Inhibition of poly(adenosine diphosphate-ribose) polymerase by the active form of vitamin D

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Abstract. Vitamin D is well characterized for its role in mineral homeostasis and maintenance of normal skeletal architecture. Vitamin D has been demonstrated to exert anti-inflammatory effects in a variety of disease states including diabetes, arthritis and inflammatory bowel disease. In these diseases poly[adenosine diphosphate (ADP)-ribose] polymerase (PARP) inhibitors have also proved effective as anti-inflammatory agents. Here we present data demonstrating that the active metabolite of vitamin D, 1α,25-dihydroxy-vitamin D3, is a PARP inhibitor. UV irradiation-mediated PARP activation in human keratinocytes can be inhibited by treatment with vitamin D, 7-dehydrocholesterol or 1α,25-dihydroxyvitamin D3. Inhibition of cytochrome P450 reversed the PARP inhibitory action of vitamin D and 7-dehydrocholesterol, indicating that conversion to 1α,25-dihydroxyvitamin D3 mediates their PARP inhibitory action. Vitamin D may protect keratinocytes against over-activation of PARP resulting from exposure to sunlight. PARP inhibition may contribute to the pharmacological and anti-inflammatory effects of vitamin D.

Introduction

Poly(ADP-ribose) polymerase-1 (PARP-1) is an abundant nuclear enzyme present in eukaryotes (1), which has been implicated in the cellular response to DNA injury. PARP-1 functions as a DNA damage sensor and as a signaling molecule binding to both single- and double-stranded DNA breaks. Upon binding to damaged DNA, PARP-1 forms homodimers and catalyzes the cleavage of NAD^+ into nicotinamide and ADP-ribose. The latter is used to synthesize branched nucleic acid-like polymers with poly[adenosine diphosphate (ADP)-ribose] units covalently attached to nuclear acceptor proteins including histones, transcription factors and PARP itself (2). This poly(ADP-ribosyl)ation of these proteins contributes to inflammatory signal trans-duction processes. In addition, oxidative stress-induced over-activation of PARP consumes NAD^+ and consequently ATP, culminating in cell dysfunction or necrosis (2).

Activation of PARP has been implicated in the patho-genesis of stroke (3), myocardial ischemia (4), diabetes (5), diabetic complications (6,7), shock (8), traumatic central nervous system injury (9), arthritis (10), colitis (11), and various other forms of inflammation (2,11). PARP is involved in the regulation of the activation of various pro-inflammatory transcription factors including NF-κB (12), AP-1 (13) ATF-2 and CREB (14). PARP regulates the cellular expression of many pro-inflammatory cytokines, chemokines and adhesion molecules (15).

Vitamin D is a lipid-soluble vitamin which is traditionally viewed as a regulator of mineral (calcium and phosphorous) homeostasis and maintenance of normal skeletal architecture (16). Additional studies have demonstrated that this vitamin is also involved in a host of cellular processes including cell differentiation, cell proliferation and immune function (16). Vitamin D, the biologically inactive moiety, is converted into the active form 1α,25-dihydroxyvitamin D3 by hydroxyl-ation reactions, which occur primarily in the liver and kidney (16), but have also been described in keratinocytes (17-20). Vitamin D has been shown to be anti-inflammatory (16) and able to protect against various inflammatory disease states including rheumatoid arthritis (21), type I diabetes (22), inflammatory bowel disease (23), transplant rejection (24) and multiple sclerosis (25). The anti-inflammatory action of vitamin D involves down-regulation of inflammatory chemokine and cytokine expression (22,26), inhibition of transcription factor activation (27), and inhibition of T cell proliferation (28). As the anti-inflammatory effects of vitamin D overlap substantially with those of PARP inhibitors, we examined whether the active form of vitamin D inhibits PARP activation.

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Materials and methods

Reagents were obtained from the following sources: ^{3}H-NAD was from Perkin-Elmer Inc. (Boston, MA, USA); vitamin D, 1α,25-dihydroxyvitamin D3, 1α-hydroxyvitamin D3, 25-hydroxyvitamin D3, 7-dehydrocholesterol and ketoconazole were from Sigma (St. Louis, MO, USA); peroxynitrite was from Calbiochem (San Diego, CA, USA), and cell-free PARP assay was from Trevigen Inc. (Gaithersburg, MD, USA). Cell culture media and supplements were from Invitrogen (Carlsbad, CA, USA), and the RAW 264.7 cell line was from ATCC (Manassas, VA, USA).

Cell-free PARP assay. The activity of vitamin D3 and its metabolites against purified human PARP enzyme was determined using a commercially available kit (Trevigen). The assay was carried out in 96-well ELISA plates following the manufacturer's instructions. Briefly, wells were coated with 1 mg/ml histone (50 μl/well) at 4˚C overnight. Plates were then washed four times with PBS and then blocked by adding 50 μl of Strep-Diluent (supplied with the kit). After incubation (1 h, room temperature) plates were washed four times with PBS. Appropriate dilutions of vitamin D3, its metabolites and a positive control (the PARP inhibitor PJ-34) were combined with a 2X PARP cocktail (1.95 mM NAD$^+$, 50 μM biotinylated NAD$^+$ in 50 mM Tris pH 8.0, 25 mM MgCl$_2$) and a high specific activity PARP enzyme (both supplied with the kit) in a volume of 50 μl. The reaction was allowed to proceed for 30 min at room temperature. After four washes in PBS, incorporated biotin was detected by peroxidase-conjugated streptavidine (1:500 dilution) and TACS Sapphire substrate.

PARP cell assay. The macrophage cell line RAW 264.7 was treated with a combination of peroxynitrite (750 μM) ± various concentrations of vitamin D3 or its metabolites for 15 min. For the measurement of PARP activity, the media was removed and replaced with 0.5 ml HEPES (pH 7.5) containing 0.01% digitonin and ^{3}H-NAD (0.5 μCi ml^{-1}) for 20 min. The cells were then scraped from the wells and placed in Eppendorf tubes containing 200 μl of ice-cold 50% TCA (w/v), and the tubes were then placed at 4˚C. After 4 h the tubes were centrifuged at 1800 x g for 10 min, the supernatant was removed, and the pellet was washed twice with 500 μl ice-cold 5% TCA. The pellet was solubilized in 250 μl NaOH (0.1 M) containing 2% SDS overnight at 37˚C, and the PARP activity was then determined by measuring the radioactivity incorporated using a Wallac scintillation counter. The solubilized protein (250 μl) was mixed with 5 ml of scintillant (ScintiSafe Plus, Fisher) before being counted for 10 min. Results were expressed as percent of PARP activity seen without the addition of compounds.

In a second series of experiments HaCaT cells (kindly provided by Dr Ulrich Rodeck, Thomas Jefferson University, Philadelphia, PA, with the permission of Professor Norbert E. Fusenig, German Cancer Research Center, Heidelberg, Germany) were cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum and 100 U/ml penicillin and 100 μg/ml streptomycin for 48 h before being incubated with vitamin D (10-300 μM) for 15 min, 4, 8, or 16 h. The treated HaCaT cells were then exposed to peroxynitrite (750 μM) for 15 min, and the PARP activity was determined as above. Finally, HaCaT cells were treated with increasing doses of vitamin D, 7-dehydrocholesterol and 1α,25-dihydroxyvitamin D3 ± the cytochrome P450 enzyme inhibitor ketoconazole and irradiated with UVA and B. Cells were irradiated with 72 mJ/cm^2 of UVA and B using a Multiport Solar UV simulator (model 601, from Solar Light Co. Inc., Philadelphia, PA) with the dose being regulated by a PMA 2100 dose control system (Solar Light Co. Inc.). Cellular PARP activation was measured 2 h after irradiation as described above.

Results

Effect of vitamin D on the activity of isolated human PARP enzyme. The active form of vitamin D, 1α,25-dihydroxyvitamin D3, dose-dependently inhibited PARP with 40% inhibition being observed at 0.1 μM increasing to 80% at 1 μM (Table I). The inactive form of vitamin D exhibited a minor PARP-inhibitory activity with only 10% inhibition observed at 100 μM. The 1α-hydroxyvitamin D3 form of vitamin D had no inhibitory activity at any of the concentrations tested (Table I). 25-Hydroxyvitamin D3 did show some PARP

Table I. Effect of vitamin D3 and its active metabolites on the activity of isolated high specific activity human poly(ADP-ribose) polymerase.

Drug	Concentration							
	0.003 μM	0.01 μM	0.03 μM	0.1 μM	1 μM	10 μM	30 μM	100 μM
Vitamin D3	-	-	-	-	87±1[a]	85±0.5[a]	84±1[a]	83±0.5[a]
1α-hydroxyvitamin D3	104±3	103±2	99±0.4	100±1	100±1	99±0.4	103±1	95±1
25-hydroxyvitamin D3	91±2	83±1[a]	75±1[a]	78±0.2[a]	77±4[a]	79±1[a]	82±2[a]	56±6[a]
1α,25-dihydroxyvitamin D3	81±0.3[a]	78±1[a]	74±1[a]	64±2[a]	24±1[a]	24±5[a]	-	-

Results were expressed as percent activity, where the activity of the untreated enzyme was considered 100%. Purified human PARP was incubated for 45 min with 250 μM NAD ± drug. Data were expressed as the mean ± SEM (n=3). Statistical analysis was carried out using one-way analysis of variance followed by the Student-Newman-Keuls multiple comparison *post-hoc* analysis where $p<0.05$ was considered significant; [a]$p<0.05$ vs. untreated cells.

Table II. Effect of vitamin D3 and its active metabolites on poly(ADP-ribose) polymerase activity in RAW 264.7 cells treated with peroxynitrite.

Drug	Concentration						
	0.03 μM	0.1 μM	0.3 μM	1 μM	3 μM	10 μM	100 μM
Vitamin D3	-	-	99±2	97±7	98±2	104±4	78±5[a]
1-hydroxyvitamin D3	-	100±3	100±6	118±8	93±3	85±2[a]	65±1[a]
25-hydroxyvitamin D3	-	95±4	96±4	90±3	74±1[a]	70±2[a]	65±3[a]
1,25-dihydroxyvitamin D3	90±5	89±3[a]	85±3[a]	72±3[a]	54±1[a]	-	-

Results were expressed as percent activity of the ONOO⁻ (750 μM)-treated cells. The mouse macrophage RAW 264.7 cell line was treated with vitamin D3 and its active metabolites for 15 min prior to the addition of ONOO⁻ for a further 15 min. The cellular PARP activity was then determined. All forms of vitamin D3 showed PARP inhibitory activity but the most active was the 1,25-dihydroxyvitamin D3. Data were expressed as the mean ± SEM (n=3). Statistical analysis was carried out using one-way analysis of variance followed by the Student-Newman-Keuls multiple comparison *post-hoc* analysis where p<0.05 was considered significant; [a]p<0.05 vs. untreated cells.

inhibitory activity, reducing the activity by 40% at 100 μM (Table I).

Effect of vitamin D on cellular PARP enzyme. Similar to the results obtained using isolated enzyme, 1α,25-dihydroxy-vitamin D3 was the most effective at inhibiting peroxynitrite-stimulated PARP activity in the RAW 264.7 macrophage cell line with 50% inhibition being observed at 3 μM (Table II). Vitamin D itself showed a 20% inhibition at 100 μM. The monohydroxylated forms of vitamin D also demonstrated PARP-inhibitory activity reduced by 45% at 100 μM (Table II).

Effect of vitamin D on PARP activity in the human keratino-cyte cell line HaCaT. Incubation of HaCaT cells with various concentrations of vitamin D (for 15 min prior to the addition of peroxynitrite) concentration-dependently inhibited PARP activation (Fig. 1). As the time of incubation of the HaCaT cells with vitamin D increased prior to the addition of the peroxynitrite, the effectiveness of vitamin D in inhibiting PARP increased, especially at the lower doses of 10 and 30 μM of vitamin D which had no inhibitory effect at 15 min but with a >4-h incubation time significantly reduced the HaCaT PARP activity in response to peroxynitrite (Fig. 1).

In a second series of experiments, HaCaT cells were exposed to 72 mJ/cm² UVA and B radiation and PARP activity was measured 2 h later. Following irradiation PARP activity in the HaCaT cells doubled (4008±252 cpm to 9893±614, p<0.001). Prior to irradiation, HaCaT cells were treated with increasing doses of vitamin D, 7-dehydro-cholesterol (provitamin D) and 1α,25-dihydroxyvitamin D3 and the PARP activity was again determined 2 h after irradiation. We found that all three forms of vitamin D concentration-dependently inhibited PARP activation (Fig. 2). To further characterize the mechanism by which vitamin D inhibits PARP, we simultaneously treated the HaCaT cells with the highest dose of vitamin D, 7-dehydrocholesterol or 1α,25-dihydroxyvitamin D3 plus the cytochrome P450 enzyme inhibitor ketoconazole at either 5 or 10 μM. Ketoconazole partially reversed the inhibition of PARP seen with both vitamin D and 7-dehydrocholesterol at 300 μM, but had no effect on inhibition by 1α,25-dihydroxyvitamin D3 implicating
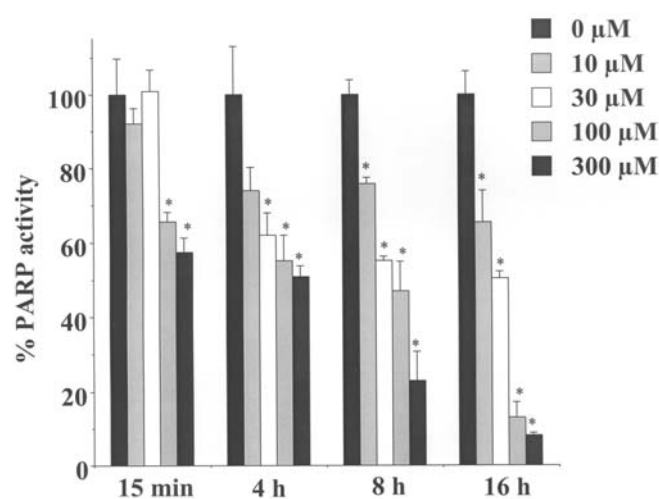

Figure 1. Treatment of human keratinocyte HaCaT cells with vitamin D3 protected against activation of PARP by peroxynitrite. HaCaT cells were incubated for 15 min, 4, 8, or 16 h with various concentrations of vitamin D3 prior to being treated with ONOO⁻ at 750 μM for 15 min. PARP activity was then measured. The longer the incubation time with vitamin D3 the more effective the inhibitory action became suggesting that the inactive vitamin D3 was being converted into the active 1,25-dihydroxyvitamin D3. Data were expressed as the mean ± SEM (n=3). *p<0.05 was considered significant vs. untreated cells.

the cytochrome P450 enzyme system in converting vitamin D and 7-dehydrocholesterol to active PARP inhibitors (Fig. 2). Ketoconazole alone at 10 μM had no effect on UV-induced PARP activity (9893±614 cpm vs. 9527±632 in the treated cells, p=0.73).

Discussion

We have demonstrated here for the first time that vitamin D exerts a concentration-dependent inhibitory effect on the nuclear enzyme poly(ADP-ribose) polymerase. Our studies determined that the dihydroxylated active form of vitamin D, (1α,25-dihydroxyvitamin D3) is a more potent PARP inhibitor than vitamin D. The monohydroxylated forms, 1α-hydroxyvitamin D3 and 25-hydroxyvitamin D3 have little or
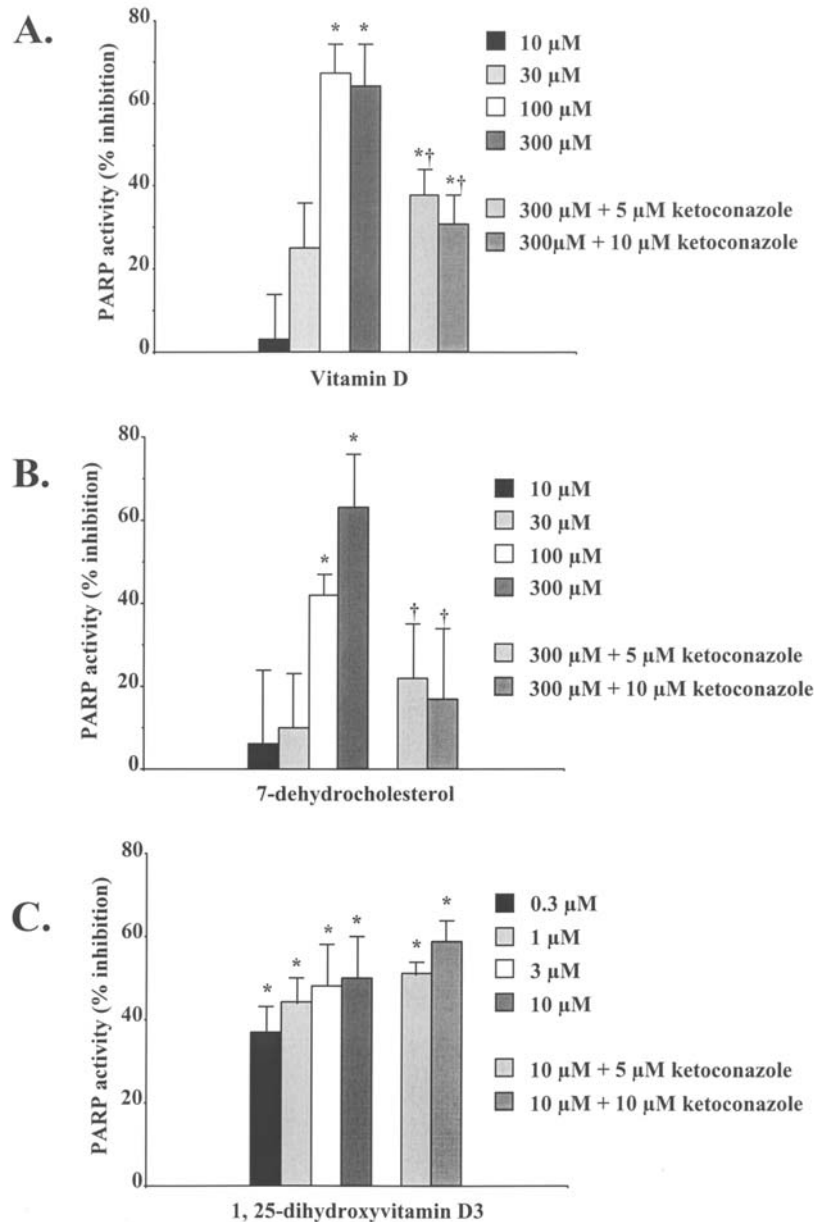
Figure 2. Effect of vitamin D (A), 3,7-dehydrocholesterol (provitamin D3) (B) and 1α,25-dihydroxyvitamin D3 (C), on poly(ADP-ribose) polymerase activity in HaCaT cells exposed to UV irradiation. Results were expressed as percent inhibition of the activity observed in untreated cells following UV exposure. The HaCaT cell lines were treated with the various concentrations of the vitamin D isoforms before being exposed to 72 mJ/cm^2 UVA and B, and 2 h after exposure the PARP activity was determined. All isoforms of vitamin D demonstrated concentration-dependent PARP inhibitory activity. The inhibitory effect of the precursors of 1α,25-dihydroxyvitamin D3, vitamin D and 3,7-dehydrocholesterol, was blocked by the cytochrome P450 enzyme inhibitor ketoconazole, indicating that their conversion to 1α,25-dihydroxyvitamin D3 was required for PARP inhibition. Results were expressed as the mean ± SEM (n=3). Statistical analysis was carried out using one-way analysis of variance followed by the Student-Newman-Keuls multiple comparison *post-hoc* analysis where p<0.05 was considered significant; *p<0.05 vs. untreated cells, †p<0.05 vs. 300 (10) μM alone.

no PARP inhibitory effect. The results of our studies in the human keratinocyte cell line, HaCaT, are consistent with the notion that UV irradiation-induced PARP activity can be inhibited by pro-vitamin D and vitamin D by a mechanism which involves their cellular conversion to 1α,25-dihydroxy-vitamin D3.

Supraphysiological doses of vitamin D exert protective effects in inflammatory and non-inflammatory conditions, including type I diabetes, arthritis and cardiac failure (29). The anti-inflammatory effects of vitamin D include inhibition of pro-inflammatory chemokine and cytokine expression (22,26) and inhibition of transcription factor activation (27),

effects duplicated by specific PARP inhibitors (13,15). Survival of keratinocytes post UV-radiation was significantly greater following treatment with the active form of vitamin D, 1α,25-dihydroxyvitamin D3 (30). We propose, therefore, that the anti-inflammatory and cardioprotective effects of vitamin D may be, at least in part, mediated by the inhibition of PARP.

Our studies used well-characterized methods to determine PARP inhibitory activity, such as the isolated enzyme in a cell-free system (31,32) and RAW 264.7 macrophages, which demonstrated considerable PARP activation following peroxynitrite treatment (32). In the cell-free assay we found that 1α,25-dihydroxyvitamin D3 was the most potent PARP

inhibitor and the only one which was active at concentrations which made it likely to be a PARP inhibitor *in vivo*. Vitamin D and its two monohydroxylated forms have some minor PARP inhibitory activity, but only at very high concentrations, which are probably unimportant for potential *in vivo* effects. In RAW 264.7 cells treated with peroxynitrite to induce PARP activation we found 1α,25-dihydroxyvitamin D3 was the most potent PARP inhibitor, but both the mono-hydroxylated forms also had some inhibitory activity, possibly due to some cellular conversion of the mono-hydroxylated forms of vitamin D into 1α,25-dihydroxy-vitamin D3.

Physiologically, vitamin D is converted to the biologically active metabolite 1α,25-dihydroxyvitamin D3 by a cascade of reactions, which include hydroxylation at the C-25 position in the liver and at the C-1α position in the kidney. In 1998 Lehmann *et al* demonstrated for the first time that the human HaCaT cell line could metabolize both vitamin D and 1α-hydroxyvitamin D3 into 1α,25-dihydroxyvitamin D3 (33,34) and later demonstrated that this occurred not only in the HaCaT cell line but also in primary human skin cells (18). In our studies, vitamin D treatment of HaCaT cells inhibited peroxynitrite-induced PARP activation. As we increased the incubation time with vitamin D prior to peroxy-nitrite treatment, the vitamin became increasingly more effective at inhibiting PARP activation, especially at the lowest concentrations studied. This effect may be due to the conversion of vitamin D to its active metabolite over time.

Lehmann *et al* also investigated the UVB-induced conversion of 7-dehydrocholesterol, a precursor of vitamin D, to 1α,25-dihydroxyvitamin D3 in HaCaT cells (17,19). 7-Dehydrocholesterol (provitamin D), when exposed to UVB radiation, was converted *in vivo* (35) and *in vitro* (36) to pre-vitamin D, which in turn was isomerized to vitamin D. They also demonstrated that the cytochrome P450 enzyme inhibitor ketoconazole (37) blocked the UVB-mediated conversion of 7-dehydrocholesterol to 1α,25-dihydroxyvitamin D3 in human skin cells (17,19). UV irradiation has been demonstrated to induce nitric oxide and peroxynitrite production in keratinocytes (38) an effect mediated by UVB-mediated activation of enzymes responsible for nitric oxide and super-oxide production (38). This increase in nitrogen reactive species following UVB exposure is of particular importance in sunburn erythema. Peroxynitrite is a potent activator of PARP (39). When considering the direct effects of UV irradiation on DNA (40), it is likely that PARP will become activated in skin cells following UV light irradiation *in vivo*. Indeed, topical application of a PARP inhibitor has been shown to protect skin from acute photodamage (41). Here we found that *in vitro* 2 h after irradiation with UVA and B there was a doubling of PARP activity in the HaCaT cells.

The human skin is continually exposed to UV radiation and though it is not surprising that PARP becomes activated in extreme circumstances such as sunburn erythema it is conceivable that even under normal exposure PARP may become activated in skin cells. Therefore, it is likely that skin cells have built-in protection mechanisms to prevent over-activation of PARP, and vitamin D may serve as one such protective mechanism. We found that exposing HaCaT cells to UV irradiation in the presence of 7-dehydrocholesterol, vitamin D or 1α,25-dihydroxyvitamin D3 significantly reduced cellular UV-induced PARP activation. In addition we found that the presence of the cytochrome P450 enzyme inhibitor, ketoconazole, significantly blunted the PARP inhibitory effect of both 7-dehydrocholesterol and vitamin D but not 1α,25-dihydroxyvitamin D3 suggesting that by blocking the conversion of vitamin D to 1α,25-dihydroxy-vitamin D3 the PARP inhibitory effect was also blocked, hence further strengthening the notion that the 1α,25-dihydroxyvitamin D3 form of vitamin D is the active PARP inhibitor.

In conclusion, we described that vitamin D has a novel pharmacological effect as a PARP inhibitor and we provided multiple lines of evidence to suggest that its active metabolite, 1α,25-dihydroxyvitamin D3, is responsible for this activity. Therefore, we speculate that at least in part vitamin D's cellular actions may be mediated by the inhibition of PARP. We also demonstrated that keratinocytes exposed to UV irradiation have increased PARP activation and that this is attenuated by 1α,25-dihydroxyvitamin D3 treatment, as well as by the active metabolite precursors, 7-dehydrocholesterol and vitamin D. Therefore, we propose that the UV-radiation-mediated vitamin D generation system in keratinocytes not only provides vitamin D for the whole body but also acts as an intrinsic self-preservation mechanism for keratinocytes to prevent pathological over-activation of PARP in response to sunlight exposure or other external sources of UV radiation.

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