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Original Contribution

UVA1 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.

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 after 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, 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 DNA repair pathways [9–11]. Novel functions and applications of NFAT signaling have been steadily surfacing [12–15].

Calcineurin is the principal target of the immunosuppressive drugs cyclosporin A (CsA) and tacrolimus (TRL) [16]. 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 erythematosis, atopic eczema or hypersensitivity reactions, by alleviating inflammatory symptoms [17–20]. By now, however, much of the initial attractivity of Cn inhibitors (CnI's) has faded away, because patients treated with CnI's exhibit a markedly increased incidence of keratinocyte malignancy on areas of their skin that receive frequent sunlight exposure [21–23]. Although CnI's have been indicated to directly promote tumor growth, metastasis, and angiogenesis by increasing TGFβ and VEGF production [24–28], suppressed Cn signaling could be an important contributing factor as well.

Depending on the intensity and duration of exposure, both stimulating and suppressing influences of UVA radiation on the immune system have been recognized [29–33]. The inflammatory skin conditions mentioned above typically benefit from UVB phototherapy [34], but, intriguingly, similarly good results can be obtained with UVA phototherapy [35]. For this, T helper cell depletion after UVA-induced apoptosis has been proposed as a plausible operative mechanism [36]. The common belief is that UVA, in contrast to UVB, exerts a significant part of its effects via reactive oxygen species (ROS)

Abbreviations: Cn, Calcineurin; PP2A, protein phosphatase 2A; UVA, ultraviolet A; UVB, ultraviolet B; PUVA, psoralen plus UVA; NDP, 3,3′-(1,4-naphthylidine)dipropionate; MOP, methoxypsoralen; CnI, calcineurin inhibitor; TRL, tacrolimus; CsA, cyclosporin A; TGFβ, transforming growth factor β; VEGF, vascular endothelial growth factor; NFAT, nuclear factor of activated T cells; NF-κB, nuclear factor κB; ROS, reactive oxygen species; CD, circular dichroism; TFA, trifluoroacetic acid; HBSS, Hanks' balanced salt solution.

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[37,38]. Although 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 [39,40]. During the past 2 decades, several studies have shed light on possible mechanisms of Cn activity modulation by a variety of reactive oxygen species [41–43]. Hydrogen peroxide has been found to precipitate calcium-dependent NFAT activation [44], and high concentrations of hydrogen peroxide and superoxide disrupt NFAT signaling by disabling Cn [45]. The enzyme's active site is known to suffer iron loss after 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 [46,47]. 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 [48–50]) irreversibly inactivate Cn [51] 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's 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 after 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 foreskins 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 were removed. Cells were passaged 1 to 5 weekly 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 a 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. Before irradiation, the DMEM was supplemented with various concentrations of TRL (Sigma). After 1 h, the DMEM was replaced by HBSS (Gibco) without further dosing of TRL and irradiation was started. Immediately afterward, the cells were washed with PBS, detached with trypsin/EDTA (0.25%, Invitrogen), collected in PBS containing 5% FCS, and centrifuged. The 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-well 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 [52], according to the manufacturer's instructions. $NDPO_2$ was then added to the medium of fibroblast cultures. Singlet oxygen was generated by thermal dissociation of $NDPO_2$ at 37 °C [50].

Activity measurements

Calcineurin assays were performed under optimal conditions (excess Ca^{2+} and calmodulin) according to the method reported by Sellar et al. [53], 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 dithiothreitol (DTT), 0.02% NP-40 (Calbiochem), and protease inhibitors and lysed by three 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 the presence or absence of excess EGTA, calmodulin, and okadaic acid. Cn activity was defined as the calcium/calmodulin-dependent okadaic acid-insensitive phosphatase activity [53]. PP2A activity, measured in the same assay, was defined as the okadaic acid-inhibited calcium-insensitive phosphatase activity [54]. 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 room temperature for 20 min. Tryptic digestion was then performed by adding 50 ng trypsin (sequencing grade modified trypsin; Promega, Madison, WI, USA) and incubating overnight 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 matrix-assisted laser desorption/ionization (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 (Bruker Daltonics). The mass spectrometry (MS) acquisitions were performed in positive-ion reflectron mode at a laser frequency of 100 Hz. The scanner *m/z* range was up to 5000 and the