Calcineurin in skin : rising star or fallen angel?

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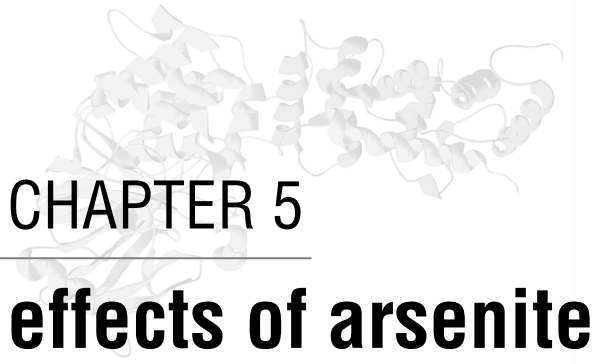

CHAPTER 5

effects of arsenite and UVAI radiation on calcineurin signaling

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ABSTRACT

Calcineurin is a Ca^{2+}-dependent serine/threonine phosphatase and the target of the immunosuppressive drugs cyclosporin and tacrolimus, which are used in transplant recipients to prevent rejection. Unfortunately, the therapeutic use of this drugs is complicated by a high incidence of skin malignancy, which has set off a number of studies into the role of calcineurin signaling in skin, particularly with respect to cell cycle control and DNA repair. Both UVA1 radiation and arsenic species are known to promote skin cancer development via production of reactive oxygen species. In light of the well-documented sensitivity of calcineurin to oxidative stress, we examined and compared the effects of UVA1 and arsenite on calcineurin signaling.

In this paper, we show that physiologically relevant doses of UVA1 radiation and low micromolar concentrations of arsenite strongly inhibit calcineurin phosphatase activity in Jurkat and skin cells and decrease NFAT nuclear translocation in Jurkat cells. The effects on calcineurin signaling could be partly prevented by inhibition of NADPH oxidase in Jurkat cells or increased dismutation of superoxide in Jurkat and skin cells. In addition, both UVA1 and arsenite caused a decrease in NF-κB activity, although at lower concentrations, arsenite enhanced NF-κB activity. These data indicate that UVA1 and arsenite affect a signal transduction route of growingly acknowledged importance in skin and that calcineurin may serve as a potential link between ROS exposure and impaired tumor suppression.

INTRODUCTION

Reactive oxygen species (ROS) have been implicated in tumorigenesis and tumor growth by direct mutagenic effects, damage to a variety of cellular components, and induction of signaling cascades (*1*). One of the cellular targets affected by ROS is the Ca^{2+}-dependent serine/threonine phosphatase calcineurin (Cn, PP3), which is best known for its role in T cell recruitment but has been found to regulate Ca^{2+} signaling in a large number of organs (*2, 3*). Cn activates several transcription factors, among which NFAT, the nuclear factor of activated T cells, has been most extensively studied (*4, 5*). The sensitivity of Cn to ROS has been well documented (*6*) (reviewed in (*7*)). Cn has received interest as the therapeutical use of the calcineurin inhibitors (CnI), cyclosporin A and tacrolimus, in transplant recipients to prevent rejection is typically accompanied by a high incidence of skin cancer. During the last decade, a number of studies have addressed the issue by

which mechanism Cn inhibitors may specifically cause skin cancer, either due to direct secondary effects (*8, 9*) or through suppression of Cn signaling. In a recent paper, strongly suppressive effects of physiologically and therapeutically relevant doses of UVA1 on Cn have been described (*10*). Both superoxide and singlet oxygen generated by photosensitization were found to contribute to Cn activity loss, which has been associated with immunosuppression, reduced apoptosis, p53 downregulation and disrupted DNA repair (*11-17*), all tumor-promoting factors.

Similar to CnI, exposure to arsenic species has been known to be an important etiological factor for malignancy formation in skin, particularly squamous cell carcinoma (*18-20*). Although arsenic exposure has limited potential to induce skin cancer on its own, its combination with a genotoxic agent such as UVA radiation produces synergistic effects (*21-23*). Arsenic poisoning mainly occurs through contaminated drinking water or via occupational routes. Trivalent arsenic species, either biomethylated or inorganic, are considered the most potent deleterious forms of arsenic (*24*). Arsenite is known to affect epidermal gene and protein expression (*25*), to cause genetic damage (*24*) and epigenetic dysregulation (*26*), and to interfere with nucleotide excision repair (*27, 28*). Interestingly, much like UVA radiation, oxidative effects are hypothesized to be paramount to its carcinogenic and tumor promoting ability (*29-31*). It has been shown that the oxidative effects of arsenic in keratinocytes can be traced back to activation of NADPH oxidase which converts molecular oxygen to superoxide using NADPH as electron donor (*22, 32*). Superoxide, although fairly unreactive by itself, can be easily converted to hydrogen peroxide and hydroxyl radical, which are the main culprits responsible for the damage to the cell and its machinery (*29*).

Moreover, recent work of Vahidnia *et al.* shows that in neuronal cells, arsenite exposure may precipitate a calpain/p25-mediated induction of CDK5, resulting in accumulation of hyperphosphorylated tau (*33, 34*), which is one of the hallmarks of Alzheimer's disease. Alzheimer's disease has recently been linked to As exposure (*35, 36*), but also to Cn inhibition, as calcineurin and its sibling protein phosphatase 2A (PP2A) are responsible for the dephosphorylation of tau (*37-42*). However, despite some indications that As species may affect Cn activity, their effects on Cn signaling have not yet been studied in detail (*43-46*).

In this paper, we show that both arsenite and UVA1 in physiologically relevant doses suppress Cn signaling in Jurkat cells as well as in skin cells, and we confirm superoxide as a mediating factor to both.

EXPERIMENTAL PROCEDURES

Cell cultures

Jurkat (T lymphoma) cells were grown in RPMI (Gibco) containing 10% FCS (Hyclone). HaCaT cells were cultured in DMEM (Gibco) containing 5% FCS. Foreskin melanocytes were prepared according to standard procedures (*47*) and kept on RPMI (Gibco) containing 2% FCS and 1% Ultroser-G (Pall Biosepra SA), supplemented with 2 ng/mL basic fibroblast growth factor (PeproTech), 2 ng/mL endothelin-1 (Sigma), 16 nM TPA (12-*O*-tetradecanoylphorbol-13-acetate; Sigma), and 0.1 mM IBMX (3-isobutyl-1-methylxanthine; Sigma). To all medium formulations 100 U/mL penicillin/streptomycin (Invitrogen) was added prior to first use. All cultures were maintained at 37°C under 5% CO_2 atmosphere in an incubator. Skin cells were weekly passaged 1 to 5 using trypsin/EDTA (1:100; Invitrogen), 8x diluted for melanocytes, and used for the irradiation experiments at near confluence, between passages 8 and 18. In the experiments involving arsenite, cells were preincubated with the NADPH/GSH:$O_2\bullet^-$ oxidoreductase MnTMPyP (Merck), superoxide dismutase (Sigma) and/or catalase (Sigma), or the NADPH oxidase inhibitors DPI (diphenylene iodonium; Aldrich) and apocynin (Aldrich) for 15 min., and subsequently exposed to different doses of sodium arsenite (Fluka) for 3 h. Jurkat cells were additionally stimulated with TPA (5 ng/mL) and PHA (2 µg/mL) to activate Cn (*48*).

UV irradiation

UVA experiments were performed using Sellas Sunlight lamps at 12.5 mW/cm^2 output, combined with UVASUN blue filter (emission spectrum: 340-400 nm). Doses were monitored using a IL700A Research radiometer with a WBS320#801 sensor. After replacement of medium with HBSS, all cell cultures were UVA1-irradiated in triplicate in Greiner Petri dishes with covers while kept on ice. Immediately afterwards, cells were washed with PBS, detached with trypsin/EDTA, 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.

Activity measurements

Calcineurin assays were performed according to the method reported by Sellar *et al.* (*49*), implementing a few modifications. Freshly harvested cell pellets were dispersed in 50 mM Tris-HCl, pH 7.7, containing ascorbic acid, DTT, 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 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 (*49*). PP2A activity, measured in the same assay, was defined as the okadaic acid-inhibited calcium-insensitive phosphatase activity (*50*). All enzyme activity values were corrected for protein content. Recombinant Cn for control experiments was purchased from Enzo Life Sciences, formerly Biomol.

Transcription factor assays

Nuclear lysates were obtained by hypotonic swelling and lysis of the outer membrane with 0.5% NP-40, followed by centrifugation and lysis of the nuclear envelope, using reagents from the Nuclear Extract Kit (Active Motif, Belgium). Spectrophotometric HRP-based ELISAs to detect the nuclear presence of dephosphorylated NFATc1 and activated p65 were performed according to the manufacturer's protocol (TransAM NFATc1 and NF-κB kits, Active Motif, Belgium), using plates to which oligonucleotides containing the consensus sequences for these transcription factors are immobilized. Absorption values at 450 nm with a reference wavelength of 655 nm were read on a SoftMax SpectraMax 190 microplate reader (Molecular Devices, USA) and corrected for sample protein content (Abs/g protein). For both assays, the allowed absorption range was defined such as to ensure linear behavior.

Statistics

Statistical operations were performed using GraphPad Prism v5. Details of analyses are provided in the figure legends.

RESULTS

Both arsenite and UVA1 cause a dose-dependent decrease in Cn activity in Jurkat cells

To see whether arsenite can influence Cn activity, Jurkat T cell lymphoma cells were subjected to treatment with micromolar concentrations of arsenite for 3 h. Doses up to 5 μM were well tolerated, as evidenced by minimal cell death directly after treatment. Cn activity was subsequently measured in the cellular lysates. Jurkat cells were chosen based on the fact that Cn signaling in lymphocytes has been thoroughly studied and documented at different levels; thus, they constitute a valuable model system for mechanistic experiments. The effects of UVA1 at the Cn activity level in Jurkat cells have recently been described (*51*). Figure 1a shows that a few micromolars of arsenite are sufficient to virtually completely disable calcineurin activity. The activity of PP2A, closely related to Cn, shows much more resistance to arsenite than calcineurin (figure 1b); at high arsenite concentrations, however, PP2A is also partly deactivated.

UVA1 suppresses both NFAT and p65 translocation, whereas arsenite only suppresses NFAT

Next, we tried to confirm the effects of UVA1 and arsenite exposure on Cn at a more downstream level in the signaling cascade. Their consequences for the nuclear translocation of NFAT and p65 were determined and are depicted in figures 2 and 3. Figure 2 shows that the activation of both transcription factors (panel A: NFAT; panel B: p65) is impaired following UVA1. Figure 3a shows that NFAT nuclear translocation is also inhibited by arsenite. However, p65 nuclear presence displays an initial rise at lower arsenite concentrations before decreasing nonetheless (figure 3b).

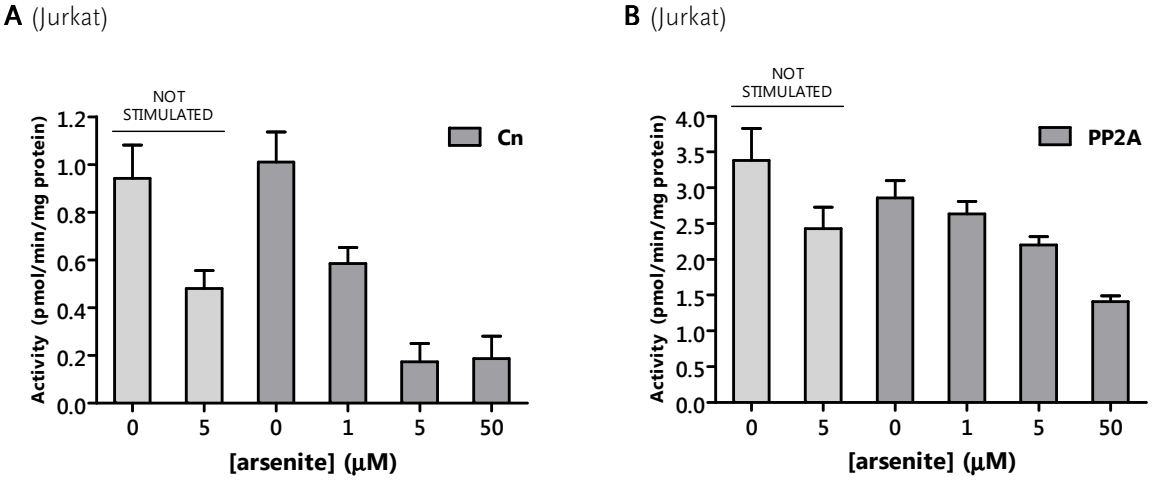


FIGURE 1 Effects of exposure (3 h) to micromolar concentrations of arsenite on [A] Cn and [B] PP2A activity in Jurkat cells (n=3, means ± SE), established using enzyme activity assays. Two unstimulated controls were included. Unpaired t-test shows the decrease in Cn activity (stimulated samples) at 1 and 5 μM vs. control as well as the difference between stimulated and unstimulated samples at 5 μM to be statistically significant ($p < 0.05$).

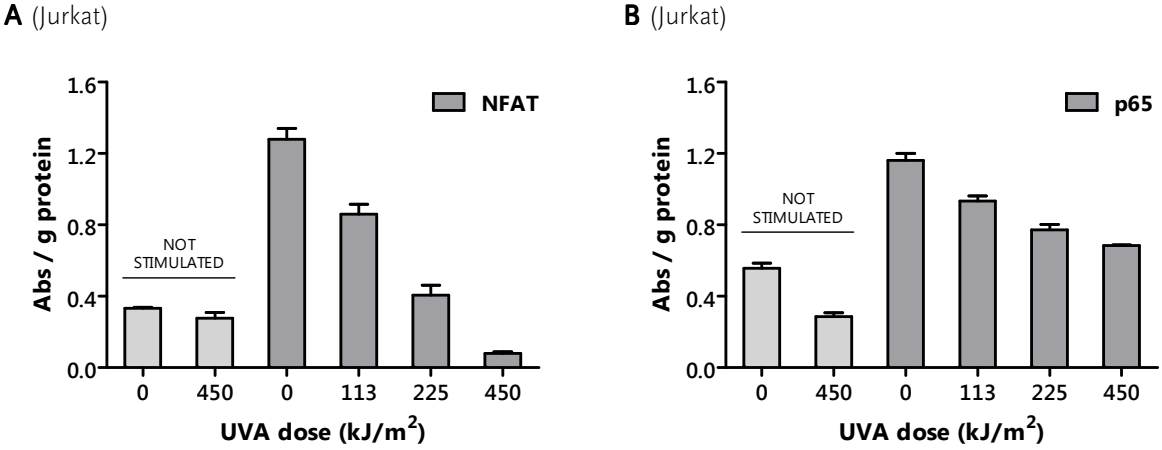


FIGURE 2 Quantification of the nuclear localisation (DNA-binding activity) of [A] NFAT and [B] p65 in Jurkat cells 3 h after exposure to UVA1 radiation (n=3, means ± SE), determined with TransAM ELISAs. Two unstimulated controls were included. One-way ANOVA yielded $p < 0.001$ for all differences among the stimulated samples in both the NFAT and p65 measurements. The Abs/g protein unit represents absorbance values corrected for protein content.

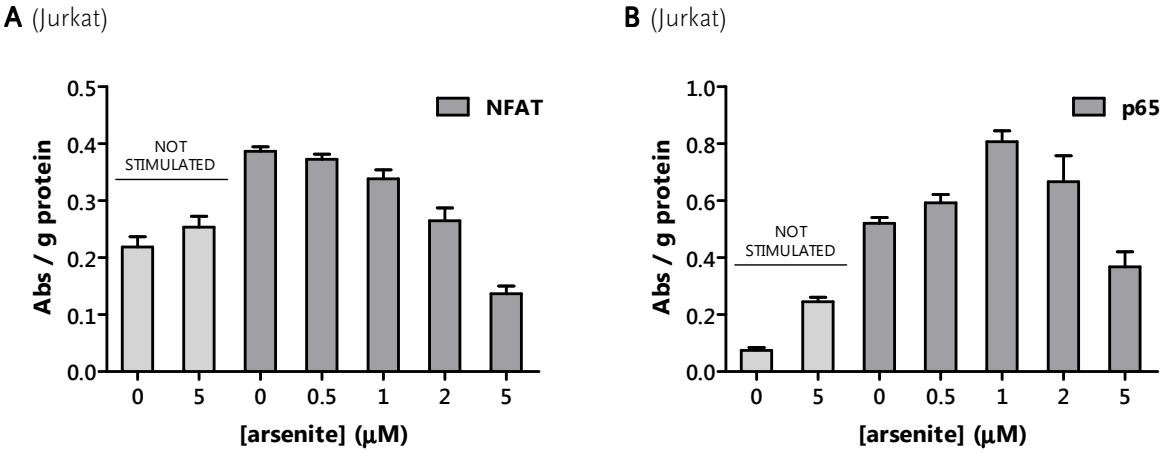


FIGURE 3 Response of [A] NFAT and [B] p65 nuclear localisation in Jurkat cells to 3 h treatment with a range of arsenite concentrations, determined using TransAM ELISAs (n=3, means ± SE). Two unstimulated controls have been included.

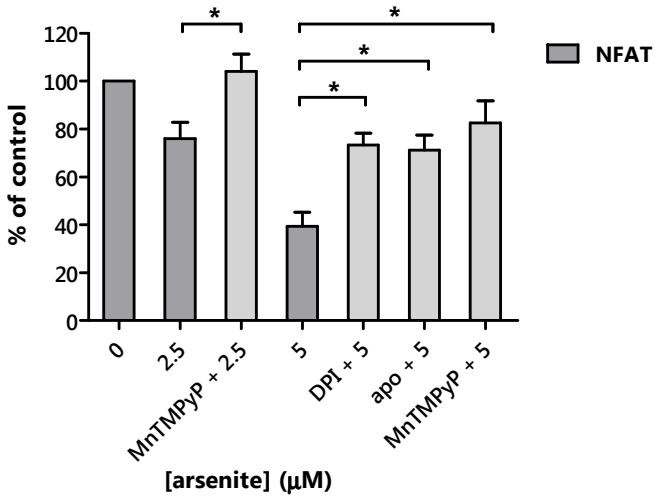


FIGURE 4 Attenuating effects of the NADPH oxidase inhibitors diphenylene iodonium (DPI, 10 µM in medium) and apocynin (apo, 10 µM in medium) and the SOD mimic MnTMPyP (5 µM in medium) on the decrease in NFAT nuclear translocation following 3 h exposure to arsenite (n=3, means ± SE). *: $p < 0.05$

Superoxide is involved in the mechanism by which arsenite deactivates Cn

To investigate whether the effects of arsenite exposure on Cn proceed through direct interaction of arsenite with the enzyme or indirectly through ROS involvement, we incubated Jurkat cells with the SOD mimic MnTMPyP (5 µM in medium) or the NADPH oxidase inhibitors apocynin and DPI 10 minutes before adding arsenite. Nuclear presence of NFATc1 was measured after 3 h, which is the time span that produces maximal translocation in stimulated arsenite-free controls, but shows limited cytotoxicity under all used conditions. We found the nuclear localization of NFAT to be significantly increased if apocynin, DPI, or MnTMPyP are present during incubation with arsenite (figure 4). This again points at superoxide, which is likely to originate from activated NADPH oxidase, as the agent responsible for decreased Cn activity. To rule out direct effects more

conclusively, recombinant Cn was incubated with sodium arsenite for 3 hours. Cn activity measured afterwards was found not to differ significantly from controls (data not shown).

Arsenite and UVA also reduce Cn activity in skin cells

In light of the present interest in the role of Cn in skin cells, we performed a series of similar experiments to investigate whether the effects of UVA1 and arsenite on Cn signaling in skin cells are comparable to those in Jurkat cells. Figure 5 depicts the effects of UVA1 on Cn activity in melanocytes and HaCaTs (panel A and B, respectively), while figure 6 displays the effects of arsenite on Cn activity and protection by MnTMPyP (panel A: melanocytes; panel B: HaCaT cells). As MnTMPyP shows some reactivity towards ROS other than superoxide, additional controls incubated with superoxide dismutase and/or catalase were used and confirmed involvement of superoxide (data not shown). Compared to fibroblasts and Jurkats, basal Cn activity levels are approximately 10 times lower in melanocytes, while levels in HaCaTs correspond to 50 - 75% (*47*). Weighed against the findings in fibroblasts and Jurkats discussed in our earlier paper (*51*) and figure 1, Cn activity in melanocytes succumbs more rapidly to either UVA1 irradiation or arsenite exposure, whereas Cn activity in HaCaTs seems somewhat more resilient.

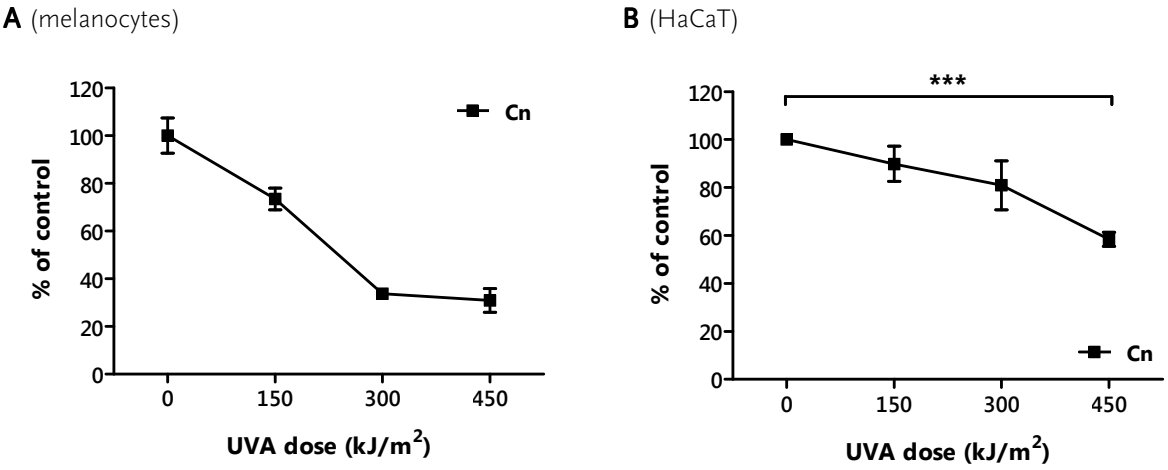
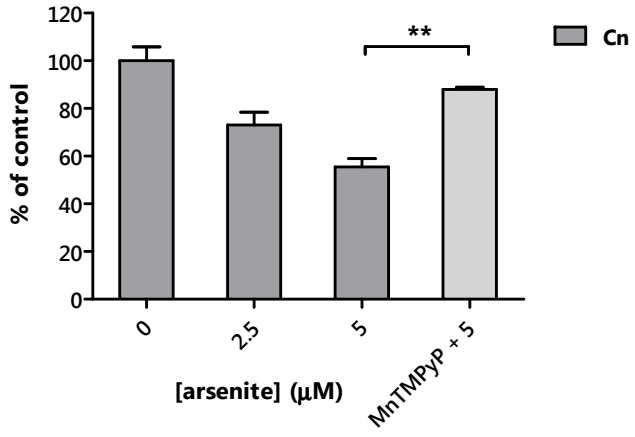


FIGURE 5 Response of Cn in [A] human primary melanocytes and [B] HaCaT cells to UVA1 irradiation up to 450 kJ/m^2 as determined using the Cn activity assay (n=3, means ± SE). ***: $p < 0.001$

A (melanocytes)



B (HaCaT)

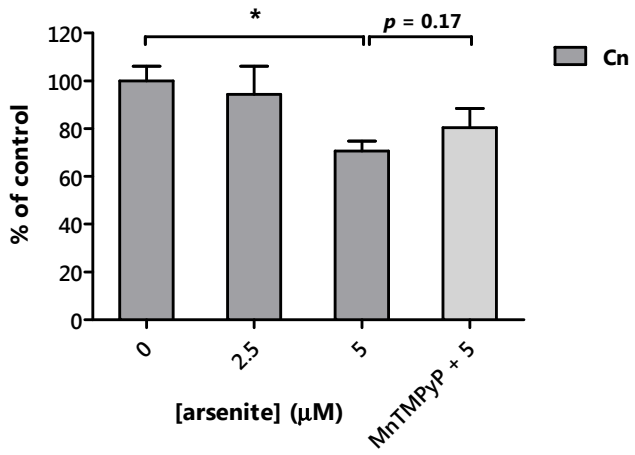


FIGURE 6 Response of Cn in [A] human primary melanocytes and [B] HaCaT cells to arsenite exposure (3 h) and effect of MnTMPyP (5 µM) on Cn activity (n=3, means ± SE). In the A panel, all relevant differences are statistically significant ($p < 0.05$). *: $0.01 < p < 0.05$; **: $p < 0.01$

DISCUSSION

In skin and skin cells, insight into the functional roles of Cn and the variety of NFAT isoforms is only just emerging (*52*). Despite sometimes paradoxical reports regarding the role of NFAT in tumor growth and development (*15, 53-56*), it has become clear that Cn signaling fulfills several tasks essential for dealing with runaway cells, such as cell cycle control, DNA repair and apoptosis (*11, 17, 57, 58*). This means that either overactivation or oversuppression of this pathway can have serious consequences. Transplant recipients treated with the calcineurin inhibitors (CnI) cyclosporin and tacrolimus to suppress their immune response and, hence, prevent allograft rejection are at high risk to develop skin cancer; they need careful therapeutic monitoring as the "safe" passage between rejection on one side and severe adverse effects on the other is very narrow (*56, 59*). In a recent paper, inhibitory effects of UVA1 on calcineurin activity were demonstrated in skin, fibroblasts and PBMC (*51*). A supplementary contribution of ROS on already suppressed

Cn could enhance the adverse profile of CnI in skin and thus increase the risk of developing malignancy.

The toxicity profile of arsenic species – for instance, their effects on brain and glucose maintenance – as well as their carcinogenic potential, primarily in skin, is reminiscent of the portfolio of side-effects displayed by CnI (*20, 24, 60*), but to a large extent based on production of ROS (*31*). This led us to believe that certain cellular effects of arsenite may be explained by suppression of Cn signaling.

Figure 1a confirms that arsenite can indeed affect Cn activity. After incubation with 5 μM arsenite for 3 hours, Cn activity shows roughly maximal inhibition. Figure 1b reveals that PP2A is less prone to deactivation than Cn but is affected more profoundly by high concentrations of arsenite than by UVA1 (*10*). As PP2A and Cn are the principal enzymes responsible for tau dephosphorylation (*37, 38, 61*), inhibition of these enzymes in neuronal cells by As-induced oxidative stress may pose an explanation for the accumulation of hyperphosphorylated tau described by Giasson *et al.* and even the very limited capability of the PP2A inhibitor okadaic acid to further enhance tau phosphorylation at an arsenite concentration of 500 μM (*62*).

It was recently demonstrated in PBMC and Jurkat cells that the production of a number of pro-inflammatory cytokines under control of Cn is also negatively affected by UVA1 (*51*). We now show that the nuclear translocation of both NFAT and NFκB in Jurkat cells following UVA treatment is strongly decreased (see figure 2), which is envisaged to be directly responsible for the diminished cytokine production. NFAT nuclear translocation following arsenite treatment is significantly lessened as well (figure 3a), which is conceivable based on the effect of arsenite on Cn activity displayed in figure 1a.

The sensitivity of Cn to reactive oxygen species is well established (*6, 63*). Inhibition by UVA is envisaged to proceed via ROS generated by photosensitization (*10*). In addition, both UVA and arsenite activate NADPH oxidase to produce large quantities of superoxide (*22, 64*), which is also likely to affect Cn activity. On the other hand, given their affinity for sulfhydryl groups, arsenic species could have a direct effect on Cn (*44, 65*). Figure 4 shows that in Jurkat cells, the cell-permeant SOD mimic MnTMPyP partly prevents the large decrease in NFAT nuclear localization due to arsenite exposure. Moreover, the NADPH oxidase inhibitors apocynin and DPI display the same effect as MnTMPyP (see figure 4). Combined with the finding that recombinant Cn is impervious to arsenite (data not shown), these results make it plausible that the suppression of Cn enzyme activity by arsenic exposure should be ascribed to superoxide.

As the most frequent skin cancers (basal and squamous cell carcinomas and melanomas) originate from either keratinocytes or melanocytes, a comparison of the effects of UVA1 and arsenite in these cell types is obviously appropriate. Figures 5 and 6 show similar effects of UVA1 and arsenite in HaCaTs and melanocytes, conforming to the tenor of earlier studies (*51*) and figure 1. Melanocytes typically feature 10x lower Cn

activity than fibroblasts, but, interestingly, show a conspicuously higher susceptibility to UVA (and As treatment) than other skin cells (see figures 5a and 6a). This may reflect the reported higher predisposition of melanocytes to oxidative stress, presumably due to photosensitizing pheomelanin (*66*, *67*). Figure 6 also shows that the cell-permeant SOD mimic MnTMPyP is capable of mitigating As-induced Cn activity loss in melanocytes and possibly in HaCaTs as well, which is in line with the attenuating influence of MnTMPyP on the effects of arsenite on other signaling cascades found in HaCaT cells by Cooper *et al.* (*22*), and again points at superoxide involvement. Unfortunately, our assay proved unsuitable to confirm these measurements at the level of NFATc1 translocation in skin cells, which could be caused by lower expression or different activation kinetics of this particular isoform in skin (*52*).

Although arsenite and UVA1 show a similar effect on Cn activity and NFAT translocation (see figures 1a, 2a and 3a), their effects on p65 contrast greatly (figure 2b and 3b). NF-κB is known to be induced by oxidative stress – whether or not generated by UVA – in several cell types (*68*, *69*), although the response of NF-κB to UVA was found to be notably slow due to transient loss of nuclear membrane integrity (*70*). Moreover, immunoreactive levels of p65 in keratinocytes 3 h after UVA exposure were even decreased compared to untreated controls. Degradation of subunits has been suggested as a probable cause (*71*). It it also known that NF-κB is partly under control of Cn, which promotes degradation of IκB-β, so that under specific circumstances, Cn activity loss may have repercussions on NF-κB status (*72*). On the other hand, constitutive NF-κB activation promotes cell survival and may contribute to malignant transformation (*73*). The characterization of the response of p65 to UVA1 and arsenite may benefit from additional measurements of intracellular ROS production levels at different time points and assessment of p65 translocation in presence and absence of ROS scavengers such as SOD and catalase, and calcineurin inhibitors such as cyclosporin A and tacrolimus, which will be the basis of future studies.

It should be noted that the Cn activity assay is performed under optimal conditions, ensuring excess substrate, calcium and calmodulin. Hence, any loss of activity measured by this assay reflects direct, physical damage to the enzyme. The fact that this assay measures Cn capacity rather than activity also explains why PHA/TPA stimulation does not have any effect at the Cn activity level, as illustrated by figure 1a. To attain NFAT translocation, however, PHA/TPA stimulation is indispensable (see figure 2a and 3a). Interestingly, figure 1a shows significantly lower Cn activity in the presence of 5 μM arsenite under stimulated conditions than in unstimulated samples. In our opinion, this discrepancy constitutes additional evidence of large quantities of superoxide being released during exposure to arsenite, since continuous operation of Cn may render its active site accessible not only to substrate, but also to superoxide, as suggested by Ullrich and coworkers (*74*).

In conclusion, we have shown interference of both UVA1 and arsenite at different levels in the calcineurin signaling cascade in Jurkat and skin cells and we have provided evidence that this interference is mediated by ROS. Inhibitory effects on Cn signaling could be a new explanation how substantial production of ROS, whether or not due to arsenic exposure, may lead to malignancy formation. Additionally, this knowledge could offer a starting point for tailored prophylaxis. One of the priorities of future studies should be to assess the significance of inhibition of Cn signaling by As species by clarifying how and how much their effects on Cn contribute to the tyical range of their pathological symptoms. For instance, more extensive studies in the field of DNA repair may reveal to what extent suppression of DNA repair by arsenite proceeds via Cn and/or NFAT, respectively.

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