springer, Cham, Cham, Germany.

9. Matsunaga, Y. and H. N. Ananthaswamy (2002) Short-term and long-term cellular and molecular events following UV irradiation of skin: Implications for molecular medicine. *Expert Rev. Mol. Med.* **4**, 1–22.

10. Peak, M. J., M. L. Fisher (1976) Immunochemical parameters of ultraviolet carcinogenesis. *J. Natl Cancer Inst.* **57**, 211–215.

11. Applegate, L. A., R. D. Ley, J. Alcalay and M. L. Kripke (1989) Identification of the molecular target for the suppression of hypersensitivity by ultraviolet radiation. *J. Exp. Med.* **170**, 1117–1131.

12. Halliday, G. M. (2005) Inflammation, gene mutation and photoimmunosuppression in response to UVR-induced oxidative damage contributes to photocarcinogenesis. *Mutat. Res.* **571**, 107–120.

13. Tu, C. L., D. A. Crumrine, M. Q. Man, W. Chang, H. Elalieh, M. You, P. M. Elias and D. D. Bikle (2012) Ablation of the calcium-sensing receptor in keratinocytes impairs epidermal differentiation and barrier function. *J Invest. Dermatol.* **132**, 2350–2359.

14. Lee, S. E. and S. H. Lee (2018) Skin barrier and calcium. *Annu. Dermatol.* **30**, 265–275.

15. Leach, K. F., M. Hannan, T. M. Josephs, A. N. Keller, T. C. Moller, D. T. Ward, E. Kallay, R. S. Mason, R. V. Thakker, D. Riccardi, A. D. Conigrave and H. Brauner-Osborne (2020) International union of basic and clinical pharmacology. CVIII. Calcium-sensing receptor nomenclature, pharmacology, and function. *Pharmacol. Rev.* **72**, 558–604.

16. Komuves, L., Y. Oda, C. L. Tu, W. H. Chang, C. L. Ho-Pao, T. Mauro and D. D. Bikle (2002) Epidermal expression of the full-length extracellular calcium-sensing receptor is required for normal keratinocyte differentiation. *J Cell Physiol.* **192**, 45–54.

17. Tu, C. L. and D. D. Bikle (2013) Role of the calcium-sensing receptor in calcium regulation of epidermal differentiation and function. *Best Pract. Res. Clin. Endocrinol. Metab.* **27**, 415–427.

18. Tu, C. L., W. Chang and D. D. Bikle (2007) The role of the calcium sensing receptor in regulating intracellular calcium handling in human epidermal keratinocytes. *J Invest. Dermatol.* **127**, 1074–1083.

19. Putney, J. W. Jr (2005) Capacitative calcium entry: Sensing the calcium stores. *J Cell Biol.* **169**, 381–382.

20. Brenner, M. and V. J. Hearing (2008) The protective role of melanin against UV damage in human skin. *Photochem. Photobiol. Photomed. 84*, 539–549.

21. Scott, T. L., P. A. Christian, M. V. Kesler, K. M. Donohue, B. Shelton, K. Wakamatsu, S. Ito and J. D’Orazio (2012) Pigment-inde pendent cAMP-mediated epidermal thickening protects against cutaneous UV injury by keratinocyte proliferation. *Exp. Dermatol.* **21**, 771–777.

22. Dixon, K. M., S. S. Deo, G. Wong, M. Slater, A. W. Norman, J. E. Bishop, G. H. Posner, S. Ishizuka, G. M. Halliday, V. E. Reeve and R. S. Mason (2005) Skin cancer prevention: A possible role of 1,25-dihydroxyvitamin D3 and its analogs. *J. Steroid. Biochem.* **97**, 137–143.

23. Gupta, R., K. M. Dixon, S. S. Deo, C. J. Holliday, M. Slater, G. M. Halliday, V. E. Reeve and R. S. Mason (2007) Photoprotection by 1,25 dihydroxyvitamin D3 is associated with an increase in p53 and a decrease in nitric oxide products. *J. Invest. Dermatol.* **127**, 707–715.
57. Zhai, T. Y., B. H. Cui, L. Zou, J. Y. Zeng, S. Gao, Q. Zhao, Y. Wang, W. L. Xie and Y. H. Sun (2017) Expression and role of the calcium-sensing receptor in rat peripheral blood polymorphonuclear neutrophils. Oxid. Med Cell Longev. 2017, 1–10.
58. Li, T. T., M. R. Sun, X. Yin, C. L. Wu, Q. Y. Wu, S. L. Feng, H. Li, Y. Luan, J. Wen, L. X. Yan, B. H. Zhao, C. Q. Xu and Y. H. Sun (2013) Expression of the calcium sensing receptor in human peripheral blood T lymphocyte and its contribution to cytokine secretion through MAPKs or NF-kappa B pathways (vol 53, pg 414, 2013). Mol. Immunol. 55, 429.
59. Wu, C.-L., Q.-Y. Wu, J.-J. Du, J.-Y. Zeng, T.-T. Li, C.-Q. Xu and Y.-H. Sun (2015) Calcium-sensing receptor in the T lymphocyte enhanced the apoptosis and cytokine secretion in sepsis. Mol. Immunol. 63, 337–342.
60. Hu, B., F. Tong, L. Xu, Z. Shen, L. Yan, G. Xu and R. Shen (2018) Role of calcium sensing receptor in streptozotocin-induced diabetic rats exposed to renal ischemia reperfusion injury. Kidney Blood Press Res. 43, 276–286.
61. Chen, Y., X. Li, X. Gan, J. Qi, B. Che, M. Tai, S. Gao, W. Zhao, N. Xu and Z. Hu (2019) Fucoidan from Undaria pinnatifida ameliorates epidermal barrier disruption via keratinocyte differentiation and CaSR level regulation. Mar Drugs 17.
62. Garnyn, M., A. R. Young and S. A. Miller (2018) Mechanisms of and variables affecting UV-induced apoptosis in human skin. Photochem. Photobiol. Sci. 17, 1932–1940.
63. Breitwieser, G. E. (2013) The calcium sensing receptor life cycle: trafficking, cell surface expression, and degradation. Best Pract. Res. Clin. Endocrinol. Metab. 27, 303–313.
64. Conigrave, A. D. and D. T. Ward (2013) Calcium-sensing receptor (CaSR): pharmacological properties and signaling pathways. Best Pract. Res. Clin. Endocrinol. Metab. 27, 315–331.
65. Broadhead, G. K., H. C. Mun, V. A. Avlani, O. Jourdain, W. B. Church, A. Christophouls, L. Delbridge and A. D. Conigrave (2011) Allosteric modulation of the calcium-sensing receptor by gamma-glutamyl peptides: inhibition of PTH secretion, suppression of intracellular cAMP levels, and a common mechanism of action with L-amino acids. J. Biol. Chem. 286, 8786–8797.
66. Centeno, P. P., A. Herberger, H. C. Mun, C. Tu, E. F. Nemeth, W. Chang, A. D. Conigrave and D. T. Ward (2019) Phosphate acts directly on the calcium-sensing receptor to stimulate parathyroid hormone secretion. Nat. Commun. 10, 4693.
67. Riccardi, D., M. Traebert, D. T. Ward, B. Kaissling, J. Biber, S. C. Hebert and H. Murer (2000) Dietary phosphate and parathyroid hormone alter the expression of the calcium-sensing receptor (CaR) and the Na+-dependent Pi transporter (NaPi-2) in the rat proximal tubule. Pflugers Arch. 441, 379–387.
68. Chattopadhyay, N., G. Legradi, M. Bai, O. Kifor, C. Ye, P. M. Vasilev, E. M. Brown and R. M. Lechan (1997) Calcium-sensing receptor in the rat hippocampus: A developmental study. Brain Res. Dev. Brain Res. 100, 13–21.
69. Riccardi, D., A. E. Hall, N. Chattopadhyay, J. Z. Xu, E. M. Brown and S. C. Hebert (1998) Localization of the extracellular Ca2+/polycation sensing-cation protein in rat kidney. Am. J. Physiol. 274, F611–622.
70. Bruce, J. L., X. Yang, C. J. Ferguson, A. C. Elliott, M. C. Stewart, R. M. Case and D. Riccardi (1999) Molecular and functional identification of a Ca2+ (polycation)-sensing receptor in rat pancreas. J. Biol. Chem. 274, 20561–20568.
71. Leach, K., K. J. Gregory, I. Kufareva, E. Khajehali, A. E. Cook, R. Abagyan, A. D. Conigrave, P. M. Sexton and A. Christopoulos (2016) Towards a structural understanding of allosteric drugs at the human calcium-sensing receptor. Cell Res. 26, 574–592.
72. Miedlich, S. U., L. Gama, K. Seuwen, R. M. Wolf and G. E. Breitwieser (2004) Homology modeling of the transmembrane domain of the human calcium sensing receptor and localization of an allosteric binding site. J. Biol. Chem. 279, 7254–7263.