CHAPTER 4

UVAI radiation inhibits calcineurin through oxidative damage mediated by photosensitization

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ABSTRACT

The protein phosphatase calcineurin has been gradually revealing itself as the central controller of our immune response, although it is involved in a wide array of signaling pathways related to cellular development and cell cycle progression. As such, calcineurin is an attractive, yet delicate therapeutic target for the prevention of allograft rejection and treatment of several inflammatory skin conditions. However, calcineurin activity is not only sensitive to immunosuppressants such as cyclosporin A and tacrolimus, but also subject to modulation by reactive oxygen species. We have recently shown, both *in vivo* and *in vitro*, that UVA1 radiation suppresses calcineurin activity. In this paper, we present evidence that this activity loss is due to singlet oxygen and superoxide generated by photosensitization, and show that a closely related phosphatase, PP2A, is not affected. Furthermore, a survey of this damage reveals oxidation of several Met and Cys residues as well as an overall conformational change. These findings provide a mechanistic basis for the hypothesis that UVA1 and calcineurin inhibitors both affect the same signal transduction pathway in skin.

INTRODUCTION

Calcineurin (protein phosphatase 3, Cn) is a calcium/calmodulin-dependent serine/threonine phosphatase enzyme, which has been recognized as a pivotal mediator of early immune response following antigen presentation to regulatory T cells. Calcineurin is activated by a rise in intracellular Ca^{2+} levels, accompanied by calmodulin binding; its primary substrate is the nuclear factor of activated T cells (NFAT). Dephosphorylation of NFAT instigates its translocation into the nucleus to arrange the transcription of several inflammation-related messenger genes. Among their products is interleukin-2 (IL-2), which plays a distinctive role in T cell recruitment (*1*). In addition, Cn has been ascribed roles in cellular growth and development (*2, 3*), mediation of apoptosis (*4-8*), and in DNA repair pathways (*9-12*). Novel functions and applications of NFAT signaling have been steadily surfacing (*13-16*).

Calcineurin is the principal target of the immunosuppressive drugs cyclosporin A (CsA) and tacrolimus (TRL) (*17*). Therapy based on these immunosuppressants has markedly reduced the incidence of transplant rejection in allograft recipients. In addition, tacrolimus and pimecrolimus have proven useful topically in patients suffering from chronic skin conditions such as psoriasis, lupus erythematodes, atopic eczema or

hypersensitivity reactions, by alleviating inflammatory symptoms (*18-21*). By now, however, much of the initial attractivity of Cn inhibitors (CnI) has faded away, due to the fact that patients treated with CnI exhibit a markedly increased incidence of keratinocyte malignancy on areas of their skin that receive frequent sunlight exposure (*22-24*). Although CnI have been indicated to directly promote tumor growth, metastasis and angiogenesis by increasing TGF-β and VEGF production (*25-29*), suppressed Cn signaling could be an important contributing factor as well.

Depending on intensity and duration of exposure, both stimulating and suppressing influences of UVA radiation on the immune system have been recognized (*30-34*). The inflammatory skin conditions mentioned above typically benefit from UVB phototherapy (*35*), but, intriguingly, similarly good results can be obtained with UVA phototherapy (*36*). For the latter, T-helper cell depletion following UVA-induced apoptosis has been proposed as a plausible operative mechanism (*37*). The common belief is that UVA, in contrast to UVB, exerts a significant part of its effects *via* reactive oxygen species (ROS) (*38, 39*). While massive bursts of ROS tend to be harmful to DNA, proteins and phospholipid membranes, smaller amounts perform notable roles as mediators and messengers in intricate signaling cascades, enabling the cell to adequately respond to situations at hand (*40, 41*). During the last two decades, several studies have shed light on possible ways of calcineurin (Cn) activity modulation by a variety of reactive oxygen species (*42-44*). Hydrogen peroxide has been found to precipitate calcium-dependent NFAT activation (*45, 46*) , while high concentrations of hydrogen peroxide and superoxide disrupt NFAT signaling by disabling Cn (*47*). The enzyme's active site is known to suffer iron loss following oxidation. There has been extensive speculation that the sensitivity of Cn to ROS constitutes a mechanism that allows for regulation of Cn phosphatase activity by the intracellular redox potential (*48, 49*). Still, the exact mode of action of and interplay between these species has not been fully elucidated. Our recent discovery, in skin and several cellular systems, that physiologically and therapeutically relevant doses of UVA1 radiation (0 - 450 kJ/m^2 (*50-52*)) irreversibly inactivate Cn (*53*), further substantiates the biological significance of Cn damage by ROS and could present an alternative explanation for the beneficial effects of UVA phototherapy on inflammatory skin disease. In addition, combined inhibition of Cn by ROS and CnI could have unanticipated effects that merit further study.

In this paper, we present a cumulative negative effect of the CnI tacrolimus and UVA1 radiation on Cn activity. We show that UVA1 radiation inhibits calcineurin specifically, albeit indirectly. Furthermore, we identify photosensitization as the primary mechanism involved in Cn inhibition following exposure to UVA1 and we chart the structural changes to the enzyme that may ensue from UVA1 irradiation.

EXPERIMENTAL PROCEDURES

Cell cultures

Fibroblasts were obtained from human foreskin and cultured on DMEM (Gibco) + 5% FCS (Invitrogen). After separation of epidermis and dermis by overnight treatment with dispase II (Roche, Mannheim, Germany) at 4°C, dermal explants were plated top-down in a small layer of DMEM + 5% FCS. The outgrowth was replated and the explants removed. Cells were weekly passaged 1 to 5 and used for the irradiation experiments at near confluence, between passages 8 and 18. Cultures were maintained at 37°C under 5% CO_2 atmosphere in an incubator.

UV irradiation

UVA1 experiments were performed using Sellas Sunlight lamps at 12.5 mW cm^{-2} output, combined with UVASUN blue filter (emission spectrum: 340-400 nm). For the UVB experiments, Philips TL12 lamps with an output of 0.56 mW cm^{-2} (emission spectrum: 275-375 nm, optimum at 312 nm) were employed. Doses were monitored using a IL700A Research radiometer with a WBS320#801 sensor. Fibroblasts were UVA-irradiated in triplicate in covered Greiner Petri dishes on ice. Prior to irradiation, DMEM medium had been supplemented with different concentrations of TRL (Sigma). After 1 hour, DMEM was replaced by HBSS (Gibco) without further dosing of TRL and irradiation was started. Immediately afterwards, cells were washed with PBS, detached with trypsin/EDTA (0.25%, Invitrogen), collected in PBS containing 5% FCS and centrifuged. Cells were resuspended in 10 mM Hepes-buffered saline (pH 7.5) and centrifuged once again. Trypan blue exclusion testing was used to evaluate cell viability. Solutions of recombinant Cn (obtained from Enzo Life Sciences, formerly Biomol, and containing 50 mM Tris pH 7.5, 100 mM NaCl, 6 mM $MgCl_2$, 0.5 mM $CaCl_2$, and 0.025% NP-40) and cellular lysates were irradiated in standard-volume 96-wells plates (50 µL / well) while kept on ice. Sodium azide (Merck) was dissolved in assay buffer and added to the enzyme solution shortly before irradiation. Directly after addition of riboflavin (Sigma), 8-methoxypsoralen (Sigma), or 5-methoxypsoralen (Aldrich, 99%), the plate was kept secluded from daylight. Deuterium oxide (Aldrich, 99.9% D) was stored under nitrogen. Manganese(II) chloride was purchased from Analar and added to the enzyme solution directly after irradiation.

Singlet oxygen generation

3,3'-(1,4-Naphthylidine)dipropionate (NDP) was purchased from Merck and converted to its endoperoxide ($NDPO_2$) by H_2O_2/MoO_4^{2-} oxidation (54), according to the manufacturer's instructions. $NDPO_2$ was then added to the media of fibroblast cultures. Singlet oxygen was generated by thermal dissociation of $NDPO_2$ at 37 °C (52).

Activity measurements

Calcineurin assays were performed under optimal conditions (excess Ca^{2+} and calmodulin) according to the method reported by Sellar *et al.* (55), implementing a few modifications. Freshly harvested cell pellets were dispersed in 50 mM Tris-HCl, pH 7.7, containing 5 mM ascorbic acid, 1 mM DTT, 0.02% NP-40 (Calbiochem) and protease inhibitors, and lysed by 3 quick freeze-thaw cycles. Protein concentration measurements in cellular lysates were based on the Bradford assay, using the Pierce Coomassie Plus Total Protein Assay (PerBio Science, Belgium). Samples were diluted to contain 200-300 mg L^{-1} protein. Lysates were stored at 4°C and kept in the dark. Enzyme activities in the diluted cellular lysates were determined by the release of phosphate from the RII substrate in presence and absence of excess EGTA, calmodulin and okadaic acid. Cn activity was defined as the calcium/calmodulin dependent okadaic acid insensitive phosphatase activity (55). PP2A activity, measured in the same assay, was defined as the okadaic acid inhibited calcium insensitive phosphatase activity (56). All enzyme activity values were corrected for protein content.

Circular dichroism

CD spectra were collected on a JASCO 715 spectropolarimeter equipped with a temperature controlled cuvette holder. Scanning range was 260 to 195 nm (50 nm/min, 0.1 nm interval, 5 nm slit width, 2 s response time, 20 iterations). Buffer spectra were subtracted from sample spectra. Protein samples (240 µL) contained 0.20 mg/mL Cn in 16 mM Tris pH 7.5, 31 mM NaCl, 1.9 mM $MgCl_2$, 1.6 mM DTT, 0.008% NP-40 and 0.16 mM $CaCl_2$.

Tryptic digestion and mass spectrometry

Calcineurin samples (5 µL) were diluted by adding 20 µL 25 mM ammonium bicarbonate. Subsequently, 3 µL 55 mM iodoacetamide was added and samples were kept at RT for 20 minutes. Tryptic digestion was then performed by adding 50 ng trypsin (Sequencing Grade Modified Trypsin, Promega, Madison, WI) and overnight incubation at 37 °C. One microliter of a tryptic digest was mixed with 1 µL of 10 mg/mL 2,5-dihydroxybenzoic acid (dissolved in acetonitrile/water 50:50 (MilliQ, Millipore) containing 0.1% TFA) directly onto a stainless steel MALDI target plate (Bruker Daltonics, Bremen, Germany) and allowed to dry.

MALDI-ToF-(ToF) mass analyses were performed on an Ultraflex II time-of-flight mass spectrometer controlled by FlexControl 3.0 software package (Bruker Daltonics). The MS acquisitions were performed in positive ion reflectron mode at a laser frequency of 100Hz. The scanner *m/z* range was up to 5000 and the matrix suppression (deflection) mode up to *m/z* 400. For the MS/MS analysis, precursors were accelerated and selected

in a time ion gate after which fragments arising from metastable decay were further accelerated in the LIFT cell; their *m/z* were analyzed after passing the ion reflector.

MALDI-FT-ICR spectra were recorded using a 9.4T Fourier Transform Ion Cyclotron Resonance mass spectrometer (Apex Q, Bruker Daltonics) equipped with a dual ESI/MALDI ion source (Apollo II), incorporating a quadrupole mass filter and a smartbeam laser system. All experiments used a laser spot size of approximately 150 µm and a laser repetition rate of 200Hz. Data were acquired using Bruker ApexControl 3.0.0 software. Spectra acquired on the MALDI-FT-ICR were internally calibrated using two trypsin autolysis products (*m/z* 1045.56370 and *m/z* 2211.10400) and four tryptic peptides from calcineurin (*m/z* 1007.5421, *m/z* 1247.6378, *m/z* 1783.8384 and *m/z* 1916.9811).

Statistics

Statistical operations were performed using GraphPad Prism v4.02. Details of analyses are provided in the figure legends.

RESULTS

Tacrolimus and UVA1 display supplementary effects on Cn activity but do not affect PP2A

To assess the specificity of UVA damage to Cn, we compared the effects of UVA1 radiation on both Cn (figure 1a) and PP2A (figure 1b), a closely related serine/threonine phosphatase, in human fibroblast cell cultures. As a reference, two series with different concentrations of the calcineurin inhibitor, tacrolimus, added to the culture medium were included in the experiment. Figure 1 shows PP2A to be affected neither by UVA radiation nor by tacrolimus (TRL), whereas Cn suffers from a cumulative decrease in activity.

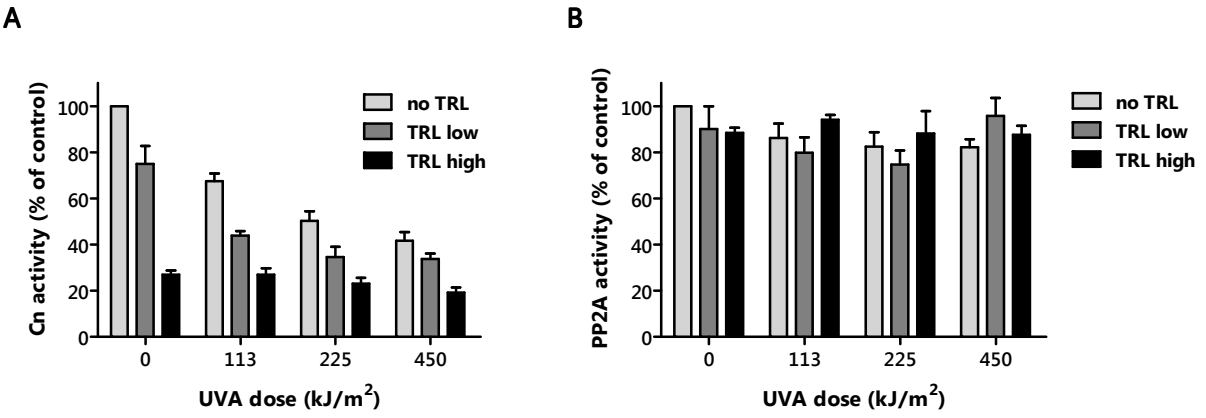


FIGURE 1 Effects of exposure of human fibroblasts to UVA1 radiation and tacrolimus on [A] Cn and [B] PP2A activity. (n=3, means ± SE) TRL low = 0.625 µg/L, TRL high = 62.5 µg/L in medium. Two-way ANOVA yielded $p < 0.0001$ for the effects of both factors (UVA and TRL) on Cn and $p > 0.2$ for their effects on PP2A.

Recombinant Cn is not directly inactivated by UVA1 radiation

In order to evaluate to what extent direct effects of UV radiation are involved in Cn deactivation, a solution of human recombinant Cn was subjected to UVA1 and UVB irradiation. Neither type of radiation showed any direct influence on enzyme activity, not even at high doses (figure 2a). As this implicates probable involvement of sensitizing species, the enzyme solution was incubated with 2 μM of riboflavin (vitamin B_2), which resulted in rapid and near-complete inhibition of enzyme activity upon UVA1 irradiation. Also, a Cn solution was treated with 1 μM of 5-methoxypsoralen (5-MOP) and 8-methoxypsoralen (8-MOP), both potent singlet oxygen generators well-known from cytotoxic PUVA therapy (*57, 58*). Both psoralens competently inhibited Cn phosphatase activity upon exposure to UVA1 (figure 2a). Effects were enhanced when experiments were performed in D_2O (figure 2b). Deuterium oxide enhances $^1O_2$ effects by allowing less of its excitation energy to dissipate to solvent molecules. Similar experiments were conducted in fibroblast lysates (figure 3). Irradiation with UVA1 doses up to 450 kJ/m^2 reduced Cn activity by nearly 50%. In presence of 2 μM of riboflavin, practically no Cn activity remained after 113 kJ/m^2 of UVA1. Again, PP2A activity was suppressed in neither case (figure 3).

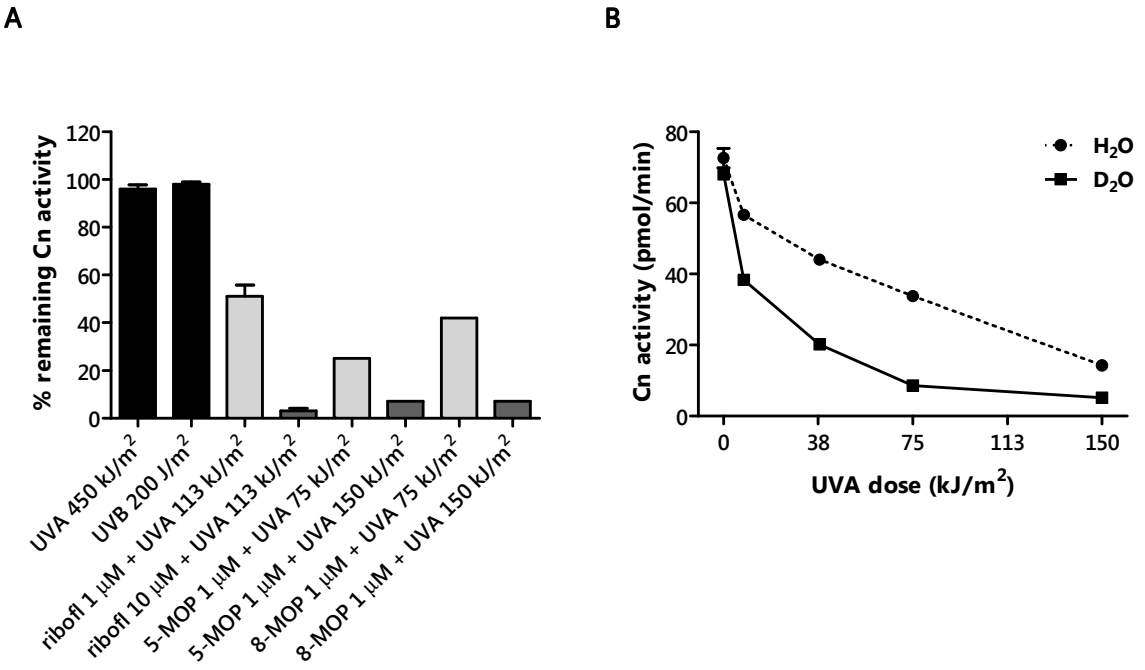


FIGURE 2 [A] Effects of UVA1, UVB and photosensitizers on recombinant Cn activity (n=3, means ± SE; psoralen data are singular). [B] Dose-response relation between UVA1 radiation and recombinant Cn activity in presence of 2 μM riboflavin in H_2O (circles) *vs.* D_2O (squares) (n=3, means ± SE). Two-way ANOVA yielded $p < 0.0001$ for all differences.

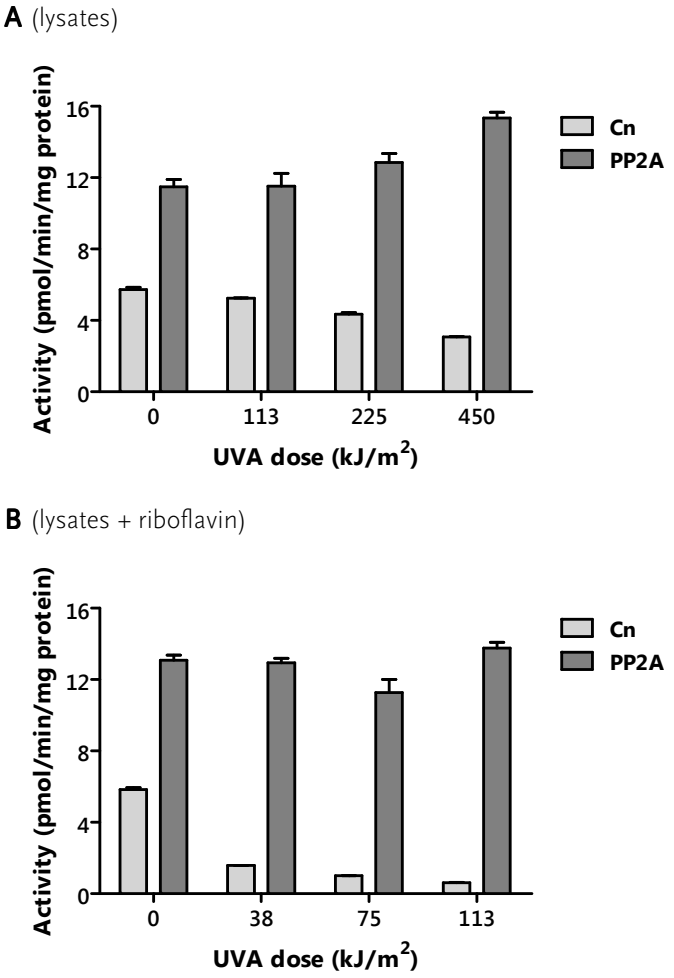


FIGURE 3 Cn and PP2A activity after exposure of human fibroblast lysates to [A] UVA1 radiation and [B] UVA1 radiation plus 2 μM riboflavin (n=3, means ± SE).

Singlet oxygen and superoxide both affect Cn activity

To confirm the involvement of singlet oxygen in Cn deactivation, fibroblast lysates were exposed to UVA1 radiation in the presence of sodium azide, a singlet oxygen quencher. Sodium azide (2.5 mM) proved protective against enzyme activity loss, as depicted in figure 4a. Addition of DTT or superoxide dismutase/catalase also attenuated activity loss, in contrast to catalase alone (data not shown). Interestingly, superoxide dismutase increased Cn activity in the unirradiated control samples by more than 75%. This may be in line with earlier findings suggesting that the protective effect of SOD on Cn traces back to partnering protein-protein interactions (59). Exposure of fibroblast cultures to singlet oxygen generated by thermal decomposition of $NDPO_2$ resulted in a dose-dependent decrease in Cn activity (see figure 4b). The other product of $NDPO_2$ decomposition, NDP, was excluded as a potential inhibiting factor to Cn. Using a lower concentration range of $NDPO_2$, Cn activity loss up to 20% (1.5 mM, 2h exposure) was found (not shown).

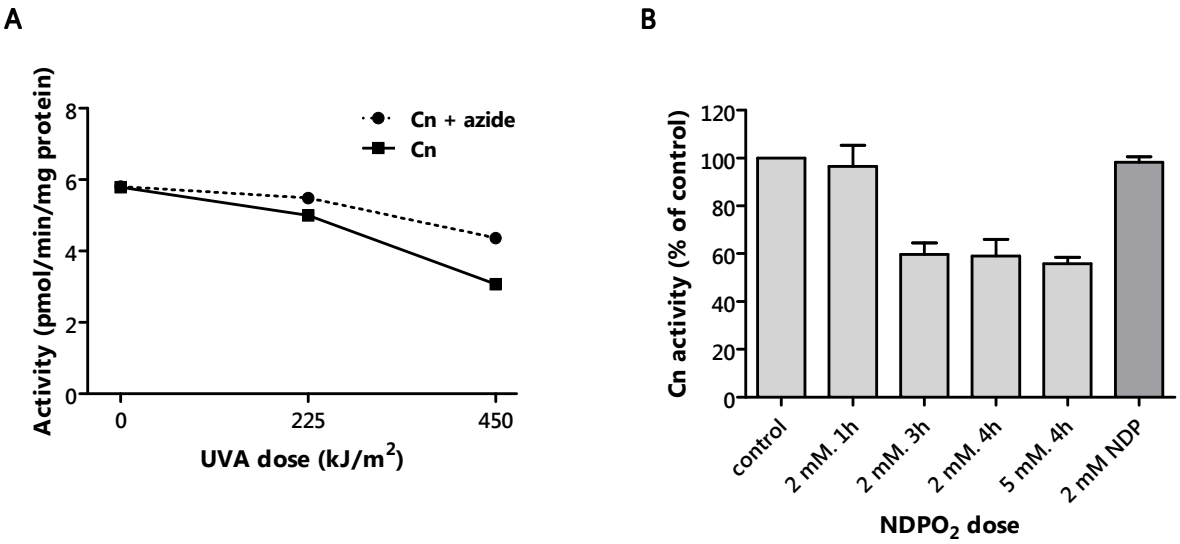


FIGURE 4 [A] Cn activity after UVA1 irradiation in both absence (squares) and presence (circles) of 2.5 mM sodium azide (n=3, means ± SE). Two-way ANOVA yielded $p < 0.0001$ for all differences. [B] Cn activity after exposure of human fibroblast cultures to singlet oxygen generated by thermal decomposition of $NDPO_2$ (n=3, means ± SE). Doses were chosen based on their effects in comparison to UVA irradiation as reported in earlier publications (*37*, *52*). NDP (2 mM, 4 h) was included as additional control. Unpaired t-test yielded $p < 0.001$ for the differences vs. control of the 3 h and 4 h samples.

Circular dichroism data reveals loss of helical content within Cn after exposure to UVA

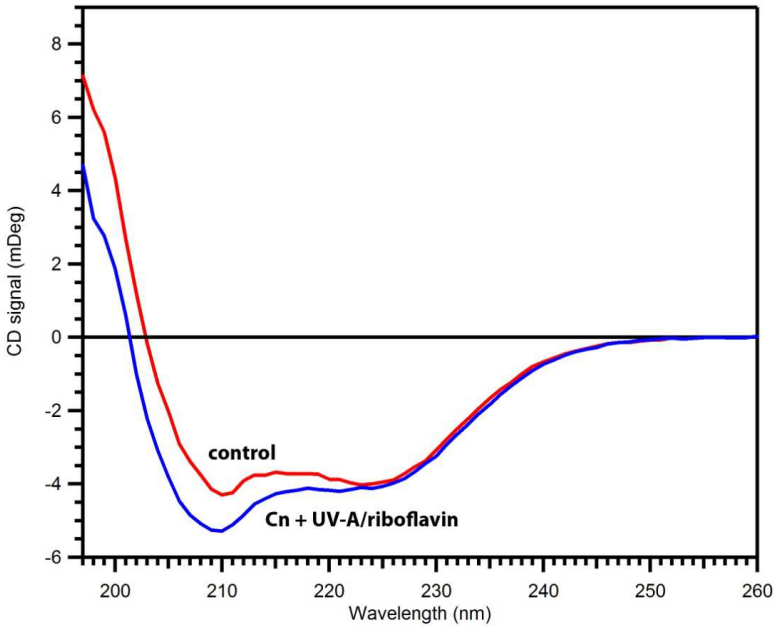
Oxidative damage to Cn may result in partial refolding of the enzyme to a different conformation. Using circular dichroism spectroscopy, we assessed the structural composition of calcineurin before and after treatment with UVA and riboflavin (1 μM). The secondary structure content was determined using the data from the CD spectra and the program CDNN (*60*). The most notable change after treatment was a reduction of the helical fraction (see figure 5). This most likely reflects modifications in or near a helical moiety of the enzyme.

UV induced oxidative modification of Cys and Met residues in recombinant Cn

In order to establish whether the loss of helical content as measured by CD spectroscopy could be due to oxidative alterations to specific amino acids, Cn was treated with 1 μM riboflavin and/or UVA1 (up to 113 kJ/m^2), and subsequently *S*-alkylated using iodoacetamide to convert unoxidized cysteines to carbamidomethylcysteines (CAM) and digested using trypsin. Mass spectrometric analysis of the digest showed that the combination of UVA and riboflavin strongly induced oxidation of several Met residues to their sulfoxides, although slight oxidation of Met residues was also observed in the control samples (data not shown). In addition, two Cys residues (CnB-Cys154 (figure 6) and CnA-Cys372 (suppl. fig. I)) oxidized to their cysteic acids were uncovered. A 3D-structure of the enzyme in which these Cys residues have been highlighted is provided as

supplementary material (suppl. fig. II). In figure 6, the loss of the signal at *m/z* 1920.95 in the UV exposed Cn sample, corresponding to the Cys_{CAM}-154 containing tryptic peptide of CnB (aa 144-168) in the control sample, coincides with the appearance of a peptide at *m/z* 1911.91, corresponding to the same peptide but containing a cysteic acid at position 154. This was confirmed using MALDI-ToF-ToF analysis and supported by similar analysis of an identical synthetic peptide (data not shown).

A



B

	control	Cn + UVA/riboflavin
helix	27.0%	22.8%
antiparallel	16.5%	18.4%
parallel	6.4%	5.9%
beta-turn	17.3%	18.0%
random coil	32.1%	32.3%
total sum	99.2%	97.4%

FIGURE 5 [A] CD spectra of untreated (red) and UVA1/riboflavin exposed (blue) Cn, and [B] calculated composition of the Cn tertiary structure.

Manganese(II) ion does not restore Cn activity after oxidative damage by UVA1

Since the oxidative inactivation of Cn can also be the result of iron loss from the active site, we assessed the significance of the found alterations of Met and Cys residues by exposing fibroblast lysates (figure 7a) and recombinant calcineurin (rCn, figure 7b) to UVA1 radiation and adding 1 mM $MnCl_2$ directly afterwards. After 10 minutes of incubation, Cn activity was determined. Mn^{2+}-ions can stimulate Cn by replacing Fe^{2+} in the active site. Although the presence of Mn^{2+} initially enhanced Cn activity, it still

underwent a dose-dependent decrease (see figure 7). These data indicate that the UVA1-induced suppression of Cn activity cannot be solely explained by oxidative release of iron from the active site.

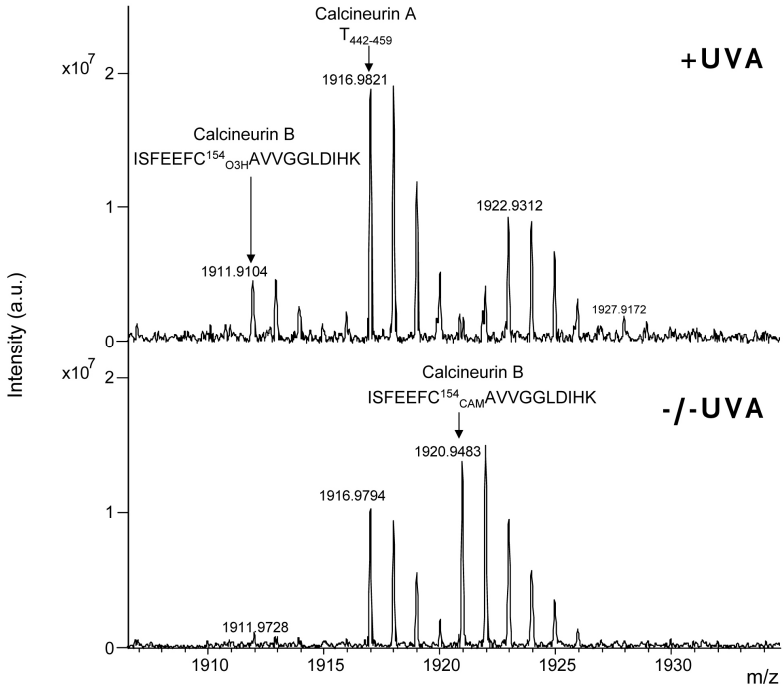


FIGURE 6 UV-induced oxidation of cysteine-154 in calcineurin B. Recombinant Cn was exposed to UVA1 in the presence of riboflavin, alkylated using iodoacetamide and digested with trypsin. MALDI-FT-ICR MS measurements revealed the oxidation of Cys-154 within the calcineurin B subunit in the UVA exposed sample (peptide at *m/z* 1911.91). In the non-exposed sample this cysteine was not oxidized and therefore converted to a carbamidomethylcysteine (CAM, peptide at *m/z* 1920.95) during iodoacetamide treatment. In both samples a normal tryptic peptide of calcineurin A (*m/z* 1916.98) was present.

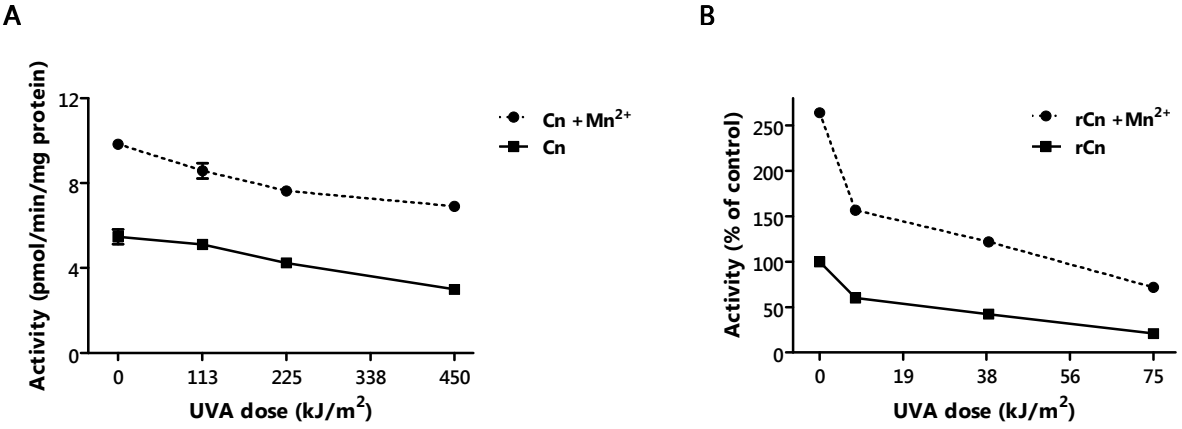


FIGURE 7 Cn activity in absence (squares) and presence (circles) of 1 mM $MnCl_2$ after exposure of [A] human fibroblast lysates and [B] recombinant Cn + 2 µM riboflavin to UVA1 radiation (n=3, means ± SE). T-test for differences between samples with and without Mn^{2+} yielded $p < 0.001$.

DISCUSSION

An increasingly popular explanation for the harmful effects of UVA on skin cells grants an important etiological role to endogenous photosensitizers such as flavins and porphyrins (*40, 61-66*). Absorbance of UVA radiation by these molecules results in formation of singlet oxygen (O_2 [$^1\Delta_g$]) *via* a type II mechanism, while superoxide ($O_2^{\bullet -}$) is generated alongside through a type I mechanism. Whereas effects of superoxide on Cn have been acknowledged, the exact response of Cn to $^1O_2$ has not yet been evaluated. In order to explore whether the drastic reductions in Cn activity following exposure to clinically relevant doses of UVA1 we encountered in a preceding study could be attributed to photosensitization and singlet oxygen, we used a model system with riboflavin as photosensitizing agent. In this system, exposure of 50 U of Cn to 113 kJ/m^2 of UVA1 radiation in the presence of 1 µM riboflavin led to 50% loss of phosphatase activity (figure 2). An even steeper decay of Cn activity was observed when experiments were performed in deuterium oxide, which serves as a singlet oxygen lifetime enhancer. Furthermore, azide ion, a powerful and selective physical quencher of singlet oxygen, displayed remarkable protection against Cn damage inflicted by UVA1. A combination of superoxide dismutase and catalase was found to boost Cn activity, while catalase by itself was ineffective, suggesting an additional role for superoxide. During photosensitization, additional contributions originating from H_2O_2 and HO• are to be expected; however, rather high concentrations of H_2O_2 are required to attain appreciable Cn activity loss (*47*), while our findings that PP2A is impervious to UVA1 radiation but is readily inactivated by HO• generated *in situ* (data not shown), makes a noteworthy role of HO• less likely as well. Finally, exposure of human fibroblasts to $NDPO_2$, a clean source of singlet oxygen, resulted in a decrease in Cn activity, which establishes a potential direct *in vivo* effect of $^1O_2$ on Cn.

To assess the damage to Cn characteristic of ROS produced through photosensitization, the UVA/riboflavin-exposed enzyme was trypsinized and subsequently analyzed by mass spectrometry, which unveiled a number of oxidative modifications to Cys and Met residues in both subunits. Met oxidation and Cys oxidation to the sulfonic acid are irreversible by mild methods. This may explain why addition of DTT after irradiation did not sort any effect. Both modified Cys residues are located in helical moieties – [CnB]Cys-154 can be found in a helix aligning one of the EF-hands, while [CnA]Cys-372 is part of the CaM binding helix – which could explain the apparent loss of helical structure in the oxidated enzyme. Further analysis and comparison of mass fragments together with molecular modeling studies might tell how the oxidative damage, conformational change and decreased enzymatic activity exactly interrelate.

Superoxide displays poor overall reactivity by itself. Most of its effects ensue from Haber-Weiss chemistry or reaction with NO radicals. Inhibitory effects of superoxide on

Cn activity have been known for several years (*42, 43*). However, the exact manner in which superoxide – and other ROS, for that matter – abolish Cn activity has been subject to debate. One of the topical issues is the configuration of the binuclear metal center of Cn (*67-69*). Bearing in mind that the electron configuration of superoxide preferably allows for one-electron reactions, such as with metal redox systems, inactivation of Cn could find its origin in alteration of the redox status of its bimetal center and subsequent loss of iron (*48*). Cu/Zn-superoxide dismutase has been found to protect the enzyme from shutting down as a consequence of the putative oxidative addition of superoxide to the active site iron following prolonged disclosure of the active site (*42, 43, 70*). Small shifts in the CD spectrum have been demonstrated upon addition or extraction of metal ions or oxidative damage to the binuclear centre (*71-73*); however, these changes do not overlap with the shift displayed in figure 5. Moreover, the limited effectiveness of Mn^{2+} in restoring full enzyme activity in irradiated samples suggests that the UVA1-induced damage to Cn extends beyond the binuclear centre and is not likely to be due to superoxide alone.

In fact, several alternative mechanisms of Cn inhibition by ROS have been proposed. One possible mode of inactivation is proteolytic cleavage, which was suggested as the principal mechanism in case of treatment of Cn with hydrogen peroxide (*74*). Inactivation could also be attained by means of oxidation of neighbouring cysteine residues to a disulfide, resulting in a conformational change (*72*) that generally may be counteracted by the enzyme thioredoxin (*75*). Carruthers *et al.* recently showed that oxidation of a crucial Met residue in the calmodulin binding domain of Cn could prevent its activation (*76*). Proteins generally constitute a major target for singlet oxygen. Owing to its electron configuration, $^1O_2$ is much inclined to participate in Diels-Alder- and ene-type reactions. Hence, amino acids featuring double bonds or aromatic systems (e.g. His, Trp, Tyr) are particularly susceptible to singlet oxygen attacks. The sulfur-containing amino acids Met and Cys readily succumb to $^1O_2$ as well, forming sulfoxides and disulfides (*77-79*). For example, oxidation of an active site cysteine is responsible for the inactivation of protein tyrosine phosphatase 1B by singlet oxygen (*80*). This underlines the capability of $^1O_2$ to interfere with signaling pathways.

The fact that malignancies in CsA-/TRL-treated patients predominantly manifest in skin has been reason to advise patients to avoid sunlight as much as possible. Although generally believed to be the result of p53 mutations caused by UVB (*81*), the incidence of non-melanoma skin cancers in these patients has been directly linked to lowered NFAT-regulated gene expression (*82, 83*). While physiological levels of Cn inhibition in lymphocytes by CnI alone typically do not exceed 30-35% in pharmacologically monitored patients (*84*), Cn inhibition levels up to 75% have been reported in skin keratinocytes using a large excess of CnI (*85*). On top of this, our results show a cumulative negative effect of UVA on Cn already inhibited by TRL. More signs of Cn vulnerability in skin cells come from PUVA therapy, in which UV radiation is

combined with extrinsic photosensitizers for treatment of psoriasis (*58*). Interestingly, PUVA is immunosuppressive (*86*); singlet oxygen is thought to be the key active agent in this therapy. Clinical cohort studies show an increased incidence of skin malignancies in patients undergoing recurrent PUVA treatment sessions (*87*), which becomes particularly apparent in patients already taking cyclosporin (*88*). This also suggests a reinforcing effect of CnI and ROS. In view of the importance of Cn as a central hub to multiple signaling cascades, low levels of Cn inhibition, attained by either CnI alone or CnI combined with UVA, as illustrated by cytokine levels in a PBMC model (*53*) could already have severe adverse effects in skin.

At the moment, however, the link between Cn/NFAT suppression and malignancy formation is still cloudy. First of all, the suppressed immune system could be directly accountable for tumor formation. Unfortunately, the precise effects of UVA radiation on the immune system remain occasionally paradoxical (*30*, *32*, *34*). Low doses of UVA have even been found to stimulate Cn (*89-91*). Alternatively, functions of Cn in the fields of apoptosis, proliferation and differentiation may be of pertinence here (*6*, *15*). The activity of the nuclear factor κB (NF-κB), another important transcription factor, is triggered by many of the same agents that modulate Cn activity and the possible mediation of NF-κB activation by Cn has already been addressed in various publications (*92-94*). Investigations into the role of Cn in the UVA-induced decrease in NF-κB activity in keratinocytes (*95*) might prove worthwhile. Very recently, Wu *et al.* showed that proper functioning of the Cn/NFAT pathway is essential for p53- and senescence-associated mechanisms that protect against the development of squamous cell carcinoma (*96*). Lastly, any secondary targets of the CnI may increase the predisposition to UVA-induced malignancy formation. CsA, for example, has been shown to interact with the mitochondrial permeability transition pore and influence DNA repair and TGF-β synthesis (*97*).

In conclusion, this work establishes a strong susceptibility of Cn activity to singlet oxygen and superoxide. We suggest that inhibition of Cn by UVA1 radiation results from oxidative damage to both the active site and other enzyme regions or residues, accompanied by a conformational change. Future studies are aimed to a more definitive characterization of the relation between cysteine oxidation, conformational change and activity loss. Cn suppression could result in impairment of a large number of important cellular processes, many of which are plausible contributors to tumor naissance. The effects of PUVA therapy on Cn-mediated cellular processes and the causal relationship between Cn/NFAT inhibition and tumorigenesis should be thoroughly held to the light. In addition, our data warrant further study into possible ways to protect against UVA-induced damage to Cn, for instance, using antioxidants.

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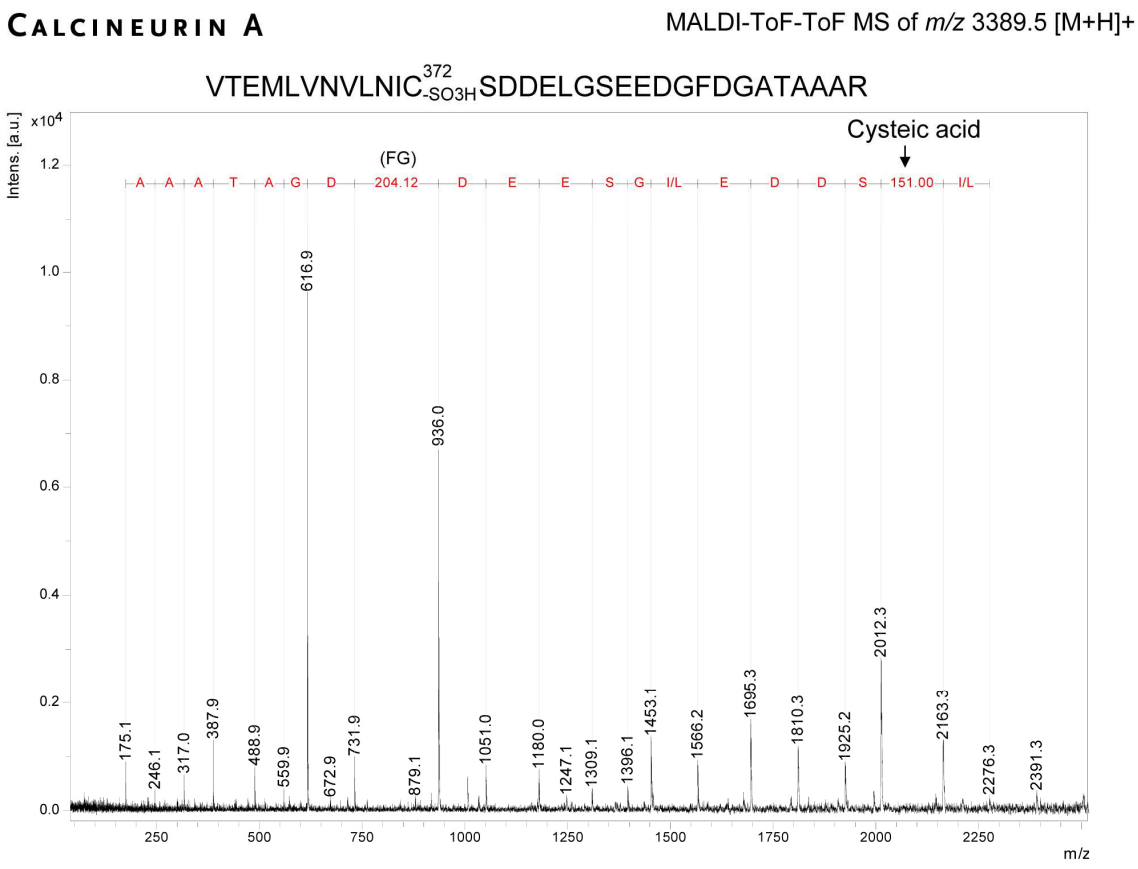
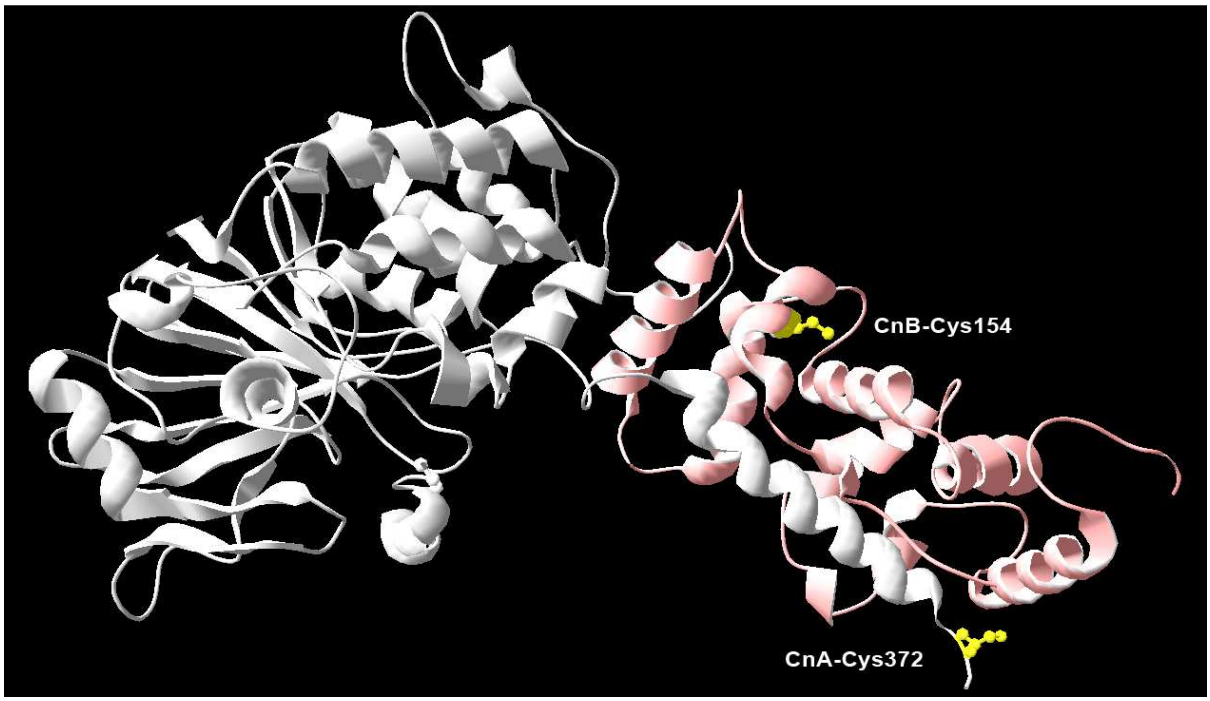
SUPPLEMENTARY FIGURES



SUPPL. FIGURE I MALDI-ToF-ToF MS of a tryptic peptide from UVA exposed calcineurin A featuring cysteine-372 oxidized to a cysteic acid.



SUPPL. FIGURE II 3D-structure of the calcineurin heterodimeric enzyme. The A subunit is displayed in grey, the B subunit in light red. The Cys residues found to be affected by oxidation have been highlighted in yellow. Image adapted from PDB entry 1AUI (*98*) and modeled using Swiss PDB viewer.