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Niacinamide mitigates SASP-related inflammation induced by environmental stressors in human epidermal keratinocytes and skin

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Abstract

OBJECTIVE: To evaluate whether niacinamide (Nam) can mitigate production of inflammatory and senescence-related biomarkers induced by environmental stressors.

METHODS: Human epidermal keratinocytes were exposed to UVB, urban dust, diesel exhaust and cigarette smoke extract and treated with Nam or vehicle control. Full thickness 3-D skin organotypic models were exposed to a combination of UVB and $PM_{2.5}$ and treated with Nam or vehicle control. Quantitation of the SASP-related inflammatory mediators PGE_2, IL-6 and IL-8 was performed on cultured media. UVB-exposed keratinocytes treated with and without Nam were immunostained for the senescence biomarker Lamin B1 (LmnB1). Transcriptomics profiling of cigarette smoke extract effects on keratinocytes was performed. A double-blind, placebo-controlled clinical was conducted on 40 female panellists that were pretreated on back sites for two weeks with 5% Nam or vehicle and then exposed to 1.5 minimal erythemal dose (MED) solar-simulated radiation (SSR). Treated sites were compared with non-treated exposed sites for erythema and the skin surface IL-1αRA/IL-1α inflammatory biomarkers.

RESULTS: Ultraviolet B induced synthesis of PGE_2, IL-8 and IL-6 and reduced LmnB1 levels in keratinocytes. Urban dust and diesel exhaust only stimulated synthesis of IL-8 whereas cigarette smoke extract only stimulated levels of PGE_2. In all exposures, treatment with Nam significantly mitigated synthesis of the inflammatory mediators and restored levels of UVB-reduced LmnB1. In the 3D skin equivalent model, Nam reduced IL-8 levels stimulated by a combination of topical $PM_{2.5}$ and UV exposure. In a UV challenge clinical, pretreatment with 5% Nam reduced erythema and skin surface IL-1αRA/IL-1α inflammatory biomarkers that were induced by SSR.

CONCLUSION: Since it is known that Nam has anti-inflammatory properties, we tested whether Nam can inhibit environmental stress-induced inflammation and senescence-associated secretory phenotype (SASP) biomarkers. We show Nam can reduce PGE_2, IL-6 and IL-8 levels induced by environmental stressors. Additionally, *in vivo* pretreatment with Nam can reduce UV-induced erythema and skin surface inflammatory biomarkers. These findings add to the body of evidence that Nam can mitigate the skin's inflammatory response elicited by environmental stressors. This supports Nam can potentially inhibit senescence and premature ageing and thereby maintain skin's functionality and appearance.

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Résumé

OBJECTIF: Évaluer si le niacinamide (Nam) peut atténuer la production de biomarqueurs inflammatoires et liés à la sénescence induits par les facteurs de stress environnementaux.

MÉTHODES: Les kératinocytes épidermiques human ont été exposés aux UVB, à la poussière urbaine, aux gaz d'échappement diesel et à l'extrait de fumée de cigarette et traités avec Nam ou à un contrôle. Les modèles organotypic de peau 3D de pleine épaisseur ont été exposés à une combinaison d'UVB et de $PM_{2.5}$ et traités avec Nam ou le contrôle. La quantification des médiateurs inflammatoires liés à la SASP PGE_2, IL-6 et IL-8 a été réalisée sur des médias cultivés. Les kératinocytes exposés aux UVB traités avec et sans Nam étaient immuno-tachés pour le biomarqueur de sénescence Lamin B1 (LmnB1). Le profilage transcriptomique des effets d'extrait de fumée de cigarette sur les kératinocytes a été exécuté. Un placebo de contrôle clinique réalisé en double aveugle a été menée sur 40 panélistes féminins qui ont été prétraités sur le dos pendant deux semaines avec 5% Nam ou par le contrôle, puis exposés à 1,5 dose érythémateuse minimale (MED) de rayonnement solaire simulé (SSR). Les sites traités ont été comparés à des sites exposés mais non traités pour l'érythème et par les biomarqueurs inflammatoires de la surface de la peau IL-1αRA/IL-1α.

RÉSULTATS: Les UVB induise la synthèse de PGE_2, IL-8 et IL-6 et réduit le niveau de LmnB1 dans les kératinocytes. La poussière urbaine et les gaz d'échappement diesel n'ont stimulé que la synthèse de l'IL-8 alors que l'extrait de fumée de cigarette ne stimulait que les niveaux de PGE_2. Dans toutes les expositions, le traitement avec Nam a significativement atténué la synthèse des médiateurs inflammatoires et ont restaurés les niveaux de LmnB1 UVB-réduit. Dans le modèle équivalent de la peau 3D, Nam a réduit les niveaux d'IL-8 stimulés par une combinaison de $PM_{2.5}$ topique et d'exposition aux UV. Dans un test UV clinique, le prétraitement avec 5% Nam réduit l'érythème et les biomarqueurs inflammatoires la surface de la peau IL-1αRA/IL-1α qui ont été induits par une source SSR.

CONCLUSION: Puisqu'il est connu que Nam a des propriétés anti-inflammatoires, nous avons testé si Nam peut inhiber l'inflammation induite par le stress environnemental et les

phénotype sécrétoire associé à la senescence (SASP). Nous montrons que Nam peut réduire les niveaux de PGE_2, IL-6, et IL-8 induits par les facteurs de stress environnementaux. En outre, le prétraitement *in vivo* avec Nam peut réduire l'érythème induit par les UV et les biomarqueurs inflammatoires de surface de la peau. Ces résultats s'ajoutent aux preuves que Nam peut atténuer la réponse inflammatoire de la peau provoquée par les facteurs de stress environnementaux. Cela soutient que Nam peut potentiellement inhiber la sénescence et le vieillissement prématuré et ainsi maintenir la fonctionnalité de la peau et son apparence.

Introduction

The skin is the largest human organ, and one of its primary functions is to protect the body from exposure to damaging environmental stressors such as solar radiation, chemicals, pollution and particulate matter. Like any organ, skin is susceptible to ageing and this process can be accelerated by cumulative acute microdamage from environmental stress exposure. This premature ageing of skin leads to cellular and structural changes that accumulates over time and ultimately affects skin's appearance, functionality and homeostatic state. Thus, it is of interest to understand these changes in order to identify mechanistic intervention targets that would prevent premature ageing and maintain skin's health and appearance.

The impact of environmental stressors on human skin health has been studied, particularly on solar radiation since it is believed that ultraviolet (UV) exposure can cause ~80% of premature ageing [1, 2]. More recently, other environmental stressors such as pollution and corresponding particulate matter have been shown to have negative impacts on skin health [3–5]. Mechanistically, the initial response in skin to environmental stressors is the generation of free radicals and reactive oxygen species (ROS) which cause DNA damage, protein structure and enzymatic activity alteration, and formation of lipid peroxides [6]. This cellular damage can be repaired via endogenous antioxidant enzymes and small molecules and, subsequently, by elevated synthesis of antioxidant enzymes under control of the Nrf2-antioxidant response element signalling pathway [7]. There is also a concurrent response in the synthesis and release of inflammatory factors to signal recruitment of an innate immune infiltrate [8]. The efficiency of these defensive responses declines with age and this leads to an accumulation of damaged cellular debris, altered gene expression patterns and changes in cellular and tissue function [9]. Phenotypically, one of the hallmarks of premature ageing is the presence of a low grade chronic inflammatory state [10]. This chronic elevated inflammation in skin tissue is hypothesized to be a significant contributor to premature ageing as suggested by the inflamm-aging theory [11]. Thus, identifying how to prevent and reduce inflammation in response to environmental stressors is proposed as a mechanistic approach to inhibit premature ageing.

Niacinamide (Nam; aka nicotinamide, vitamin B_3) has been used for decades in cosmetic and pharmaceutical products for the treatment of acne, skin photoageing attributes and barrier integrity improvement [12–14]. It has been reported that Nam can protect cells and the skin from oxidative stress, UV-induced immunosuppression and metabolic disruption [15–18]. However, it is not clear whether Nam can protect against an inflammatory immune response induced by environmental stressors. Mechanistically, this is of interest since it is known that chronic inflammation can lead to cellular senescence which can be characterized by the presence of the senescence-associated phenotype (SASP) [19]. Components of SASP factors secreted by senescent cells include the cytokines IL-1α, IL-6 and IL-8 [20]. We had previously shown that Nam can reduce senescence induced by NAD+ metabolism modulation in keratinocytes [21]. Thus, we asked whether Nam can mitigate stress-induced inflammation in skin *in vitro* and *in vivo* models. Using immortalized keratinocytes (hTERT), we tested whether Nam can mitigate the induction of the prostaglandin PGE_2 and the SASP-related biomarkers IL-6 and IL-8 after exposure to UVB, urban dust, diesel exhaust and cigarette smoke extract. Additionally, we used 3D full thickness skin equivalents to treat topically with particulate matter ($PM_{2.5}$) and ultraviolet B (UVB) exposure to more closely mimic "real-world" exposure conditions and asked what impact Nam has on mitigating SASP-associated inflammation. Finally, a double-blind, placebo-controlled UV challenge study was conducted to test whether a 5% Nam skin care emulsion system applied to female subjects' backs could reduce inflammation from exposure to solar simulated radiation at 1.5 minimal erythemal dose (MED).

We present here a body of evidence that Nam mitigates the inflammatory signal response induced by environmental stressors *in vitro* and *in vivo*. We propose that Nam can inhibit premature ageing and thereby help maintain skin's functionality and appearance. It should also be noted that while the response to Nam in these models was significant, it was partial in some instances. Additional strategies in combination with Nam will be needed to prevent the inflammatory cascade triggered by environmental stressors and thereby protect skin from cumulative damage and premature ageing.

Materials and methods

Cells and reagents

Telomerase-transfected keratinocytes (hTERT) were expanded in a CO_2, 37°C incubator using Epilife™ (Thermo Fisher Scientific, Waltham, MA) media with human keratinocyte growth supplement and gentamicin/amphotericin B. Cells never exceeded 70% confluency nor passage 5. UVB stress was administered using a BioSun (Vilber Lourmat, Germany). Urban dust was purchased from the National Institute of Standards & Technology (NIST), as atmospheric material collected in the Washington DC area during a 12-month period from 1976-77 (NIST 1649b). Diesel exhaust was also purchased from NIST, as a collection from a filtering-system designed for diesel-powered forklifts (NIST 2975). $PM_{2.5}$ was collected from quartz tape environmentally exposed in Beijing, China, in 2014 and was extracted in an aqueous solvent. A cigarette smoke extract (CSE) was prepared from burning commercial unfiltered cigarettes in a modified apparatus from a previously described set-up [22]. The process was controlled by the number of cigarettes, the length of time to burn each cigarette and the volume of collection fluid (PBS). Briefly, filters were removed from cigarettes, cigarettes were burned at a rate of 2 min/cigarette and smoke phase passed through 60 μL of PBS buffer. After collecting the smoke from 4 cigarettes, the smoke extract was removed from the capture flask and used within 2 h of production. Treatments with stressors with and without Nam were further cultured for 20 h before harvest.

A 3D full thickness human skin organotypic model (EpiDermFT™, MatTek Corporation, Ashland, MA) was used to assay the effects of Nam and stressors. Briefly, epidermal/dermal cultures

were received and allowed to acclimate overnight in Dulbecco's Modified Eagle's Medium (DMEM) with epidermal growth factor, insulin, hydrocortisone and proprietary stimulators of epidermal differentiation supplement, and gentamicin/amphotericin B in a humidified atmosphere with 5% CO_2 at 37°C. Prior to treatments, cultures were lifted to the air-liquid interface as per manufacturer's instructions. After treatment, media was collected and analysed for IL-8. Cultures were manually separated into epidermal and dermal fractions and subjected to microarray profiling on the GeneTitan U219 array platform (Affymetrix, Santa Clara, CA) as previously described [23].

ATP measurement

ATP was quantified from cells in culture by CellTiter-Glo® assay (Promega, Madison, WI). The media was removed, cells were washed with 200 μL of PBS, and 100 μL of media was added to each well. CellTiter-Glo reagent was prepared according to manufacturer's instructions, and 100 μL were added to cells. Cells were incubated with reagent per manufacturer's instructions, and 200 μL were transferred from the 24 well V7 plate to a black clear bottom 96-well plate in order to quantify ATP via luminescence. A minimum of 3 separate experiments were performed, and data were analysed from either 3 replicates. Student's *t*-test was used for statistical analysis with data presented as average value and standard deviation.

PGE_2, IL-6 and IL-8 measurements

For PGE_2 experiments, the media was supplemented with 2.5 μg mL^{-1} arachidonic acid. PGE_2 was measured from culture supernatants using an HTFR kit (CisBio, Bedford, Massachusetts) and normalized to ATP values derived from the cells. The cytokines IL-6 and IL-8 were measured using Mesoscale Discovery platform and kits.

Lamin B1 and Ki-67 immunofluorescence staining

Usage of primary human keratinocytes was approved by A*STAR Institutional Review Board (A*STAR IRB reference: 2019-53) and the National University of Singapore (NUS) Institutional Review Board (NUS-IRB reference: B-14-257E).

Human Primary Keratinocytes (HPKs) were obtained from healthy human skin samples (6-year-old male donor, foreskin) from de-identified surplus surgical waste with written informed patient consent and ethical clearance. HPKs were isolated as previously described [21]. For maintenance, HPKs were cultured on lethally irradiated murine 3T3-J2 feeder cells in cFAD medium (3:1 DMEM/Ham's F-12) supplemented with 10% foetal calf serum (FCS), 1% penicillin/streptomycin and 10 ng mL^{-1} epidermal growth factor as previously described [21]. The medium was replaced every 2–3 days.

One day before irradiation, HPKs were seeded in a feeder-free system in Dermalife® medium (LL-0007; Lifeline Cell Technology, Oceanside, CA). Medium was changed just before irradiation (the volume was reduced by 5 times for irradiation), and cells were irradiated with 25 mJ cm^{-2} UVB using a BioSun (Vilber-Lourmart, Germany). The medium was changed again immediately after irradiation and cells were maintained for 72 h in medium with or without 1.5 mM Nam (for Nam-treated cells, 1.5 mM Nam was also added to the medium just before irradiation).

Seventy-two hours after irradiation, cells were fixed in 2% paraformaldehyde at 4°C for 30 min, permeabilized with PBS/0.1% Triton, blocked with PBS/10% goat serum, incubated with primary antibodies (Lamin B1 (1/800): Novus NBP2-48966; Ki-67 (1/100): DAKO M7240), washed in PBS and incubated with secondary antibodies (Alexa Fluor, Thermo Fisher Scientific, Waltham, MA) and DAPI (1 μg mL^{-1}) before washing and mounting (Vectashield®; Vector Laboratories, Burlingame, CA). Images were acquired on an Olympus FLUOVIEW FV3000 RS inverted confocal microscope and quantitated in ImageJ.

Eigene gene analysis

Cells were lysed (RLT Buffer, Qiagen, Germantown, Maryland), and RNA was isolated using Agencourt magnetic beads (Beckman Coulter, Indianapolis, IN). Targets were prepared according to manufacturer's instructions and analysed on Affymetrix U219 GeneChips® (Affymetrix, Santa Clara, CA). Gene expression changes of Apoptosis, DNA repair and Cell Cycle biological processes in GeneOntology (GO:0006915, GO:0006281 and GO:0007049, respectively) were assessed by using Eigenegene expression [24]. The Eigenegenes of Apoptosis, DNA repair and Cell Cycle consisted of 415, 243 and 352 genes expressed in keratinocytes, respectively, and capture ~30% of the variances for the changes between 0.5% CSE treatment and vehicle control.

UV challenge study

A four-week, double-blind, placebo-controlled study in 40 healthy female subjects, ages 18–50, with Fitzpatrick skin types II-III, that employed a randomized, complete block design, in which 12 treatments were evaluated on twelve separate 3 cm × 3 cm sites in the middle region of subjects' backs. The study was performed between January and February 2012 at the Consumer Product Testing Company (Fairfield, NJ, USA). Good clinical practices were followed, and all subjects gave their informed consent for inclusion before they participated in the study. Because of the cosmetic nature of this study, an IRB approval was not obtained. The subjects were divided into 2 groups (one group with 31 enrolled, and one with 32 enrolled) that were staggered by 1 week. Fifty-nine subjects completed the study. There was a 1-week washout period, during which time each subject's minimal erythemal dose (MED) was determined for calculating the UV exposure dose to deliver on study day 16. Washout was followed by 2 weeks of daily test product application (3 μL cm^{-2}) conducted at the study facility before a single controlled UV exposure of 1.5 MED or target a* value between 13 and 19 on each site. Daily product applications were continued on the day of UV exposure and the next 2 consecutive days. Some treatment applications occurred on Saturdays because of scheduling logistics. At evaluation visits, all measurements and imaging were completed before treatments were applied. Measurements were obtained in a controlled temperature and humidity environment (70 ± 2°F and 30%–45% R.H.). Measurements taken on each treatment site included live visual redness grading by 2 trained graders, Chromameter and full back images at baseline, day 15 (pre-UV exposure), days 16–18 (post-UV exposure). Additionally, Chromameter measurements were taken at the time of MED determination as an exploratory measure to compare with visual grading. Four separate D-Squame® tape strips were collected from the non-treated and treated sites at baseline (day 0), at end of pretreatment prior to UV exposure (day 15), and at 7, 10 and

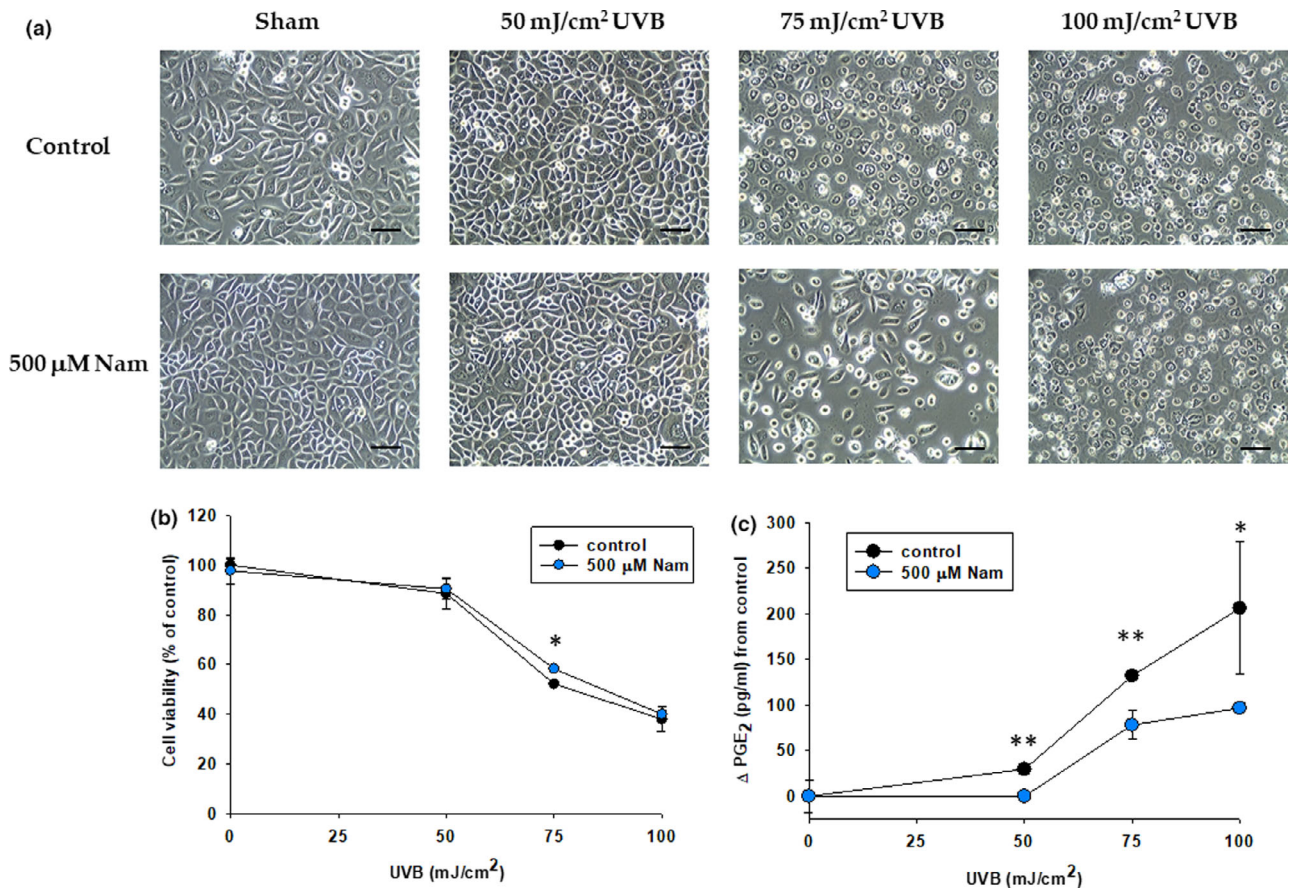

Figure 1 Nam partially protected keratinocyte viability and reduced PGE_2 levels induced by UVB exposure. Keratinocytes were incubated for 24 h with or without 500 μM Nam and exposed to 50, 75 and 100 mJ cm^{-2} of UVB after media was changed to PBS. Keratinocytes were then incubated for 24 h in full media with or without 500 μM Nam. (a) Bright field image capture across treatment groups shows significant cell morphology changes with increasing UVB fluency. Treatment with 500 μM Nam partially protected cell morphology changes from 75 mJ cm^{-2} UVB. Scale bar = 50 μm. (b) Cell viability (ATP quantitation) was lower across all fluencies, and there was a statistically significant mitigation by Nam at 75 mJ cm^{-2} UVB exposure. (c) Quantitation of inflammatory response showed a significant reduction in secreted PGE_2 levels by 500 μM Nam at all UVB fluencies tested. A replicate of $n = 4$ were averaged and Student's *t*-test was performed to calculate statistical significance ($^*P < 0.05$, $^{**}P < 0.01$).

14 days post-UV exposure (days 23, 26 and 30). Total protein was extracted from the tapes and analysed for IL-1αRA and IL-1α via ELISA as previously described [25]. Repeated-measures ANOVA was used for statistical analysis, modelling the original baseline as a covariate on original values and change from baseline.

Results

Nam mitigates UV-induced inflammatory response

Human hTERT keratinocyte cultures were used to determine the impact of Nam on mitigating UVB-induced inflammatory response. Cultures were pretreated with and without 500 μM Nam prior to UVB exposure. Cell morphology and viability were evaluated along with quantitation of PGE_2 levels following exposure to a UVB dose response range between 50 and 100 mJ cm^{-2}. Bright field images along with cell viability showed that Nam had a detectable protective effect at 75 mJ cm^2 UVB (Fig. 1a and b, $^*P < 0.05$). Treatment with Nam significantly reduced PGE_2 levels induced at 50 (32%, $^{**}P < 0.01$), 75 (22%, $^{**}P < 0.01$) and 100 (40%, $^*P < 0.05$) mJ cm^{-2} UVB (Fig. 1c).

We then tested whether Nam can impact the inflammatory response post-UVB exposure as well as measured additional inflammatory signalling agents. hTERT keratinocytes were exposed to 50 mJ cm^{-2} of UVB and incubated overnight with and without 500 μM Nam. Nam treatment after UVB exposure showed an increase in UVB-induced PGE_2 as well as the SASP-associated inflammatory cytokines IL-6 and IL-8. Treatment with Nam resulted in a 30% reduction in PGE_2 levels ($^{**}P < 0.01$), 31% reduction in IL-6 levels ($^{**}P < 0.01$) and 21% reduction of IL-8 levels ($^*P < 0.05$) (Fig. 2a). Since SASP induction has been reported to correlate with an onset of senescence, we measured changes in Ki-67 as an indicator of proliferation and Lamin B1 (LmnB1), a nuclear scaffold protein known to be lost during the senescence process [26]. To better simulate senescence response, we utilized primary human keratinocytes in place of the immortalized hTERT keratinocytes. We had found that UVB exposure of primary keratinocytes causes a similar increased PGE2 that is

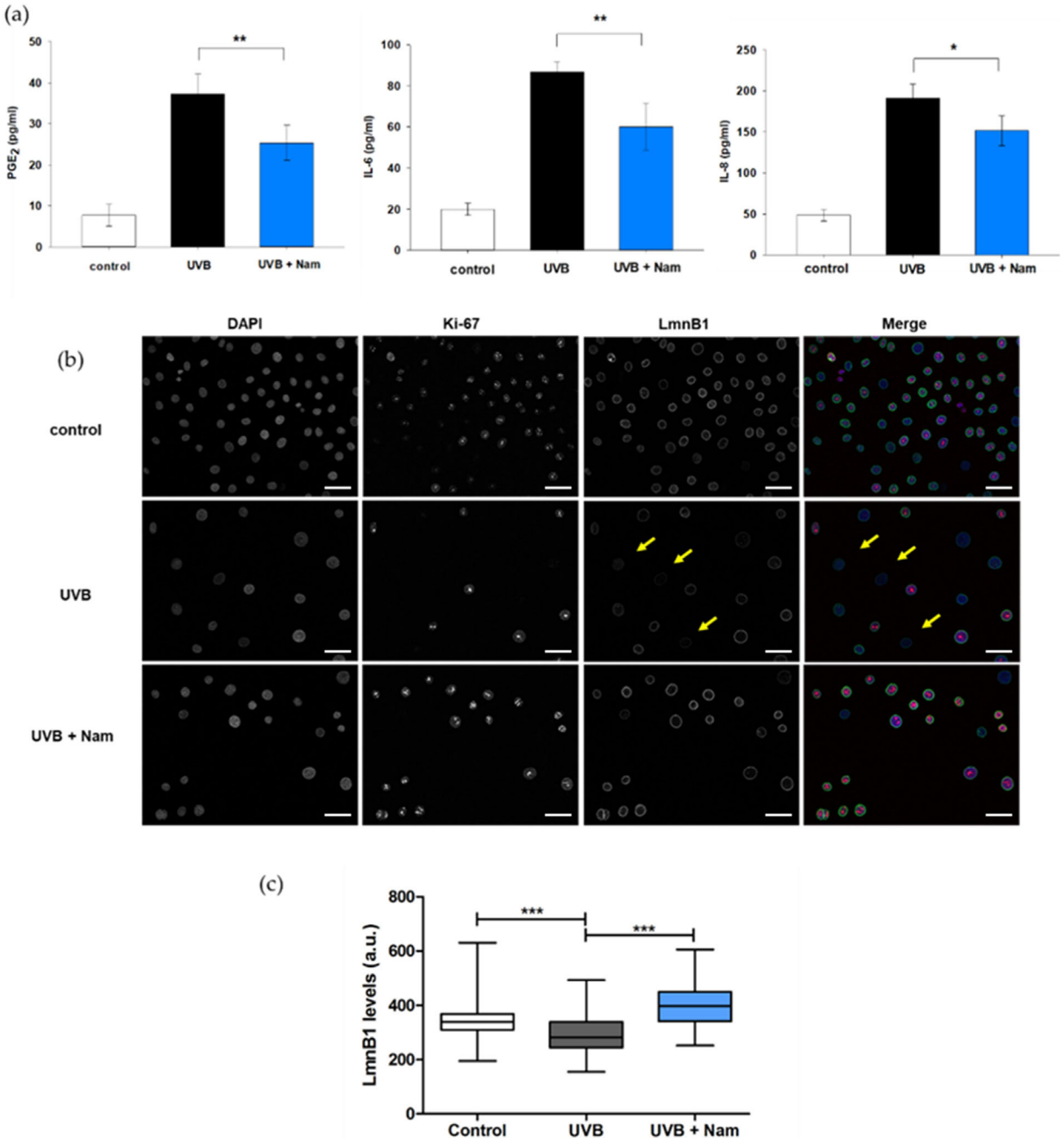

Figure 2 Nam reduced synthesis of inflammatory signals and restored Lamin B1 levels in keratinocytes after exposure to UVB. (a) hTERT keratinocytes were cultured for 2 days in full media and switched to PBS prior to being exposed to 50 mJ cm^{-2} of UVB radiation. After irradiation, 500 μM Nam was added to the culture wells in full media, incubated for 24 h, and PGE_2, IL-6 and IL-8 media levels quantitated. Nam-treated cells showed a significant decrease in levels of PGE_2, IL-6 and IL-8. A replicate of $n = 4$ were averaged, and Student's t-test was performed to calculate statistical significance ($^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$). (b) Immunofluorescence staining for the senescence biomarker Lamin B1 (LmnB1) showing protective effect of 1.5 mM Nam on human primary keratinocytes exposed to 25 mJ cm^{-2} UVB. Nam treatment also partially protected against loss of cell proliferation triggered by UVB as measured by Ki-67 and DAPI staining. Scale bar = 50 μm (c) Quantitation of LmnB1 immunofluorescence images showed that 25 mJ cm^{-2} of UVB significantly decreased LmnB1 levels per cell whereas treatment with Nam significantly prevents the LmnB1 drop. GraphPad Prism version 5.03 was used for statistical analyses. A one-way ANOVA test ($^{***}P < 0.001$) was applied, whiskers represent Min to Max values, horizontal lines represent the median value per cell for each condition, $n > 150$ cells per condition, a.u. = arbitrary units.

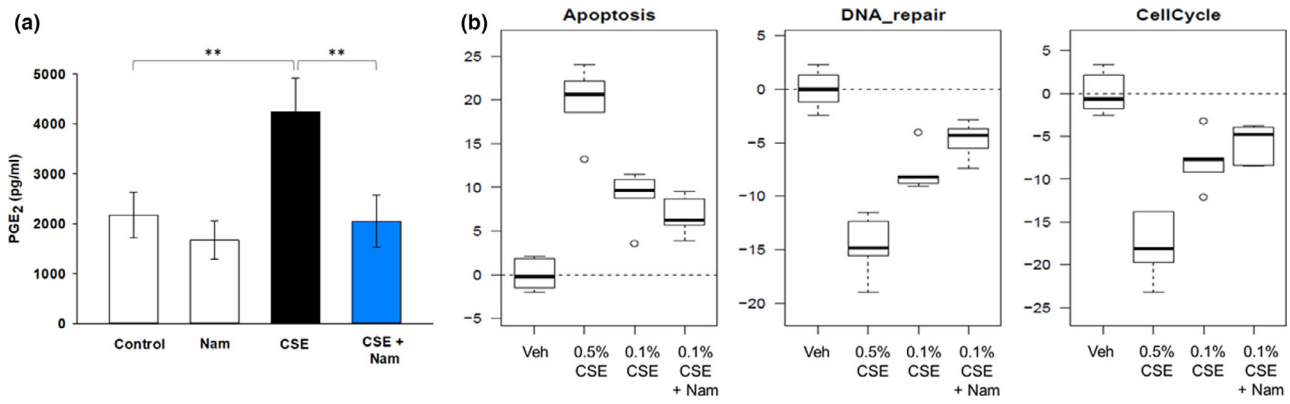

Figure 3 Nam reduced PGE_2 levels induced by cigarette smoke extract (CSE) in keratinocytes. hTERT keratinocytes were incubated in full media for 24 h and then exposed to CSE with or without 500 μM Nam for 24 h. PGE_2 levels in media were quantitated via ELISA. (a) 500 μM Nam prevented induction of PGE_2 by 0.5% cigarette smoke extract (CSE). A replicate of $n = 4$ were averaged and Student's *t*-test was performed to calculate statistical significance (**$P < 0.01$). (b) Transcriptomics profiling of changes in top bio-themes in hTERT keratinocytes following exposure to 0.1% and 0.5% CSE showed a significant increase in apoptosis and decreases in DNA repair and cell cycle-related genes in a dose-dependent manner. Concurrent treatment with 500 μM Nam and 0.1% CSE showed that Nam was able to partially prevent the degree of response to CSE in keratinocytes.

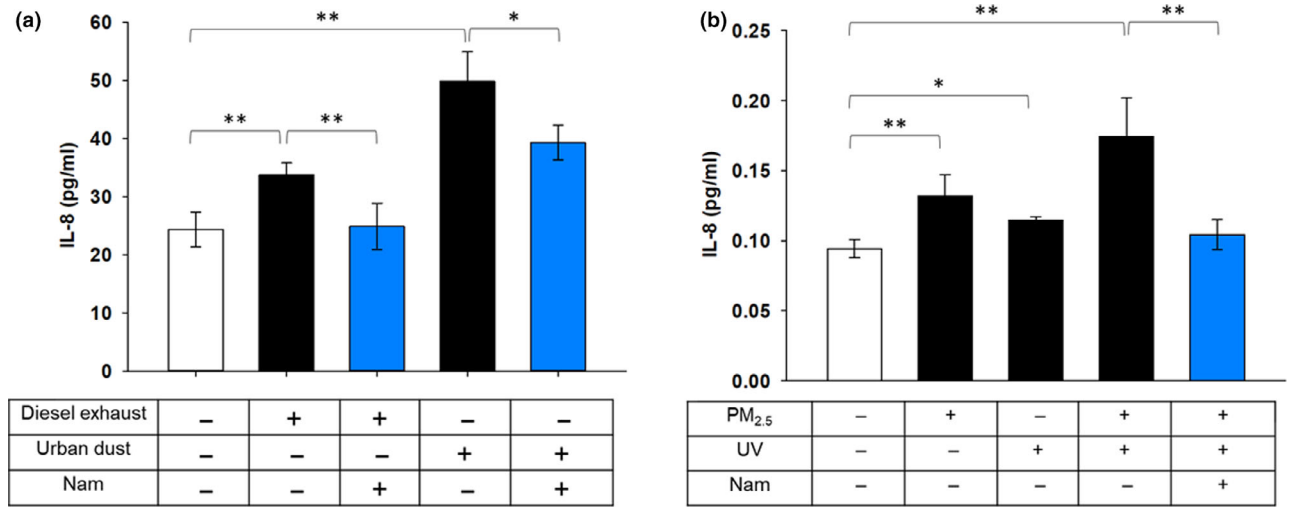

Figure 4 Nam reduced IL-6 and IL-8 cytokine levels induced by environmental stressors in keratinocytes and 3D skin equivalent model. (a) hTERT keratinocytes were incubated in full media for 24 h and then exposed to diesel exhaust, urban dust or cigarette smoke extract (CSE) without (black bars) or with 500 μM Nam (blue bars) for 24 h. IL-8 and PGE_2 levels in media were quantitated via ELISA and compared with media alone control samples (white bar). 500 μM Nam co-treatment limited induction of IL-8 levels by both diesel exhaust and urban dust. (b) Nam limited IL-8 induction by particulate matter alone and in combination with UV in 3D skin equivalents. EpiDermFT™ 3D skin equivalent cultures were acclimated for 24 h in full media and then exposed to topical particulate matter ($PM_{2.5;}$ 0.12 μg mL^{-1}) alone and in combination with 250 mJ cm^{-2} UVA/UVB (89.5:10.5) (black bars). 500 μM Nam was added to the media of select treatment groups and incubated for 24 h prior to stress and immediately following stress (blue bar). IL-8 levels were quantitated via ELISA and compared with media alone control samples (white bar). The combination of UV and $PM_{2.5}$ additively induced IL-8, whereas Nam treatment significantly prevented IL-8 induction. A replicate of $n = 4$ were averaged and Student's *t*-test was performed to calculate statistical significance (*$P < 0.05$, **$P < 0.01$).

inhibited by Nam exposure. UVB treatment at 25 mJ cm^{-2} induced a dramatic increase in cell size and a significant loss of both LmnB1 and Ki-67, all indicative of senescence (Fig. 2b). A substantial number of cells (shown by yellow arrows) expressed levels of LmnB1 barely detectable by immunofluorescence 72 h after UVB irradiation. The addition of Nam partially prevented the drop of LmnB (although most cells still appeared larger than control cells and the confluency was lower than in the control population). Accordingly, proliferation was partially preserved by Nam as shown by maintenance of numerous Ki-67-positive cells as compared to UVB alone. Of note, all Ki-67-positive cells were strongly positive for LmnB1 as expected (consistently, cells negative for Ki-67 expressed no or very low levels of LmnB1). Quantitation of immunofluorescent images confirmed the significant decrease in

LaminB1 with UVB exposure and restoration with Nam treatment (Fig. 2c, $^{***}P < 0.001$).

Secretion of inflammatory signals and gene expression patterns induced by environmental stressors in keratinocytes is mitigated by Nam

In addition to UVB, we tested whether other environmental stressors can induce SASP-related inflammation in hTERT keratinocytes at non-cytotoxic levels. A cigarette smoke extract (CSE) was made using cigarette smoke percolated through a water capture system [23]. Exposure to 0.05% CSE showed a 95% increase in PGE_2 levels and treatment with 500 μM Nam caused a complete rescue to basal levels (Fig. 3a, $^{**}P < 0.01$). In a separate experiment, hTERT keratinocytes were exposed to 0.5% CSE and 0.1% CSE with and without 500 μM Nam, incubated overnight and processed for microarray analysis. Transcriptomics profiling of key bio-themes showed that CSE induced gene expression patterns associated with apoptosis, DNA repair and cell cycle regulation in a dose-dependent manner (Fig. 3b). Nam treatment showed a partial reversal of the 0.1% CSE-induced changes.

hTERT keratinocytes were exposed to 0.00028% diesel exhaust and 0.0075% urban dust in media with and without 500 μM Nam. After 24 h of exposure, PGE_2 and IL-8 levels were quantitated from culture media (Fig. 4a). Interestingly, we did not see any detectable increase in PGE_2 by either diesel exhaust or urban dust (data not shown). Compared with basal levels, both diesel exhaust and urban dust stimulated a 39% and 100% increase in IL-8 levels, respectively. Nam treatment showed a complete inhibition of IL-8 induction by diesel exhaust ($^{**}P < 0.01$) and a 21% reduction in levels induced by urban dust ($^{*}P < 0.05$).

Particulate matter ($PM_{2.5}$) is comprised of carbon-based matter that ranges in particulate size and is bound with various chemical agents [27]. While the chemical agents can be extracted and tested, we wished to more closely simulate physiological exposure of $PM_{2.5}$ that comes in direct contact with skin. Additionally, we tested the combination effect of $PM_{2.5}$ with UV exposure by using an equivalent to solar radiation of UVA/UVB at the ratio of 89.5:10.5. $PM_{2.5}$ sample was kindly provided by Professor Jiang Jingkun (Tsinghua University, Beijing, China) after manually collecting from air by quartz tape in Beijing, China. 3D full thickness skin equivalent cultures (EpiDermFT™, MatTek Corporation, Ashland, MA) were stressed with topically applied $PM_{2.5}$ and with and without exposure to 250 mJ cm^{-2} of UVB. Cultures were also treated with 500 μM Nam in full media and incubated overnight (24 h) prior to stress and immediately following stress. $PM_{2.5}$ and UVB exposures alone caused a 40% ($^{**}P < 0.01$) and 21% ($^{*}P < 0.05$), respectively, increase in secreted IL-8 levels whereas the combination led to a synergistic increase of 85% ($^{**}P < 0.01$) compared with basal levels (Fig. 4b). Treatment with Nam caused a complete inhibition of IL-8 induction stimulated by the combination of $PM_{2.5}$ and UV ($^{**}P < 0.01$).

Treatment with topical 5% Nam can reduce UV-induced erythema and the ratio of the skin surface inflammatory biomarkers IL-1 αRA/IL-1 α

Based on the *in vitro* UVB-induced inflammation data, we then asked whether Nam could impact UV-induced inflammation in skin. A double-blind, placebo-controlled UV challenge study was designed with controlled on-site daily application (minus weekends) of an oil-in-water emulsion skin care formulation with or without 5% Nam. After 2 weeks of product application, all sites including a non-treated site were exposed to a single 1.5 MED dose of solar simulated radiation (SSR; UVA and UVB proportion of 89.5:10.5). After exposure, panellists continued to have product applied for an

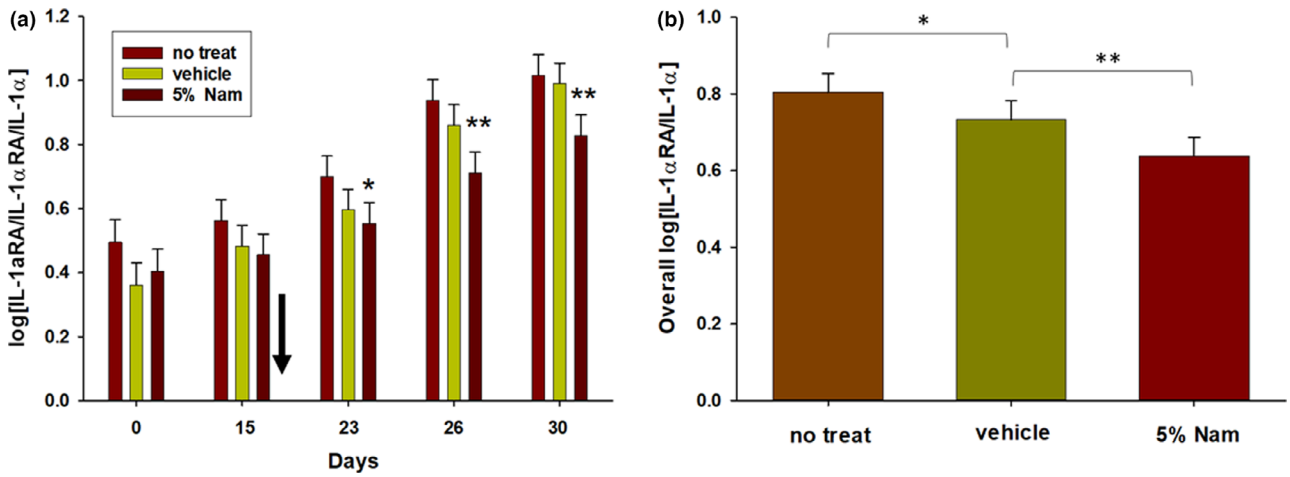

Figure 5 Topical Nam reduced the ratio of the skin surface inflammatory biomarkers IL-1αRA/IL-1α induced by solar simulated radiation. Female panellists were treated on a 3 cm × 3 cm site on their backs for 14 days with a skin care emulsion vehicle with or without 5% Nam. At day 16, the treatment sites, including a non-treated site (no treat) were exposed to solar simulated radiation (UVA and UVB proportion of 89.5:10.5) to induce a 1.5 minimal erythema dose (MED). Four separate D-Squame® tape strips were collected from each site at baseline (day 0), after pretreatment phase (day 15) and 7, 10 and 14 days after UV exposure (days 23, 26 and 30). (a) Quantitation of extracted IL-1αRA/IL-1α protein ratio showed a significant reduction in 5% Nam versus no treat at day 7 post-UV exposure (day 23, $P = 0.010$) and versus vehicle control at days 10 and 14 post-UV exposure (day 26, $P = 0.008$; day 30, $P = 0.005$). (b) Overall IL-1αRA/IL-1α protein ratios showed that 5% Nam induced a ratio significantly reduced compared with vehicle control ($P = 0.001$), whereas vehicle control also showed a ratio reduced compared with non-treated sites although less significant (no treat, $P = 0.012$).

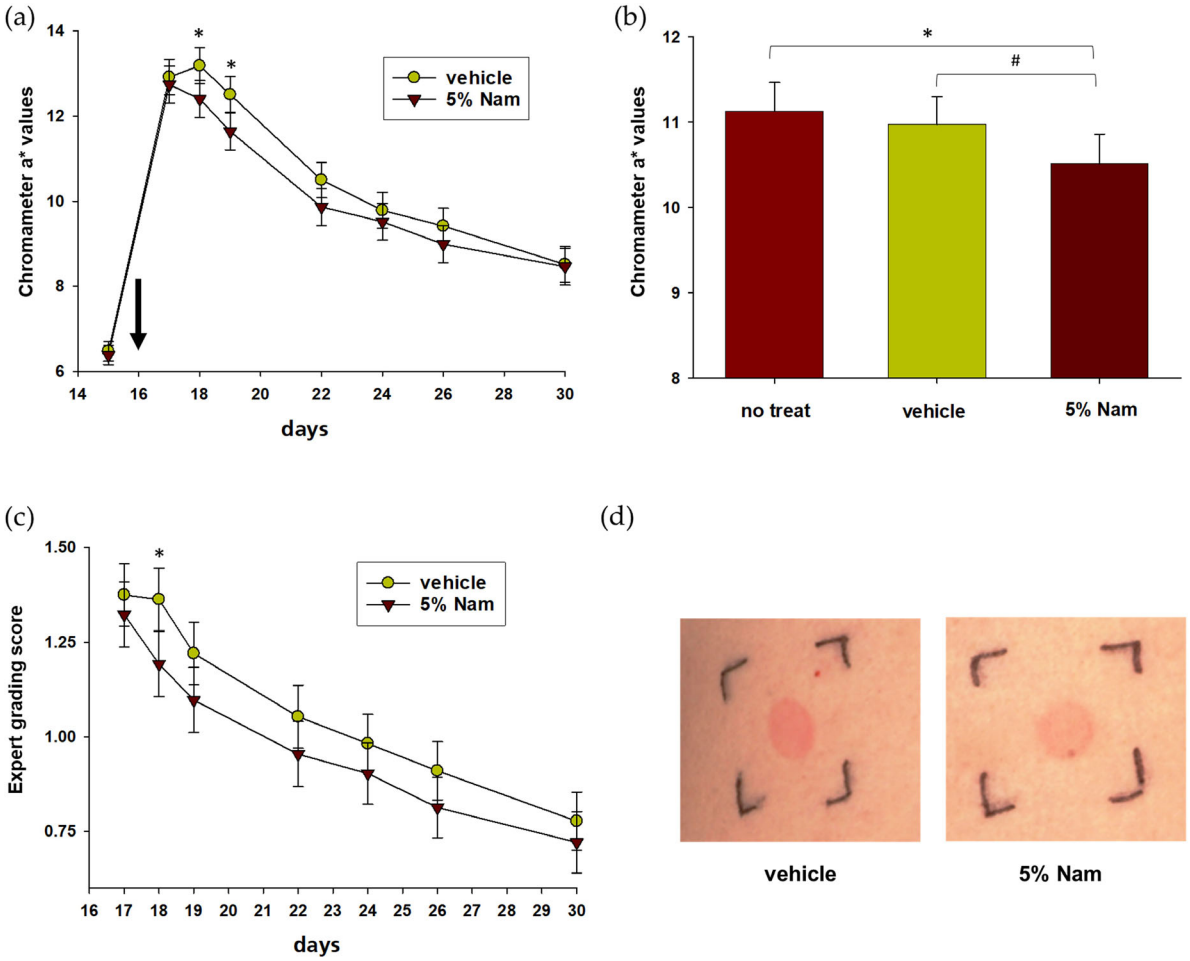

Figure 6 Treatment with Nam reduced erythema induced by solar simulated radiation. Female panellists were treated on a 3 cm × 3 cm site on their backs for 14 days with an emulsion vehicle control and an emulsion containing 5% Nam. At day 16, the treatment sites, including a non-treated site (no treat), were exposed to a 1.5 minimal erythema dose (MED) with solar simulated radiation (UVA and UVB proportion of 89.5:10.5). (a) Chromameter a* value readings were collected at the end of the pretreatment phase (day 15) and at 7 separate visits post-UV exposure (arrow denotes day 16 of MED exposure). Treatment with 5% Nam showed directional reduction in a* values compared with vehicle control 2 and 3 days after UV exposure (day 18, #$P = 0.085$; day 19, #$P = 0.057$). (b) Overall Chromameter a* values calculated post-UV exposure showed a significant reduction by the 5% Nam treatment compared with the non-treated sites (no treat, *$P = 0.027$) and vehicle control (#$P = 0.083$). (c) Visual grading for erythema utilized a 6-point redness scale to calculate redness between treatment groups during resolution phase. Treatment with 5% Nam resulted in an overall redness profile that was directionally lower than vehicle control 2 days after UV exposure (day 18, $P = 0.065$). (d) A representative set of digital colour images comparing vehicle control and 5% Nam treatment 2 days after UV exposure (day 18).

additional 2 weeks. Clinical measures were collected at varying time points before and after SSR exposure.

To measure the effect of 5% Nam treatment on UV-induced inflammatory response, D-Squame® tape strips were used to collect skin surface material for quantitation of the inflammatory biomarkers IL-1αRA and IL-1α. The ratio of these cytokines has been previously reported to provide a measure of the underlying inflammatory state of skin in such conditions as UV-induced sun damage, dandruff and diaper dermatitis [28]. Comparison across the various sites and time points showed a significant increase in the relative ratio of IL-1αRA to IL-1α after SSR exposure (Fig. 5a, $P = 0.001$). Comparing not treated and vehicle treated sites with 5% Nam, treated sites showed a significant reduction of the IL-1αRA/IL-1α ratio at all time points after SSR exposure (Fig. 5a, arrow). At 14 days after SSR exposure, 5% Nam had a maximal inhibitory effect of 18% and 16% compared with non-treated ($P = 0.0046$) and vehicle treated-($P = 0.008$) sites, respectively (Fig. 5a, day 30). Additionally, an overall analysis showed a 16% reduction by 5% Nam compared with vehicle control ($P = 0.001$) (Fig. 5b). These data support the erythema measures where treatment with 5% Nam can partially mitigate the inflammatory response induced by UV. Interestingly, while there were no significant differences between non-treated sites and vehicle control at individual time points, overall analysis showed vehicle control sites had a significantly lower level of the inflammatory ratio ($P = 0.012$).

Relative to SSR-induced erythema, 5% Nam showed a numerically directional effect in reducing chromameter a* values compared with no treatment and vehicle control sites (Fig. 6a, b). Visual grading for redness showed a similar effect of Nam compared with vehicle sites (Fig. 6c). Digital images captured two days after UV exposure further confirmed the ability of 5% Nam to partially prevent SSR-induced redness (Fig. 6d). There was no significant difference between no treat and vehicle control sites (data not shown).

Discussion

Cumulative exposure of human skin to environmental stressors is one of the biggest factors that leads to molecular reprogramming and structural changes associated with premature ageing. In order to block the negative impact and prevent premature ageing, it is of importance to mechanistically understand the immediate effects from acute exposure that can serve as target(s) of an intervention strategy. Historically research has focused on the role of solar UV radiation on premature skin ageing classified as photoageing [1, 2]. Over the past 10 years, there has been increasing research efforts to better understand the impact of other environmental stressors such as pollution emissions and urban residency on skin [29]. It is known that the major impact of emitted gases and particulate matter from carbon emissions on human health include respiratory effects because of direct inhalation contact and recent reports suggest that these stressors can also affect secondary organs [30, 31]. This can manifest in skin as atopic dermatitis, psoriasis and eczema flareups [32, 33] and can also negatively impact skin ageing and appearance [5]. Cigarette smoke exposure is similar in that the cardiorespiratory health risks are established and secondary effects on skin appearance have also been confirmed [34].

To better understand the molecular response to these environmental stressors, we utilized both *in vitro* and *in vivo* models to assess the inflammatory response to controlled non-toxic exposure conditions. We further investigated whether Nam, a known vitamin that has been previously shown to have antioxidant and anti-inflammatory properties, can protect against these inflammatory responses.

To study this mechanistically, we used UVB, urban dust and diesel exhaust exposure to measure the impact on keratinocyte SASP-related inflammatory response. We found that all these stressors can induce an inflammatory response with varying agents. Since Nam was previously reported to downregulate gene expression of several cytokines, including IL-6, in response to UVB [35], we wished to further explore this effect at the protein level. Nam treatment significantly inhibited the increase of cytokines and prostaglandins synthesized after stress and significantly restored levels of Lamin B1 (a biomarker known to be lost during senescence) that were reduced by UVB exposure. Relative to cigarette smoke, it has been reported that exposure to keratinocytes can elicit a strong oxidative stress response based on proteomics [36]. We measured an elevated response in PGE_2 production and Nam treatment inhibited these induced levels and also partially reversed the most significant gene expression patterns that were related to apoptosis, DNA repair and cell cycle regulation.

Similar to what is known with UV exposure, pollution (particulate matter) has been reported to trigger production of inflammatory mediators by keratinocytes [37, 38]. In testing pollution-related stressors such as urban dust and diesel exhaust, we did not observe a significant induction of PGE_2 or IL-6 but rather an induction of IL-8. It is not clear whether this is due to a technical limitation in the testing because of solubilization or a true cellular response differential. It has been reported that pollution-related stressors such as $PM_{2.5}$ can elicit apoptosis and ROS formation in keratinocytes and the authors showed that this can be prevented by Nam [39]. Since these experiments were performed under *in vitro* conditions in which keratinocytes came directly in contact to a suspension of $PM_{2.5}$ we wished to test under more closely simulated *in vivo* skin exposure conditions. A 3D skin equivalent model that has an established barrier and intact stratum corneum would more closely simulate physiological exposure conditions of $PM_{2.5}$ coming in direct contact to skin with subsequent release of chemical agents bound to the particles [40]. We used a 3D skin equivalent model to evaluate the impact of a combination of topical $PM_{2.5}$ with UVB radiation. The combination of topical $PM_{2.5}$ with UVB showed a synergistic increase of IL-8 levels that were the completely inhibited by Nam treatment. This supports that Nam usage may provide a level of protection when skin is exposed to particulate matter during daytime.

While an inflammatory response can resolve post-stress, it has been suggested that this resolution becomes less efficient with ageing and leads to a persistent sub-chronic inflammatory state. This has been proposed as the basis for "inflamm-ageing" that leads to premature ageing of tissue and disease [11, 38, 41]. A persistent inflammatory state can also lead to senescence that is hallmarked by SASP [20]. Major components of SASP include the cytokines IL-1α, IL-6 and IL-8 [19]. Thus, we wished to understand the impact of environmental stressors on the release of these SASP-associated cytokine biomarkers as well as the prostaglandin PGE_2, a marker of acute UV-induced inflammatory response [42]. Additionally, we analysed the impact of UVB on the known senescence biomarker Lamin B1 [26].

Finally, we show that treatment of skin with Nam prior to UV exposure can help to partially mitigate induction of inflammation via quantitation of the ratio of the skin surface biomarkers IL-1αRA and IL-1α. It is important to point out that PGE_2, IL-6 and IL-8 could not be detected in tape strip extractions (data not shown). Since it is known that IL-1α and its receptor antagonist reside in stored pools in the epidermis, these inflammatory components have been well established to correlate with inflammation in various skin conditions, including being induced by UV radiation [28, 43]. Additionally, IL-1α is known to be a component of SASP, along with IL-6 and IL-8 [44]. This measure of reduced inflammation by Nam was supported by a lower quantitated degree of erythema induced by UV. We do not believe that this affect was because of physical or chemical absorption of UV by Nam because there was no product application on the day of UV exposure and Nam does not have strong absorption in the UVA and UVB wavelength region.

In this work, we show that a SASP-related inflammatory response can be simulated in skin models when exposed to environmental stressors. Keratinocytes exposed to UVB release elevated levels of PGE_2, IL-6 and IL-8 and reduced levels of Lamin B1. In contrast, CSE exposure only triggered an increase in PGE_2 levels whereas urban dust and diesel exhaust only increased IL-8. Finally, the topical combination UVB and PM2.5 in a 3D skin model lead to significant increases in IL-8 and treatment with Nam was able to significantly inhibit these stress-induced inflammatory and senescence-related responses. It should be noted that IL-1α was not induced by UVB and thus was not tested with the other stressors (data not shown). Additionally, we report that treatment of skin with Nam could partially mitigate the level of IL-1α-related inflammation and erythema elicited by solar simulated radiation.

These findings support the hypothesis that Nam could help mitigate premature ageing of skin and maintain overall skin homeostasis and health. Future work is needed to more clearly refine the differentials in response of keratinocytes and 3D skin models to the range of environmental stressors tested in this work as well as the impact of Nam on mitigating the response. In total, this work contributes to the growing fundamental understanding of the impact of environmental stressors on skin biology. Additionally, the data presented here provides further proof to the growing body of evidence that inflammation induced by environmental stressors can be mitigated by Nam to help prevent premature ageing and maintain skin's overall homeostasis.

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Conflicts of Interest

All authors, except C.Y.R.T., M.M. and S.B., are employees of The Procter & Gamble Company. All authors declare no conflict of interest.

Author Contributions

Conceptualization of this work was led by J.E.O. and S.B., methodology by J.C.B., T.L., B.C.H., T.M., S.B. and C.E.M..; validation by T.M.; formal analysis by T.M., J.D.S. and S.B.; investigation by J.E.O., J.C.B., T.L., B.C.H., J.D.S., T.M., S.B., C.Y.R.T., M.M. and C.E.M.; data curation by J.E.O., J.C.B., T.L., T.M. and S.B.; writing–original draft preparation by J.E.O; writing–review and editing by J.E.O., J.C.B., T.L., B.C.H., T.M., J.D.S., C.E.M. and S.B.; visualization by J.E.O.; and project administration by J.E.O., C.E.M. and S.B.

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