Published in final edited form as:

Photochem Photobiol. 2017 July ; 93(4): 990–998. doi:10.1111/php.12720.

The B₆-vitamer Pyridoxal is a Sensitizer of UVA-induced Genotoxic Stress in Human Primary Keratinocytes and Reconstructed Epidermis[†]

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Abstract

UVA-driven photooxidative stress in human skin may originate from excitation of specific endogenous chromophores acting as photosensitizers. Previously, we have demonstrated that 3-hydroxypyridine-derived chromophores including B₆-vitamers (pyridoxine, pyridoxamine, and pyridoxal) are endogenous photosensitizers that enhance UVA-induced photooxidative stress in human skin cells. Here, we report that the B₆-vitamer pyridoxal is a sensitizer of genotoxic stress in human adult primary keratinocytes (HEKa) and reconstructed epidermis. Comparative array analysis indicated that exposure to the combined action of pyridoxal and UVA caused upregulation of heat shock (*HSPA6*, *HSPA1A*, *HSPA1L*, *HSPA2*), redox (*GSTM3*, *EGR1*, *MT2A*, *HMOX1*, *SOD1*), and genotoxic (*GADD45A*, *DDIT3*, *CDKN1A*) stress response gene expression. Together with potentiation of UVA-induced photooxidative stress and glutathione depletion, induction of HEKa cell death occurred only in response to the combined action of pyridoxal and UVA. In addition to activational phosphorylation indicative of genotoxic stress [p53 (Ser15) and γ -H2AX (Ser139)], comet analysis indicated the formation of Fpg-sensitive oxidative DNA lesions, observable only after combined exposure to pyridoxal and UVA. In human reconstructed epidermis, pyridoxal pre-incubation followed by UVA exposure caused genomic oxidative base damage, procaspase 3 cleavage, and TUNEL-positivity, consistent with UVA-driven photooxidative damage that may be relevant to human skin exposed to high concentrations of B₆-vitamers.

Graphical abstract

UVA-driven photooxidative stress in human skin may originate from excitation of specific endogenous chromophores acting as photosensitizers. Here, evidence is presented suggesting that the B₆-vitamer pyridoxal is a sensitizer of genotoxic stress in human adult primary keratinocytes. In human reconstructed epidermis, pyridoxal pre-incubation followed by UVA exposure caused genomic oxidative base damage (8-oxo-dG), procaspase 3 cleavage, and TUNEL-positivity.

[†]This article is part of the Special Issue honoring Dr. Hasan Mukhtar's 70th Birthday and his outstanding contributions to various aspects of photobiology research, including photocarcinogenesis and chemoprevention.

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consistent with UVA-driven photooxidative damage that may be relevant to human skin exposed to high concentrations of B₆-vitamers.

INTRODUCTION

In human skin, various chromophores may act as endogenous photosensitizers, potentiating photodamage induced by exposure to solar ultraviolet (UV) and visible radiation. A number of endogenous chromophores displaying activity as UV-sensitizers has been identified including protoporphyrin IX (1), urocanic acid (2), riboflavin (3), B₆-vitamers (4), melanin precursors (5), collagen crosslinks (6), advanced glycation and lipid peroxidation endproducts (7, 8), tryptophan-derived photoproducts including the nanomolar UV-sensitizer 6-formylindolo[3,2-b]carbazole (9), and pterin (10), all of which have been associated with photon-driven excited state chemistry, causing the formation of specific photoproducts and reactive oxygen species (ROS) including singlet oxygen (¹O₂) as key mediators of cutaneous photooxidative stress (11–13). The process of photosensitization seems to be of particular relevance to the causation of UVA (320 – 400 nm) radiation-induced skin photodamage, since UVA exposure results in little photoexcitation of DNA directly, and sensitizer-dependent photochemistry leading to the cutaneous generation of reactive excited states, organic free radicals, and ROS is now a widely accepted mechanism of UVA-phototoxicity targeting human skin cells with potential relevance to cutaneous photoaging and carcinogenesis (9, 12–19).

In addition to playing a functional role as a class of metabolic coenzymes involved in the catalysis of oxidative transaminations and other vital biochemical reactions, cumulative evidence suggests that B₆-vitamers [including pyridoxine (PN), pyridoxamine (PM), and pyridoxal (PL)] play an important non-vitamin role as potent sacrificial antioxidants and ¹O₂-quenchers, metal chelators, carbonyl scavengers, and UVA-photosensitizers (20–22). Prior research has identified the 3-hydroxypyridine moiety shared among B₆-vitamers as the minimal photoactive chromophore conferring UV-sensitizer activity (4). Phototoxicity of B₆-vitamers has been investigated before in the context of clinical photodermatitis and hypervitaminosis, and molecular evidence obtained from simple chemical reaction systems has been presented demonstrating the ability of B₆-vitamers to potentiate UVA-induced photo-oxidation of biomolecules including DNA bases and nucleotides, amino acids and peptides, and plasmid DNA, observable at concentrations that might be relevant to

phototoxicity of endogenous cutaneous B₆-vitamers (4, 21–26). Interestingly, it has also been observed that vitamin B₆ supplementation (using PN) enhances tumorigenesis in a hairless mouse model of UV-induced carcinogenesis (27).

In continuation of these previous studies we decided to explore the specific molecular consequences of B₆-vitamer-dependent photosensitization in relevant epidermal model systems. Here, we report for the first time that the B₆-vitamer PL is a micromolar sensitizer of UVA-induced genotoxic stress, presenting experimental evidence derived from comparative gene expression array analysis, alkaline single cell gel electrophoresis, and immunohistochemical analysis that demonstrates the occurrence of PL-sensitization of UVA-induced photodamage and genotoxicity in primary human keratinocytes and reconstructed epidermis.

MATERIALS AND METHODS

Chemicals

The cell-permeable pancaspase inhibitor Z-VAD-fmk was from Calbiochem-Novabiochem (San Diego, CA, USA). All other chemicals were from Sigma (St. Louis, MO).

Cell culture

Primary human epidermal keratinocytes [adult HEKa (C-005-5C)] were cultured on collagen matrix protein coated dishes using Epilife medium (EDGS growth supplement; Life Technologies, Carlsbad, CA). Dermal neonatal foreskin Hs27 fibroblasts from ATCC were cultured in DMEM containing 10% fetal bovine serum (9, 28).

Human epidermal skin reconstructs

EpiDerm™ tissues (EPI-200, 9 mm diameter, 6-well format; MatTek, Ashland, MA) were treated with PL (500 μM final concentration in 0.9 ml EPI-200-ASY media per well), followed by culture at 37 °C for 12 h, followed by UVA exposure (6.6 J/cm²) in PBS. After irradiation, tissue inserts were cultured for another 6 h in media. Tissue was then processed for paraffin embedment followed by H&E staining and immunohistochemical analysis as described recently (9, 29).

Irradiation with solar simulated UVA

Irradiation of cells in the absence or presence of B₆ vitamer was performed in PBS. A KW large area light source solar simulator, model 91293, from Oriel Corporation (Stratford, CT) was used, equipped with a 1000 W Xenon arc lamp power supply, model 68920, and a VIS-IR bandpass blocking filter plus UVB and C blocking filter (output 320–400 nm plus residual 650–800 nm, for UVA) (6, 9). The output was quantified using a dosimeter from International Light Inc. (Newburyport, MA), model IL1700, with a SED033 detector for UVA (range 315–390 nm, peak 365 nm), at a distance of 365 mm from the source, which was used for all experiments. Using UVB/C blocking filter, the dose at 365 mm from the source was 5.39 mJ/cm² sec UVA radiation with a residual UVB dose of 3.16 μJ/cm² sec.

Flow cytometric analysis of cell viability

Induction of cell death was confirmed by annexin-V-FITC/propidium iodide (PI) dual staining of cells using an apoptosis detection kit according to the manufacturer's specifications (APO-AF, Sigma, St. Louis, MO).

Cell proliferation assay

Cells were seeded at 10,000 cells/dish on 35-mm dishes. After 24 h, cells were exposed to the isolated or combined action of UVA and vitamin B₆ test compound in PBS. After exposure, cells were placed under media and cultured for another 72 h. Cell numbers at the time of treatment (and 72 h later) were determined using a Z2 Analyzer (Beckman Coulter, Inc., Fullerton, CA, USA). Proliferation was compared with cells that received mock treatment. The same methodology was used to establish IC₅₀ values (drug concentration that induces 50% inhibition of proliferation of treated cells within 72 h exposure $\pm$ SD, n = 3) of anti-proliferative potency.

Human Stress and Toxicity Pathfinder RT²Profiler™ PCR Expression array analysis

Preparation of total cellular RNA, reverse transcription, and Expression Array (SuperArray, Frederick, MD, USA) profiling was performed as published recently (9). Total cellular RNA was prepared according to a standard procedure using the RNeasy kit (Qiagen, Valencia, CA, USA). Reverse transcription was performed using the RT² First Strand kit (SuperArray) and 1 μ g total RNA. The Human Stress and Toxicity Pathfinder RT²Profiler™ PCR Expression Array (SuperArray) profiling the expression of 84 stress-related genes was employed as described before (30). Gene-specific product was normalized to *ACTB* and quantified using the comparative (C_t) Ct method following the ABI Prism 7000 sequence detection system user guide. Expression values were averaged across three independent array experiments followed by statistical analysis.

DDIT3, GADD45A, CDKN1A, HMOX1, and HSPA6 expression analysis by real time RT-PCR

For expression analysis by real time RT-PCR, total cellular RNA (3 $\times$ 10⁶ cells) was prepared using the RNeasy kit from Qiagen (Valencia, CA, USA). Reverse transcription was performed using TaqMan Reverse Transcription Reagents (Roche Molecular Systems, Branchburg, NJ, USA) and 200 ng of total RNA in a 50 μ l reaction. Reverse transcription was primed with random hexamers and incubated at 25 °C for 10 min followed by 48 °C for 30 min, 95 °C for 5 min, and a chill at 4 °C. Each PCR reaction consisted of 3.75 μ l of cDNA added to 12.5 μ l of TaqMan Universal PCR Master Mix (Roche Molecular Systems), 1.25 μ l of gene-specific primer/probe mix (Assays-by-Design; Applied Biosystems, Foster City, CA) and 7.5 μ l of PCR water. PCR conditions were: 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s, alternating with 60 °C for 1 min using an Applied Biosystems 7000 SDS and Applied Biosystems' Assays On Demand primers specific to *CDKN1A* (assay ID Hs00355782_m1), *DDIT3* (assay ID Hs00358796_g1), *GADD45A* (assay ID Hs00169255_m1), *HSPA6* (assay ID Hs00275682_s1), *HMOX1* (heme oxygenase-1, assay ID Hs00157965_m1), and *ACTB* (β -actin, assay ID Hs99999903_m1). Gene-specific product was normalized to *ACTB* and quantified using the comparative (C_t) Ct method as described before (9).

Flow cytometric detection of intracellular oxidative stress

Induction of intracellular oxidative stress by photosensitization was analyzed by flow cytometry using 2',7'-dichlorodihydrofluorescein diacetate (DCFH-DA) as a non-fluorescent precursor dye following a published standard procedure (4, 9).

Quantification of cellular reduced glutathione (GSH) content

After treatment, cells were harvested by trypsinization and counted using a Z2 Coulter counter. Intracellular reduced glutathione was determined per 10,000 viable cells using the ultrasensitive luminescent GSH-Glo™ glutathione assay (Promega, Madison, WI). Luminescence reading was performed using a BioTek Synergy 2 Reader (BioTek, Winooski, VT, USA). Data represent relative levels of glutathione normalized for cell number comparing treated versus untreated controls (means $\pm$ SD; n=3).

Immunoblot analysis

Immunoblot analysis was performed following our published standard procedures (31). The following primary antibodies were used: mouse anti-p21 monoclonal antibody (2946; Cell Signaling Technologies, Danvers, MA); rabbit anti-p-p53 (Ser15; sc-101762, Santa Cruz, Dallas, TX); mouse anti-p53 monoclonal antibody (Pab 240, Santa Cruz). The following secondary antibodies were used: HRP-conjugated horse anti-mouse IgG (7076; 1:2000; Cell Signaling, Danvers, MA) for p21, and HRP-conjugated goat anti-rabbit antibody or HRP-conjugated goat anti-mouse antibody (1:10,000; Jackson Immunological Research, West Grove, PA) for all others, followed by visualization using enhanced chemiluminescence detection reagents. Equal protein loading was examined by β -actin-detection using a mouse anti-actin monoclonal antibody (Sigma; 1:1500 dilution).

Comet assay (alkaline single cell gel electrophoresis)

The alkaline Comet assay was performed according to the manufacturer's instructions (Trevigen, Gaithersburg, MD, USA) as published recently (9). Cells were then stained with SYBR™ Green and visualized with a fluorescence microscope (fluorescein filter) followed by analysis using CASP software. At least 100 tail moments for each group were analyzed in order to calculate the mean $\pm$ S.D. for each group. The Fpg-FLARE assay for assessment of Fpg-induced strand cleavage at oxidized purine bases was performed according to the manufacturer's instructions (Trevigen).

Phospho-H2A.X detection by flow cytometry

Treatment-induced accumulation of nuclear phosphorylated histone variant H2A.X (γ -H2AX) was examined in keratinocytes using a phospho-H2A.X (Ser139) monoclonal antibody (Alexa488-conjugate, Cell Signaling, Danvers, MA) followed by flow cytometric analysis as published before (31).

In situ-Terminal dUTP Nick End Labeling (TUNEL), cleaved caspase 3, and 8-oxo-dG detection in epidermal reconstructs

Unstained tissue sections were visualized by differential interference contrast (DIC) microscopy employing an Olympus IMT-2 inverted microscope. Moreover, tissue sections (5

µm) from formalin fixed, paraffin embedded EpiDerm™ reconstructs were collected onto slides, deparaffinized, rehydrated, and analyzed for DNA fragments using the DermaTACS™ *in situ* terminal deoxynucleotidyltransferase (TdT) kit (Trevigen) according to the manufacturer's instructions as published before (29). Sections were treated with proteinase K and then incubated with TdT enzyme and brominated dNTP mixture (37 °C, 30 min). Afterwards, samples were labeled with biotinylated anti-BrdU antibody (37 °C, 30 min), followed by streptavidin-conjugated HRP and incubation with TACS Blue Label™ substrate. Slides were dehydrated, clarified (ethanol, p-xylene), and mounted for viewing. Images were captured using an Olympus BX50 with an Olympus Dp72 camera and CellSense Digital Image software. The number of TUNEL-positive cells per viewing field (200x) was counted in six random fields, and percentage TUNEL positive cells was calculated. For detection of cleaved caspase 3, a rabbit polyclonal primary antibody [cleaved caspase 3 (Asp175); Cell Signaling, Danvers, MA] with visualization employing alkaline phosphatase/fast red chromogen was used following the manufacturer's protocol. For 8-oxo-dG staining of epidermal tissue, a mouse monoclonal antibody (clone 2E2, Trevigen) was used followed by fluorescence visualization using a secondary goat anti-mouse IgG conjugate [Alexa Fluor 488 (Molecular Probes)] with DAPI nuclear counterstain following the manufacturer's protocol.

Statistical analysis

The results are presented as means ($\pm$ SD) of at least three independent experiments. Selected data sets were analyzed employing *one-way* analysis of variance (*ANOVA*) with Tukey's *post hoc* test using the Prism 4.0 software. In all bar graphs, means without a common letter differ ($p < 0.05$).

RESULTS

B₆-vitamers are sensitizers of UVA-induced phototoxicity in primary human keratinocytes, and PL-sensitization of UVA-induced keratinocyte death that can be antagonized by pharmacological caspase inhibition or antioxidant intervention

First, following our earlier experiments on UVA-induced photodynamic effects of B₆-vitamers on immortalized HaCaT keratinocytes, we examined if B₆-vitamer induced photodynamic effects might also be observable in primary human keratinocytes (HEKa) (4). To this end, the dose-response relationship of UVA-induced anti-proliferative effects sensitized by B₆-vitamers [pyridoxine (PN), pyridoxamine (PM), and pyridoxal (PL; Fig. 1a)] was established in HEKa cells. Cells were irradiated delivering a moderate dose of UVA (6.6 J/cm²) using a solar simulator source, and irradiation was performed in the presence of increasing concentrations of B₆-vitamer (0.1 – 100 µM). After UVA exposure cells were cultured for three days, and proliferation was then determined relative to control cells that were UVA-exposed in the absence of sensitizer (Fig. 1b). Over the time of proliferative capacity assessment (72 h), UVA exposure in the absence of sensitizer test compound induced a moderate inhibition of proliferation by 12.3 ± 2.3 % versus mock-irradiated cells (data not shown), an observation consistent with the published literature (4). Moreover, no 'dark toxicity' impacting cell proliferation was observed when anti-proliferative activity of the test compounds was assessed over the specified concentration range in the absence of

UVA exposure (data not shown). Among the three B₆-vitamers, photo-activated PL displayed the most potent anti-proliferative activity ($IC_{50} = 3.8 \pm 0.5 \mu M$), followed by PM ($IC_{50} = 27.3 \pm 2.9 \mu M$) and PN ($IC_{50} = 33.6 \pm 5.2 \mu M$). At higher concentrations ($> 100 \mu M$), PL ($ED_{50} = 149.4 \pm 5.8 \mu M$) displayed activity as an efficient sensitizer of UVA-induced ($6.6 J/cm^2$) cell death observed in HEK293 cells (Fig. 1c), whereas PM ($ED_{50} = 741.9 \pm 31.7 \mu M$), and PN ($ED_{50} = 1115 \pm 88.2 \mu M$) displayed diminished photodynamic potency (dose response relationship not shown), a differential cytotoxicity that is consistent with earlier studies on phototoxic effects of B₆-vitamers that indicated the superior photodynamic potency of PL (4). Strikingly, rescue of HEK293 cells from PL-sensitization of UVA-induced cell death was observed in cells co-treated with either zVADfmk, a pancaspase inhibitor and antagonist of apoptotic cell death, or NAC (N-acetyl-L-cysteine), a thiol-based sacrificial antioxidant (Fig. 1d). Likewise, UVA-induced photodynamic activity of low micromolar concentrations of PL was also observed when human dermal Hs27 fibroblasts were exposed to the combined action of PL and UVA ($6.6 J/cm^2$), suggesting that PL photosensitizer activity is not confined to HEK293 cells (Fig. 1e).

Comparative array analysis identifies PL as a sensitizer of UVA-induced heat shock, redox, and genotoxic stress response gene expression in primary human keratinocytes

Using the Human Stress and Toxicity RT² Profiler™ PCR Expression Array technology we then explored the possibility that PL treatment may modulate UVA-induced stress response gene expression (Fig. 2). Array analysis compared transcriptional profiles elicited in HEK293 cells in response to the combined or isolated exposure to PL ($250 \mu M$) and solar simulated UVA ($6.6 J/cm^2$). When analysis was performed using cells harvested 6 h after exposure, pronounced stress response gene expression was detectable only in the treatment group subjected to the combined action of PL and UVA. Genes upregulated in response to combined PL/UVA treatment were indicative of induction of heat shock [*HSPA6* (184 fold), *HSPA1A* (14 fold), *HSPA1L* (10 fold), *HSPA2* (10 fold)] and oxidative/electrophilic [*GSTM3* (49 fold), *EGR1* (36 fold), *MT2A* (4 fold), *NOS2A* (4 fold), *SOD1* (3 fold)] stress responses. In addition, expression of DNA-damage response genes including *growth arrest and DNA-damage-inducible, alpha* [*GADD45A* (19 fold)], *DNA-damage-inducible transcript 3* [*DDIT3* (16 fold)], *cyclin-dependent kinase inhibitor 1* [*CDKN1A* (5 fold)] was strongly upregulated (31). A summary of genes that were at least threefold up- or downregulated over untreated control is presented in Fig. 2d. In addition, expression changes of selected key target genes was also assessed using array-independent primer sets that confirmed a pronounced degree of PL/UVA-induced upregulation (*HSPA6*, *DDIT3*, *GADD45A*, *CDKN1A*), including the oxidative stress response gene *HMOX1* (*heme oxygenase-1*), a gene not contained on our commercial gene array (Fig. 3A). As expected, moderate induction of *HSPA6* and *HMOX1* expression, fundamental components of the cellular heat shock and oxidative stress response, was already observable in response to UVA-exposure performed in the absence of sensitizer, an effect further potentiated if UVA exposure occurred in the presence of PL (32).

PL causes UVA-induced oxidative stress and impairment of genomic integrity in primary human keratinocytes

Next, the occurrence of oxidative stress in HEKa cells exposed to the isolated or combined action of PL and UVA was assessed by flow cytometric analysis of DCFH loaded cells (Fig. 3b). As documented before in HaCaT keratinocytes and other target cells exposed to B₆-vitamers and UVA (4), an almost fourfold increase in DCF fluorescence intensity was observed in HEKa cells, indicative of the generation of reactive species of sufficient longevity, such as protein peroxides (33), capable of oxidizing the indicator dye DCFH during cell loading after irradiation (9). Likewise, a significant depletion of the cellular reduced glutathione (GSH) pool by almost 20% was detectable 6 h after exposure, an effect consistent with treatment-induced redox dysregulation confined to cells exposed to combined PL/UVA treatment (Fig. 3c).

In HEKa cells undergoing PL/UVA cotreatment, genotoxic stress response signaling was detectable within 1h after exposure. Immunoblot detection indicated the activational phosphorylation of p53 (Ser15), a crucial molecular component of the genotoxic stress response in keratinocytes (34). This effect that was suppressed if PL/UVA exposure occurred in the presence of the antioxidant NAC, suggesting that photooxidative stress might occur upstream of p53 activation (Fig. 3D). In addition, upregulation of cellular protein levels of p21 (*cyclin-dependent kinase inhibitor 1* encoded by the p53 target gene *CDKN1A*) was observable exclusively in response to PL/UVA cotreatment (35). Flow cytometric analysis using a γ -H2AX-directed Alexa-488 antibody conjugate revealed that PL/UVA coexposure increased phosphorylation of H2AX by almost 5 fold over control, representing an established hallmark of genotoxic stress associated with double strand breaks (and also elicited by UVB exposure), which was not observed in response to the isolated exposure to either PL or UVA (Fig. 3E) (36).

The genotoxic consequences of PL/UVA exposure were further substantiated in HEKa cells employing alkaline single cell gel electrophoresis (comet assay), enhanced by oxidative base damage-specific endonuclease digestion using Fpg [formamidopyrimidine DNA glycosylase](9). Comet analysis was performed at 1 h after exposure to the combined or isolated treatment with B₆-vitamers (PN, PM, PL; dose range: 10–250 μ M; Fig. 3f). Consistent with photodynamic introduction of oxidized DNA base lesions downstream of PL/UVA interactions, HEKa cells exposed to the combined (but not the isolated) action of PL and UVA displayed a pronounced increase in comet tail moment (up to tenfold), further enhanced (by almost twofold) by Fpg-digestion. Remarkably, a statistically significant increase in Fpg-enhanced comet tail moment was already detectable at PL concentrations as low as 10 μ M. It was also observed that PN and PM display inferior sensitizer potency over the low micromolar dose range employed, and only PM (250 μ M)/UVA combination treatment followed by Fpg digestion was associated with a statistically significant increase in average tail moment over untreated control. Taken together, these data indicate that PL is a sensitizer of UVA-induced genotoxic stress, causing an impairment of genomic integrity in primary keratinocyte culture that occurs downstream of photodynamic induction of oxidative stress.

PL is a genotoxic UVA-photosensitizer in reconstructed human epidermis

After demonstrating photodynamic effects of PL in primary human epidermal keratinocytes, follow up prototype experiments tested the possibility that comparable PL/UVA interactions might be observable in reconstructed human epidermis after short term culture in growth medium supplemented with PL (Fig. 4). To this end, phototoxic effects of PL supplementation (500 μ M; 12h incubation) followed by UVA exposure (6.6 J/cm²) were examined employing IHC analysis of epidermal tissue specimens. After UVA exposure, only the tissue reconstructions that had received PL/UVA combination treatment displayed pronounced phototoxic effects as evident from detection of pycnotic/eosinophilic features (H&E stain; Fig. 4a). Moreover, basal keratinocytes displayed pronounced staining for cleaved caspase 3 and TUNEL-positivity, characteristic of more than 95% of the cells situated in the basal layer (Fig. 4a&c). In addition, staining for nuclear DNA (DAPI) with immunofluorescent detection of oxidized DNA lesions (8-oxo-dG) revealed that PL/UVA combination treatment caused the introduction of oxidative DNA damage in more than 60% of all epidermal keratinocytes (Fig. 4b&c). Taken together, these findings demonstrate the occurrence of photooxidative stress and genotoxic insult as a consequence of PL/UVA photodynamic interactions in reconstructed human epidermis.

DISCUSSION

Cumulative chemical and biological evidence suggests a mechanistic role of B₆-vitamers as endogenous UVA-photosensitizers, and the potential importance of B₆-vitamer-induced phototoxicity in human skin, particularly in the context of photosensitive dermatitis, has been reported before (4, 23–25). The photophysical and photochemical properties of B₆-vitamers have been examined in much detail, and a role of UV-driven electron transfer reactions in B₆-vitamer sensitized photooxidative modification of amino acids and DNA bases has been substantiated (26). Our own research has documented the ability of B₆-vitamers and other related photoactive 3-hydroxypyridine chromophores (including the collagen crosslink pyridinoline) to cause the UVA-driven photooxidation of macromolecular targets including peptides and plasmid DNA (4). In addition, rate constants for singlet quenching of B₆-vitamers by nucleotides have been established, and it has been shown that GMP (tested versus TMP, UMP, and CMP) is the most oxidizable nucleotide, engaged in electron transfer from nucleotide to excited B₆-vitamer (26).

Based on this prior experimental evidence, we selected PL as the most phototoxic UVA-activated B₆-vitamer and explored its potential photodynamic effects on human primary keratinocytes and reconstructed epidermis for the first time. Comparative array analysis substantiated the efficient induction of genotoxic (*GADD45A*, *DDIT3*, *CDKN1A*) stress response gene expression further supported by immunodetection of p53 (Ser15) phosphorylation and γ -H2AX formation, hallmarks of the cellular response to genotoxic insult (34, 36). Follow up comet analysis indicated the formation of Fpg-sensitive DNA lesions, a finding consistent with the established photochemical reactivity of B₆-vitamers as UV-activated electron transfer reagents involved in DNA base oxidation and plasmid cleavage (4, 26). Importantly, the specific photochemical mechanism underlying the pronounced cellular phototoxicity of PL (as compare to PN and PM) remains to be

elucidated, a phenomenon that has been attributed tentatively to excited triplet state photochemistry associated with the aldehyde-function of PL (4). Strikingly, our data demonstrate for the first time that UVA-induced PL phototoxicity is observable in human reconstructed epidermis pre-incubated with this specific B₆-vitamer, causing genotoxic stress with oxidative DNA damage and keratinocyte death. It remains to be seen if the substantial photodynamic damage observed here in reconstructed epidermis, achieved at sub-millimolar concentrations of PL excited by a moderate dose of UVA, is of pathomechanistic relevance to human skin photodermatology, either in the context of photosensitivity or photocarcinogenesis.

Acknowledgments

Parts of this research were presented at the biannual meeting of the American Society for Photobiology (ASP), Tampa, FL, May 21–26, 2016. Supported in part by the University of Arizona Cancer Center Support Grant, NIH CA023074 and the following NIH grants: R03CA167580, R03CA212719, ES007091, ES006694.

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Figure 1. The B_6 -vitamer pyridoxal as a micromolar sensitizer of UVA-induced cytotoxicity in primary human keratinocytes and dermal fibroblasts
 (A) Chemical structures of B_6 vitamers [pyridoxine (*PN*), pyridoxamine (*PM*), pyridoxal (*PL*). (B) Dose response relationship of B_6 vitamer-induced inhibition of proliferation. Cells were exposed to UVA (6.6 J/cm²) in combination with test compound (0.1–100 μ M), and proliferation was determined by cell counting at 72 h after exposure. Proliferation of cells treated exposed to UVA only was used as a control, and proliferation of treatment groups was expressed as % relative to control (mean $\pm$ SD, n=3; ***p< 0.001; **p< 0.01; *PL* versus *PN*). (C). Modulation of cell viability in response to combined *PL* (500 μ M)/UVA (6.6 J/cm²) exposure was assessed 24 h after treatment using flow cytometric analysis [annexin V-PI staining; numbers in quadrants indicate viable (AV-negative, PI-negative) in percent of total gated cells (mean $\pm$ SD, n=3)]. (D) Modulation of cell viability in response to combined *PL* (250 μ M)/UVA (6.6 J/cm²) exposure performed in the absence of presence of the pancaspase inhibitor zVADfmk (40 μ M) and the antioxidant NAC (10 mM) was assessed using flow cytometric analysis as in panel C. (E) Modulation of cell viability in response to combined *PL* (500 μ M)/UVA (6.6 J/cm²) exposure was assessed in Hs27 human dermal fibroblasts using flow cytometric analysis as in panel C.

Figure 2. Pyridoxal as a sensitizer of UVA-induced stress response gene expression in primary human keratinocytes

After exposure to the isolated or combined action of UVA (6.6 J/cm²) and PL (250 μM), cells were rinsed and then cultured in medium (6 h) followed by Stress and Toxicity Pathfinder RT²Profiler™ PCR Expression array analysis. Scatter blots depict differential gene expression (cut-off lines: threefold up- or down-regulation). Arrays were performed in

three independent repeats and analyzed using the two-sided Student's *t* test. (A) PL-induced gene expression (versus untreated). (B) UVA-induced gene expression (versus untreated). (C) PL/UVA-induced gene expression (versus untreated). (D) Numerical expression changes (PL/UVA versus untreated) [$n=3$, mean $\pm$ SD; ($p<0.05$)].

Figure 4. Pyridoxal as a sensitizer of UVA-induced genotoxic stress and cytotoxicity in reconstructed human epidermis

Epidermal reconstruct (Epiderm™) specimens were cultured in growth medium supplemented with or without PL (500 μ M; 12 h) followed by UVA (6.6 J/cm²) or mock-irradiation. After 6 h, reconstructs were processed for IHC. Per treatment group, representative images taken from three repeat samples are displayed. (A) H&E and IHC (cleaved caspase 3, TUNEL) analysis revealed keratinocyte death in the basal epidermis. (B) Comparative imaging employing differential interference contrast (DIC) microscopy, DAPI staining of nuclei, and fluorescence of oxidative base damage (8-oxo-dG). (C) Bar graphs display the quantitative analysis of TUNEL positivity (upper panel; basal epidermis), cleaved caspase 3 expression (middle panel; basal epidermis), and 8-oxo-dG-positive cells as a fraction of all DAPI-stained cells (lower panel; all epidermal layers). [n = 6 specimens per group; mean $\pm$ S.D.; means without a common letter differ (ANOVA with Tukey's *post hoc* test)].