Cover Page

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CHAPTER 3

effects of UVAl radiation on calcineurin activity and cytokine production in (skin) cell cultures

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ABSTRACT

Calcineurin (Cn) is the target of immunosuppressive drugs used for maintenance therapy of transplant patients. Ultraviolet radiation is also known to be immunosuppressive and, similar to the Cn inhibitors, UV has been shown to positively influence various inflammatory skin diseases. Recently, Cn activity has been demonstrated in skin and skin cell cultures. In the present study, we have investigated the effects of UV(A1) irradiation on Cn activity in skin.

In total skin, we found a significant reduction of Cn activity after exposure to 450 kJ/m^2 of UVA1 (340-400 nm). In repeated experiments, cultures of fibroblasts and keratinocytes also showed dose-dependent and selective reduction of Cn activity after UVA1 irradiation. UVB irradiation caused a decrease in Cn activity in one of two fibroblast cultures and was ineffective in keratinocytes. In Jurkat cells and PBMC, UVA1 reduced Cn activity as well as the production of cytokines that are controlled by the Ca^{2+}-Cn pathway, such as IL-2, IFN-γ, IL-4, and IL-10. These results indicate that UV(A1) irradiation may lead to inactivation of Cn in the skin; next to the calcineurin inhibitors, UVA1 may target Cn and suppress the skin immune system.

INTRODUCTION

Calcineurin (Cn), also called protein phosphatase 3 serves as a central regulating enzyme in the calcium signaling pathway. It is responsible for the dephosphorylation of important signal transduction molecules like the nuclear factor of activated T cells (NFAT) or the inhibitor of nuclear factor kappa B (IκB-β) (*1, 2*). In T cells, the dephosphorylation of NFAT results in its nuclear translocation, the subsequent transcription of various cytokines (e.g. IL-2 and IFN-γ) and ultimately in T cell activation (*3*). Cn is the target of the immunosuppressive drugs cyclosporin A and tacrolimus (CsA, TRL), which are used in transplantation medicine for prevention of allograft rejection.

Different functions for Cn have been described in various other eukaryotic cells. In keratinocytes, for instance, Cn plays a role in cell signaling and differentiation (*4, 5*). Recently, we were able to demonstrate Cn activity in the skin and in cultures of skin fibroblasts, keratinocytes, and melanocytes as well as the inhibition of Cn in these cells by calcineurin inhibitors (CnI) (*6, 7*). A role for Cn in skin disease is implicated by the successful application of the CnI, both systemically (CsA) or topically (TRL and pimecrolimus, PRL), for treatment of psoriasis, atopic dermatitis, or vitiligo (*8-12*). In

keratinocytes, the nuclear translocation of NFAT1 induced by TPA or calcium was shown to be prevented by pretreatment with CsA (*13*). Interestingly, a variety of inflammatory skin diseases also benefit from therapy with different types of ultraviolet (UV) irradiation. Narrow-band and broadband UVB, PUVA, UVA and UVA1 are all used for the treatment of e.g. psoriasis, atopic dermatitis, vitiligo, SLE and scleroderma (*14-17*). For this reason we were interested whether Cn might be influenced directly by exposure to ultraviolet radiation. Since Cn is known to be sensitive to oxidation (*18, 19*), we mainly focused on UVA irradiation, which is generally considered to be the oxidizing component of sunlight (*17*). Most experiments were performed using doses of UVA1 (340-400 nm) in a therapeutically relevant range (0-450 kJ/m^2) (*15*). We have tested the effects of UV irradiation on Cn activity in skin and skin cell cultures (fibroblasts and keratinocytes) as well as its downstream effects in an established system of the Jurkat (T lymphoma) cell line and peripheral blood mononuclear cells (PBMC), in which cytokine production can be easily induced via the Ca^{2+}-Cn pathway (*20*).

EXPERIMENTAL PROCEDURES

Cell cultures

Keratinocytes were isolated from foreskin after washing with phosphate buffered saline (PBS) and removal of subcutaneous fat with scissors. Epidermis and dermis were split by dispase II (Roche, Mannheim, Germany) treatment at 37 °C and the epidermis was subsequently treated for 15-20 minutes with trypsin/EDTA (1:100; Invitrogen) to obtain a single cell suspension. Cells were centrifuged in PBS + 5% FCS for 5 min. at 250 *g*, plated in KD-SFM (Invitrogen) and grown to confluence. The keratinocytes were replated and used for UVA1 irradiation after the first passage.

Fibroblasts were obtained by outgrowth of the dermal explants after separation of dermis and epidermis with dispase. Explants were plated top-down in a small layer of DMEM + 5% FCS (Invitrogen). Primary cultures of fibroblasts were replated and the explants were removed. Cells were weekly passaged 1:5 and used for the irradiations at near confluence.

Jurkat (T-lymphoma) cells were grown in RPMI medium with 10% heat inactivated fetal calf serum and penicillin/streptomycin (100 U/mL, Invitrogen) and grown in the presence of phytohemagglutinin (PHA 1:100, Invitrogen) and 20 ng/mL phorbol 12-myristate 13-acetate (PMA/TPA, Sigma, Belgium) in order to induce the production of IL-2 and other cytokines (*21*).

PBMC were isolated according to a standard procedure. Briefly, 15 mL of whole blood was separated on 15 mL Ficoll in 50 mL Leucosep tubes (Greiner Bio One, The

Netherlands). Isolated PBMC were seeded in 25 cm² culture flasks (Greiner Bio One) and grown in the same medium as Jurkat cells.

All cultures were maintained in their culture media at 37 °C in a CO_2-incubator at 5% CO_2. Blood samples were obtained from volunteers for the studies on PBMC cultures and foreskin samples from surgery for cultures of keratinocytes and fibroblasts. All materials were obtained with informed consent. Experimental procedures are approved by the Leiden University Medical Center and are in agreement with the Declaration of Helsinki Principles.

UV irradiation

For irradiation with UVA1, foreskin was washed and prepared as described above. The skin was cut in equal parts and duplicate pieces were irradiated on ice in Petri dishes with UVA1 doses of 0, 225, and 450 kJ/m². After the irradiation, dermis and epidermis were separated overnight (20 h) with dispase; the dermis and epidermal skin were subsequently homogenized in Cn lysis buffer (see below) (7).

For the experiments irradiating cells in culture with UVB and UVA1, non-toxic dose ranges were used based on experience from our previous studies (*22-24*). Cell survival of irradiated cells was tested using trypan blue exclusion after harvesting and by measuring LDH release in the culture medium. Both were negative for the dose ranges discussed in the results. Keratinocytes showed LDH release after irradiation with 300 and 400 J/m² UVB and fibroblasts after the irradiation with 900 kJ/m² of UVA1. As the keratinocyte and fibroblast cultures were irradiated at near confluency with minimal proliferation, variation in cell density after UV irradiation was limited for the different samples.

Doses of UVB and UVA1 were monitored using a IL700A Research radiometer with a WBS320#801 sensor. UVA1 irradiation was performed using Sellas Sunlight lamps with an output of 12.5 mW/cm² in combination with UVASUN blue sheet and UVASUN blue film with an emission spectrum in the range of 340-400 nm (*25*). Cells were irradiated on ice in PBS supplemented with 5% FCS in 6 cm ø Petri dishes with covers. After irradiation, the cells were kept overnight in culture medium in the CO_2-incubator and harvested after 20 hours. For the UVB irradiation, Philips TL12 lamps with a broad emission spectrum (275-375 nm) and an optimum at 312 nm (output 0.56 mW/cm²) were used (*24*). Cells were irradiated in Petri dishes without covers.

In case of the non-adherent Jurkat cells and PBMC, the cell suspensions (2×10^6 cells/dish) were collected directly from the dishes and centrifuged. Keratinocytes and fibroblasts were first detached with trypsin/EDTA (1:100; Invitrogen), collected in PBS with 5% FCS, and centrifuged. Cells were washed with 10 mM Hepes-buffered saline (pH 7.5) before centrifugation. After removal of supernatant, the pellets were solubilized in lysis buffer for enzyme and protein measurements.

Enzyme activity measurements

Cn measurements were performed in the lysates as described earlier for leukocytes isolated from blood (*6*). Briefly, cells were lysed by 3 freeze-thaw cycles in 50 mM Tris-HCl, pH 7.7, containing dithiothreitol, ascorbic acid, NP-40 and protease inhibitors. Samples were snap frozen and stored at -80 °C before use. Activity was measured by the release of phosphate from the RII phosphopeptide substrate. Other protein phosphatases were inhibited by excess okadaic acid and Cn activity was defined as the calcium/calmodulin-dependent phosphatase activity, which was obtained by completing the assay both in presence and absence of EGTA (*6*). Lactate dehydrogenase (LDH) was measured in the same cellular lysates as used for the Cn assay. The LDH assay (optimized LDH kit, Roche, Mannheim, Germany) measures the decrease of NADH (at 340 nm) accompanying conversion of pyruvate to lactate and was performed on a Roche Modular P800.

Protein concentrations were determined using the Pierce Coomassie Plus Total Protein Assay (PerBio Science, Belgium) based on the method of Bradford (*26*). Enzyme activities in the samples could thus be corrected for differences in protein content.

Measurements of cytokines and growth factors

The Cytokine Array I (Randox, Antrim, UK) was used in order to measure 12 cytokines/growth factors simultaneously using the Randox Evidence Investigator (*27*). Supernatants were collected from the cultures just before the cells were harvested for measurement of enzyme activities. Influence of CsA or UVA1 irradiation on cytokine production was measured in 100 µL of cell supernatants. Detection limits (pg/mL) according to the manufacturer (Randox) were: IL-2 (4.8); IL-4 (6.6); IL-6 (1.2); IL-8 (7.9); IL-10 (1.8); vascular endothelial growth factor (VEGF) (14.6); IFN-γ (3.5); TNF-α (4.4); IL-1α (0.8); IL-1β (1.6); MCP-1 (13.2) and EGF (2.9). The effects of UVA1 irradiation were compared to those of CsA at 25, 250, and 1000 µg/L. These concentrations represent the minimal-to-high concentration range in patient blood after drug intake (*6*).

Statistics

Student t-tests were performed to test for statistical significance as defined by $p < 0.05$. Details of comparisons are mentioned in the figure legends.

RESULTS

In our recent studies, Cn activities were measured in skin, typically featuring a higher Cn activity in epidermis than in dermis (*7*). In the present study, we irradiated skin obtained from circumcisions with UVA1 doses of 225 and 450 kJ/m^2 before dermis and epidermis

were separated by overnight dispase treatment. Cn activity was measured in the homogenates. Significant reductions were found in both the epidermal and the dermal fraction after irradiation with 450 kJ/m^2 (33 and 51% reduction, respectively). No changes were found when the skin was irradiated with the lower UVA1 dose (figure 1).
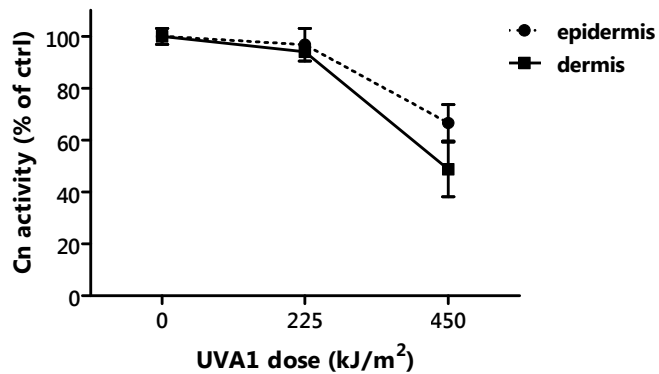
FIGURE 1 Calcineurin is reduced by UVA1 irradiation of total skin. Foreskin samples were exposed to UVA1 with 0 (sham), 225 and 450 kJ/m^2. After the separation of dermis and epidermis, samples were lysed and Cn activity measured in both epidermis and dermis. Cn activity is expressed as % of sham-irradiated control and was significantly reduced for the irradiation with 450 kJ/m^2 in both epidermis and dermis ($p < 0.05$). Results show the average of four experiments with foreskin from three different donors. Error bars represent SE ($n=4$).
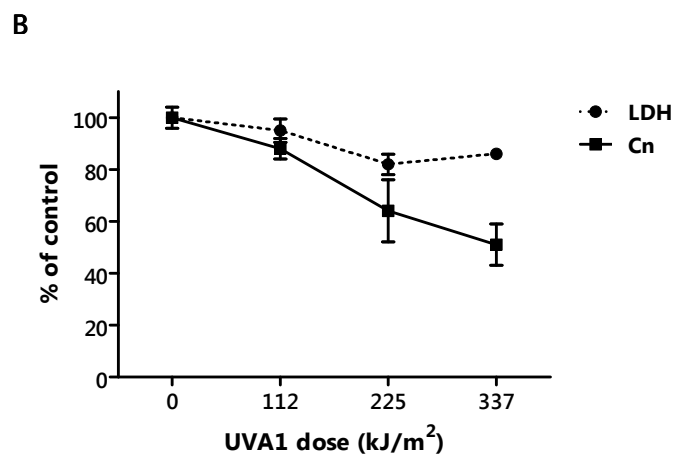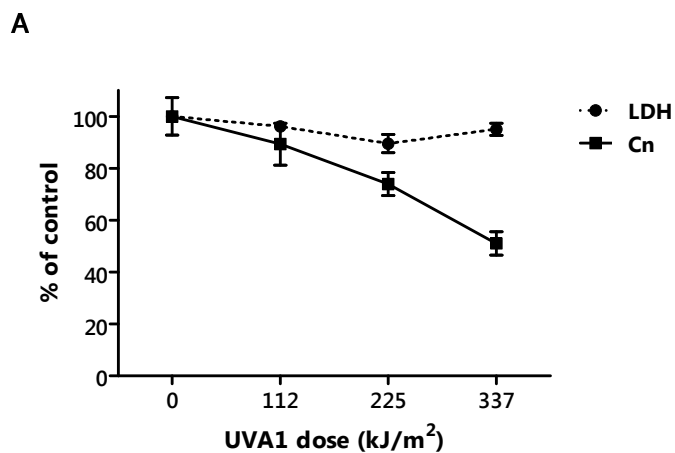

A

B

FIGURE 2 Calcineurin activity decreases as a result of UVA1 irradiation in both fibroblasts [A] and keratinocytes [B]. Both calcineurin and LDH activity were measured in cells harvested 20 hrs after irradiation. Cn and LDH are presented as % of sham irradiated controls. Cn was significantly reduced compared to control for both cultures at 337 kJ/m^2 ($p < 0.05$). Error bars represent range in duplicates ($n=2$).

Irradiation of skin fibroblasts and keratinocytes in culture with UVA1 resulted in a dose-dependent decrease in Cn activity 20 hours after irradiation (see figure 2). The reduction of Cn activity was significantly stronger than that of lactate dehydrogenase (LDH), included as a control enzyme. In the fibroblast culture, Cn activity was reduced to 51% and LDH to 95% of unirradiated control at the maximal dose of 337 kJ/m^2 and for the keratinocyte culture to 51 and 86% for Cn and LDH, respectively.

Our findings regarding Cn inactivation were confirmed for another fibroblast culture in two subsequent experiments. Figure 3a shows the average result of the UVA1 irradiations of fibroblasts in all 3 experiments. Cn activities were reduced by 28.5 (±5.9) and 41.5 (±3.0)% for Cn at 225 and 337 kJ/m^2, respectively. LDH activity was also reduced when compared to the unirradiated control, but the reduction after irradiation with 225 and 337 kJ/m^2 was limited to 13.7 (±3.0) and 11.2 (±2.3)%, respectively.
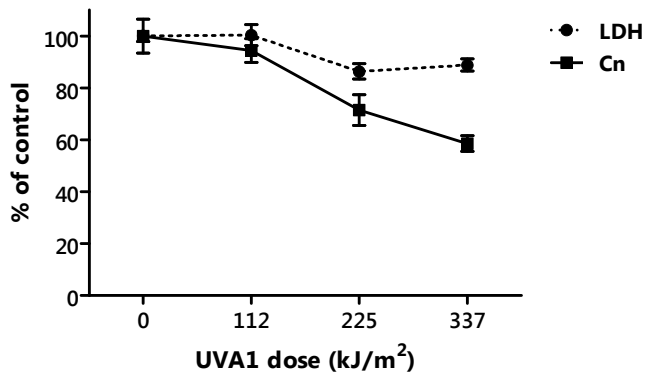
A

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FIGURE 3 Calcineurin activity after [A] UVA1 and [B] UVB in fibroblast cultures. [A] With increasing doses of UVA1, reduction of Cn activity is stronger than that of LDH. Both Cn and LDH were significantly reduced for the doses of 225 and 337 kJ/m^2 compared to the controls (0 kJ/m^2). Bars represent the average of 3 experiments with different fibroblast cultures performed in duplicate (error bars represent SE (n=6); t-test showed significant differences with $p = 0.048$ and 0.0011 for Cn and $p = 0.022$ and 0.018 for LDH at 225 kJ/m^2 and 337 kJ/m^2, respectively). [B] With increasing doses of UVB, Cn activity is significantly reduced at 100 and 200 J/m^2 compared to LDH ($p < 0.05$). Bars represent the average of triplicate measurements in one fibroblast culture, error bars represent SE. Cn and LDH activities are presented as % of sham-irradiated controls.
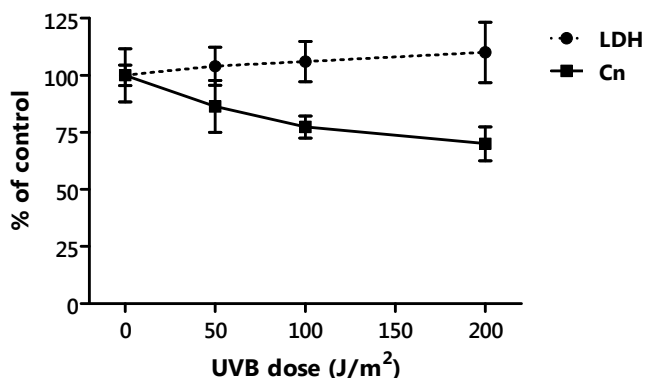
Effects of UVB irradiation were tested on two different fibroblast cultures and one keratinocyte culture. For one of the two fibroblast cultures, the UVB irradiation caused a gradual decrease in Cn activity compared to LDH with increasing doses at 20 hours after irradiation (Figure 3b). Reduction of Cn activity was 22.6 (±4.9)% and 30.0 (±7.5)%, for

doses of 100 and 200 J/m^2 of UVB, respectively. For the other fibroblast culture, no change was found for Cn activity as compared to LDH. Keratinocytes were relatively sensitive to the UVB irradiation resulting in cell death at 300 and 400 J/m^2 and release of LDH into the medium. No significant changes were found for Cn in the keratinocytes at the doses of 100 and 200 J/m^2 in comparison to LDH.
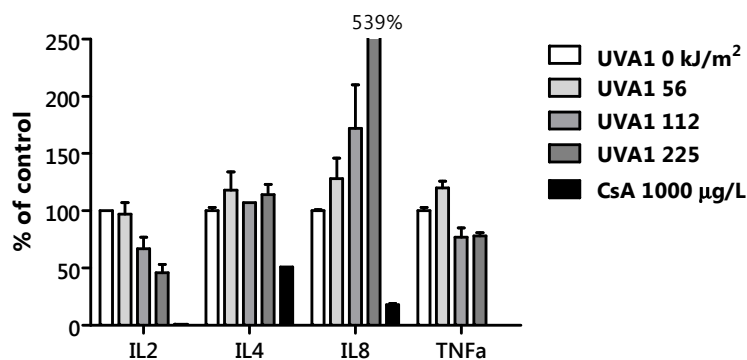
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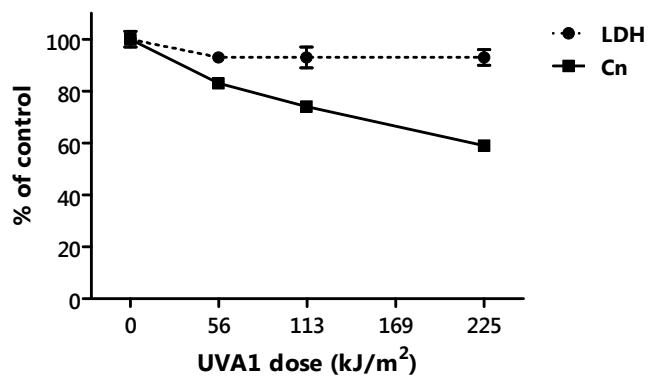
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FIGURE 4 UVA1 reduces calcineurin activity in Jurkat cells and influences their cytokine production. [A] Calcineurin and LDH enzyme activities in Jurkat cells 20 hrs after UVA1 irradiation. Cn and LDH are presented as % of sham irradiated controls and results are shown as the average of triplicate cultures with error bars representing SE. Cn activity was significantly reduced ($p < 0.05$) compared to LDH at all doses. [B] Cytokines measured in the culture supernatant of the Jurkat cells 20 hours after UVA1 irradiation or after 20 h incubation with 1000 µg/L CsA. Error bars represent SD for two of the triplicate cultures (in [A]). Jurkat cells were cultured in the presence of PMA and PHA in order to stimulate cytokine production (see Methods). In a subsequent experiment, similar results were obtained, showing reductions in IL-2 and TNF-α, a clear increase in IL-8 and almost no change for IL-4.

In order to further explore the effects of UVA1 on the Ca^{2+}-Cn pathway, we used Jurkat cells and PBMC to investigate the influence of UVA1 on Cn activity in these cells and to see how this affects the production of various cytokines. Figure 4a depicts the results of Cn activity measurements in cell lysates 20 hours after the irradiation of Jurkat cells with UVA1 doses varying from 0 to 225 kJ/m^2. A dose-dependent decrease in Cn activity was found whereas the LDH activity in the same lysates did not show significant changes for any of the UVA1 doses used. Cn activity of the cells irradiated with the highest dose (225 kJ/m^2) was reduced by 41 (±2)% compared to the unirradiated control. For comparison, overnight treatment of the Jurkat cells with a relatively high concentration of 1000 µg/L

CsA, resulted in a 57 (±0.3)% reduction in Cn activity in the cells. As shown in Figure 4b, the UVA1 irradiation also caused a (dose-dependent) decrease in IL-2 production as measured in culture supernatants (up to 54% at the maximum dose). The treatment with 1000 µg/L CsA almost completely (99%) shut down IL-2 production by the cells. No effects of UVA1 irradiation were found on IL-4 production. Treatment with CsA, on the other hand, caused a 49% reduction in IL-4 production in the Jurkat cells (Figure 4b). UVA1 caused a strong increase in IL-8 production, whereas an 82% reduction for IL-8 was found after CsA treatment. TNF-α release was reduced by 23 and 22% at UVA1 irradiations of 112 and 225 kJ/m^2, respectively. After CsA treatment, TNF-α was not detectable in the culture supernatants of the Jurkat cells.
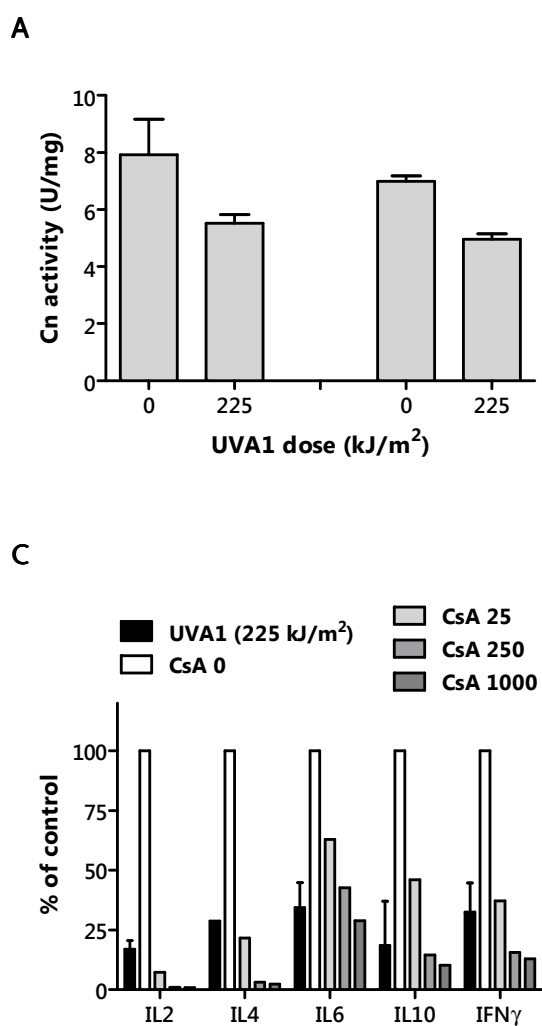
FIGURE 5 Effects of UVA1 irradiation on calcineurin activity in PBMC cultures and on the production of different cytokines in comparison to cells treated with cyclosporin A. [A] Cn activity in two different PBMC cultures, showing 30 and 29% reductions, respectively, at 20 hours after irradiation with UVA1. Error bars represent SD of duplicate experiments. [B] IL-2 production in the supernatants of the same two cultures is reduced (error bars represent SD (n=2)) after irradiation. [C] Different cytokines were measured by a multicytokine array. Cytokine production is shown as % of control for PBMC irradiated with 225 kJ/m^2 of UVA1 or by treatment with increasing concentrations of CsA (0, 25, 250 and 1000 µg/L). Error bars show the range obtained for the two UV-irradiated cultures. Single measurements were performed in the culture media of another PBMC culture treated with increasing CsA concentrations. IL-4 was not detectable for one culture and was 71% reduced for the other. All PBMC cultures were grown in the presence of PMA and PHA in order to stimulate cytokine production.

The effect of UVA1 on Cn activity and cytokine production was also studied in three different PBMC cultures. In two cultures, 225 kJ/m^2 of UVA1 caused reductions in Cn activity of 30 and 29%, respectively, at 20 hours after irradiation (Figure 5a). This reduction in Cn activity was accompanied by decreases in IL-2 production of 80 and 87%, respectively (Figure 5b). Next to the reduction for IL-2 in the two PBMC cultures, the production of interleukins-4, -6, and 10 and IFN-γ were decreased as well, by 65% or more (Figure 5c). PBMC cultures treated with increasing concentrations of CsA exhibited a 31% inhibition of Cn activity at the highest concentration used. Similar to the UVA1 irradiation, the CsA treatment resulted in a (dose-dependent) reduction of the interleukins 2,-4, -6, and 10 and IFN-γ (> 71% at the maximal CsA concentration).

DISCUSSION

Calcineurin is an important target for immunosuppression. Inhibitors of Cn are not only used for the longterm maintenance therapy of transplant patients, but also for topical treatment of various skin diseases. Next to its important role in T cells, the Ca^{2+}-Cn pathway has been shown to be involved in several regulatory functions in other eukaryotic cells. Downstream of Cn, the role of NFAT in T cell activation has been widely studied and its nuclear translocation has been used to demonstrate activation of Cn in keratinocytes and fibroblasts (13, 28-30). The results of our present studies demonstrate the inactivation of Cn in skin as a result of irradiation with therapeutically used doses of UVA1. These results were found both in epidermis and dermis, confirming that UVA1 radiation penetrates the skin into the dermal layer. In addition to the irradiation of total skin, experiments were performed on monolayer cell cultures of fibroblasts and keratinocytes. Again, a dose-dependent reduction in Cn activity was found for all cultures irradiated with UVA1 (Figures 2 and 3). Enzyme inactivation proved selective for Cn, as the effects were much more prominent for Cn than for LDH. Another protein phosphatase (PP2A), closely related to Cn, was insensitive to the inactivation by UVA1 irradiation as well (see chapter 4 of this dissertation). Next to the skin cell cultures, the effects of UVA1 on Cn were investigated in Jurkat cells and PBMC, offering the possibility to measure the downstream influences on cytokine production regulated by calcineurin. The reduction in Cn activity found for the Jurkat cells and PBMC after the irradiation with UVA1 (figures 4a and 5a) was accompanied by a reduction in production of the cytokines, IL-2 and IFN-γ, which are often described as the downstream products of the Ca^{2+}-Cn pathway in T cells (3, 31). Treatment of the cells with CnI such as CsA (Figure 4b, 5c) and TRL (20) also results in the reduction of IL-2 and IFN-γ production. Next to Il-2 and IFN-γ, IL-4 and IL-10 have been described to be under control of Cn (31, 32); both were reduced by the UVA1 treatment and by CsA (Figure 5c). IL-6 production in PBMC

was only partly inhibited by CsA and also reduced by UVA1, which may also be attributed to the inhibition of Cn activity (33). In the Jurkat cells, some differences in production of other cytokines (TNF-α, IL-8) were observed between the treatment with UVA and the CnI (Figure 4b). For example, UVA1 caused a strong increase in IL-8 production in Jurkat cells, whereas IL-8 secretion was strongly reduced by CsA (Figure 4b). The effects of the UVA1 treatment on IL-8 production may be regulated by multiple or different pathways. In this respect, examples of IL-8 induction have been described for stress factors such as UV radiation and ROS in tumor cells (as reviewed by Shi *et al.* (34)) but also for UV radiation in keratinocytes and fibroblasts (35, 36).

Thus, in Jurkat cells and PBMC, UVA1 irradiation and the use of CnI both lead to inactivation of Cn and corresponding downstream effects on cytokine production. Nevertheless, some differences between both treatments have been demonstrated. In skin, it is still unclear which cytokines are regulated by Cn, and so far, we have not been able to demonstrate any effects of CnI on cytokine production by keratinocytes using our cytokine array (see the experimental procedures).

Activation of Cn is known to induce the nuclear translocation of NFAT. Different reports have demonstrated influences of UV irradiation on nuclear localization of NFAT (28, 29, 37). Most of the results reported on NFAT localization are, however, difficult to compare to our data because of differences in experimental conditions with regard to UV sources and doses used. In the study by Mazière and coworkers (29), an increase in NFAT DNA binding activity was found at low UVA doses (< 45 kJ/m^2), whereas at higher doses (135 kJ/m^2) of UVA a reduction to 60% of control was found, correlating with our findings of reduced Cn activities at similar doses and higher.

In patients treated with CnI, maximal decrease in Cn activity is usually found 2 hours after drug intake; this initial decrease normally recovers within several hours and correlates with falling concentrations of the CnI after reaching optimal drug levels in blood (6, 38). So far, most of our studies concentrated on the effects of UVA1 irradiation on Cn activity 20 hours after exposure. UVA is known to generate reactive oxygen species (ROS), while Cn is highly sensitive to oxidation (6, 39). Wang and coworkers described the inactivation of Cn by paraquat, which is a notorious generator of superoxide anion. They showed that superoxide dismutase was able to protect Cn against spontaneous inactivation (39). Sommer *et al.* (19) also noticed the sensitivity of Cn to oxidants such as superoxide and the protective effect of superoxide dismutase and various antioxidants. Both research groups indicated that alterations to the iron-zinc cluster (i.c. oxidation of the ferrous iron) may likely be responsible for inactivation of Cn. Bogumil *et al.* (18) observed inhibition of Cn by H_2O_2 and proposed that this inhibition could be caused by formation of a disulfide bond between two closely spaced cysteine residues in the active site of the CnA subunit. A report by Morita and colleagues (40) grants an important role to singlet oxygen in the effects of UVA phototherapy on T cell apoptosis. Von Montfort *et al.* (41) have demonstrated that singlet oxygen is responsible for the inactivation of

another enzyme, protein tyrosine phosphatase 1B, through oxidation of its active site cysteine (Cys215). Thus, different mechanisms towards inactivation of Cn or other proteins by ROS may occur due to UVA irradiation. The inhibition of Cn by UVA could result in long-lasting conformational changes to the enzyme whereas the inhibition by CnI is only transient. Since the Cn assay measures Cn activity under optimized conditions, deviations from activity in control samples will reflect enzyme modification or complexation or binding of an activator or inhibitor. Our present and future investigations are therefore aimed to further clarify the influence of ROS on Cn inactivation (as compared to PP2A, see above) and on the modifications to the Cn protein (see chapter 4). Furthermore, studies on other downstream processes, such as NFAT localization, are in progress.

Several mechanisms have been described that may give an explanation how UV-radiation may induce immunosuppression in the skin (*42, 43*). In general, an important role for UVB is suggested in these mechanisms. Some effects of UVB irradiation on Cn were found in our experiments, but results were less consistent than those associated with UVA1 irradiation. Nevertheless, UVB influences on Cn could be highly important and need to be studied in more detail. Most of our experiments were performed using intermediate UVA1 doses used in therapeutic regimens (*15*). The results presented here may contribute to improving our understanding of the beneficial effects of UVA1 treatment on several skin diseases and may shed new light on mechanisms of immunosuppression in the skin with a central role for Cn as a target of UVA1 radiation. The UVA wavelengths are able to reach the deeper layers of the skin, including the dermal fibroblasts, but also the subepidermal capillary network of the skin and may thus influence blood cells in arterial and venous vessels and infiltrating T cells (*17, 40, 44*). Theoretically, the suppression of the production of certain cytokines, e.g. IL-2 and IFN-γ, could influence T cell activation in the local microenvironment of the skin.

Both inhibition of Cn with CnI and exposure to UVA1 radiation can cause immunosuppression. Both treatments influence Cn activity by different mechanisms and first results show that the inhibitory effects are supplementary (see chapter 4). A major drawback of lifetime immunosuppressive treatment with CnI is the increased overall risk of carcinogenesis (*45*). Transplant patients receiving CnI in combination with sunlight exposure exhibit a particularly dramatic increase in the risk of skin cancer development (*46-48*). In this respect, it will be of great importance to define the role of Cn (in)activation in the regulation of immunosuppression in skin, while strategies may be developed in order to circumvent the negative side-effects.

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