An Efficient Means to Mitigate Skin Inflammaging by Inhibition of the NLRP3 Inflammasome and NfKb Pathways: A Novel Epigenetic Mechanism

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INTRODUCTION

Inflammaging refers to chronic and low-grade inflammation that contributes to aging and tissue damage [1]. Growing evidence suggests that aging is driven by proinflammatory cytokines [2] (*e.g.* IL-1β, IL-18, etc.) produced by the innate immune system that involves the inflammasomes [3-4] and the nuclear factor kappa light chain enhancer of activated B cells (NfKb) pathway [5]. These inflammatory pathways are interconnected and have PKR as a common inducer. PKR is also referred to as double-stranded RNA-dependent protein kinase [6-8]. Recently, we showed that skin inflammation is also regulated by an epigenetic process involving PKR and the long noncoding RNA lnc886. This RNA transcript is a 101 nucleotide long noncoding RNA ubiquitously expressed and cytosolic [9]. Indeed, we showed that when lnc886 is downregulated, PKR is no longer suppressed and the inflammatory state of the cells is increased (overexpression of COX-2) [10]. Lnc886 regulates inflammation by direct inhibition of PKR activity *via* the suppression of PKR phosphorylation [10]. PKR expression also increases with aging in neurodegenerative diseases associated with a strong release of proinflammatory cytokines.

Abstract

Inflammaging refers to chronic, low-grade inflammation that contributes to the aging process. A growing number of references specify that aging is driven by proinflammatory cytokines produced by the innate immune system which involve the inflammasome and NfKb pathways. These two pathways are interconnected and share a common inducer, the double-stranded RNA-dependent protein kinase PKR. Recently, we showed that inflammation is controlled by an epigenetic process involving PKR and the long noncoding RNA lnc886. Lnc886 regulates inflammation by the direct inhibition of PKR phosphorylation. Although the literature suggests that excessive inflammasome activity leads to inflammaging in age-related pathologies, this has not been not demonstrated for skin aging. The present paper effectively illustrates that along with aging and concomitantly with decreased expression of lnc886 (the PKR inhibitor), the inflammasome is activated. Using several innovative inflammasome models, we demonstrate that a seaweed extract (Laminaria Japonica) efficiently inhibits both the inflammasome and NfKb pathways. With a multifaceted mechanism of action, it stimulates lnc886 expression, inhibits PKR, decreases reactive oxygen species and reduces cleavage of caspase-1 and proinflammatory cytokine release. As a result, this extract is an innovative and efficient solution to address skin inflammaging.

Inflammasomes play a crucial role in physiological conditions. They are essential components of the innate immune system and requisite for the clearance of pathogens and damaged cells in addition to the fight against metabolic diseases (*e.g.* type 2 diabetes, obesity), neurological disorders (*e.g.* Alzheimer's), autoimmune diseases (*e.g.* vitiligo, rheumatoid arthritis, lupus erythematosus), neoplasia (*e.g.* gastric, colorectal, breast, melanoma) and cutaneous fibrosis (*e.g.* acne, contact dermatitis and rosacea) [11-12]. Inflammasomes are intracellular multiprotein complexes consisting of three elements: a molecular pattern recognition receptor (PRR), an apoptosis-associated speck-like protein containing a caspase-

recruitment domain (ASC) adaptor protein, and a caspase-1 enzyme. Inflammasomes are named in accordance with their intracellular receptor's nomenclature. Two receptor classes have already been identified: NLR-NOD-like receptors and ALR-AIM2-like receptors. At least 6 types of inflammasomes have been described. Five belong to the nucleotide-binding oligomerization domain (NOD)-like receptor-containing pyrin domain (NLR) family (NLRP1, NLRP3, NLRP6, NLRP12, NLRC4/IPAF), and one to the AIM-2 is an ALR receptor.

Inflammasome assembly and activation are initiated by the recognition of stimuli by PRR receptors, which interact with the ASC adaptor molecule. PRR is a sensor able to recognize pathogen- and damage-associated molecular patterns (PAMPs and DAMPs). The ASC protein then recruits and links the pro-caspase-1 to the inflammasome complex, which is then cleaved to activate caspase-1. Activation of caspase-1 leads to the cleavage of pro-IL-1β and pro-IL-18 to their active forms IL-1β and IL-18, respectively, which stimulate the inflammatory response [13]. Both interleukins IL-1β and IL-18 are members of the same structural family, the (IL)-1 family. IL-1 is known for its ability to affect various and different biological properties associated with infection, inflammation and autoimmune processes [14].

IL-1β and IL-18 possess a similar three-dimensional structure. IL-1β has several functions, such as inducing fever and increasing both cortisol and IL-6 concentrations. IL-18 is produced by dendritic cells, macrophages and epithelial cells. IL-18 uses the same signaling pathway as IL-1 to activate NfKb and induce inflammatory mediators, such as adhesion molecules, nitric oxide, chemokines and Fas ligand. Interleukin 18 induces both T helper 1 and T helper 2 cytokines [15]. Unlike interleukin 1, IL-18 does not induce fever or cyclooxygenase-2 (COX-2). Moreover, it has been demonstrated that an overexpression of mature IL-18 in skin results in worsening of both allergic and nonallergic inflammation *via* Th2 cytokines [16]. These cytokines participate in fundamental inflammatory processes that increase during the aging process [14].

Recent papers suggest that in age-related pathologies inflammasome activity becomes excessive and may contribute to inflammaging, but this effect has yet to be demonstrated in the skin aging process [3 - 4]. The aim of this work was therefore to determine first, if along with aging in human facial skin the NLRP3 inflammasome is effectively activated (overexpression of NLRP3 and accumulation of activated caspase-1) and secondly, if lnc886, the inhibitor of PKR, is downregulated with age. In such situations the increase in inflammasome activity resulting from a decrease in lnc886 may contribute to the aging process and subsequently serve as grounds for inflammaging. Inflammaging results from the chronic overexpression of proinflammatory cytokines (*e.g.* IL-1β, IL-18) in addition to enzymes involved in the inflammatory process (*e.g.* COX-2, MMP-9). Therefore, it appears logical that the development of an active ingredient that can inhibit the excessive and uncontrolled inflammasome and NfKb activation which occurs during aging, after UV exposure and in the presence of DAMPs, such as the HMGB1 (high-mobility group box-1) molecule, is an innovative strategy to cope with skin inflammaging. For this reason, we designed models in which the inflammasome is activated following stress, such as UVB or DAMP exposure. Then we developed a 3D skin model using UV irradiation (UVA + UVB) to activate the inflammasome. We present results that demonstrate that the brown seaweed *(Laminaria Japonica)* extract is an efficient skin anti-inflammaging active ingredient. Indeed, we could show that the active is a potent inhibitor of the NLRP3 inflammasome and NfKb pathways in skin, which as previously described are two connected pathways involved in skin inflammaging. Its mechanism of action is multifaceted and includes the capability to stimulate the synthesis of Inc886 while limiting the accumulation of cleaved caspase-1 and reactive oxygen species, COX-2, MMP-9, and IL-1 and IL-18 release. As previously described, Inc886 is directly linked to the inhibition of PKR, which contributes to inhibition of the inflammasome and NfKb pathways. This paper will further present and discuss these points.

EXPERIMENTAL

Raw material

The active ingredient (AI) called Epseama™ was obtained after water extraction of the dried brown seaweed *Laminaria Japonica* followed by an ethanolic precipitation.

Skin explants

The skin samples were obtained from patients undergoing cosmetic surgery according to the French law L.1245 CSP "Products and elements of the human body taken during surgical procedures and used for scientific research". Each patient was fully informed and provided written consent before donating their tissue. Cervicofacial face lift skin samples were supplied by Alphenyx (Marseille, France). Five caucasian skin samples, 16, 22, 43, 52 and 60 years old, were used. Cervicofacial face lift skin samples (NativeSkin® model) from a 64-year old Caucasian woman were supplied by Genoskin (Toulouse, France).

Quantification of NLRP3 inflammasome markers and lnc886 in skin explants of different age

Histological processing and image analysis of skin samples to analyze NLRP3, cleaved caspase-1 protein expressions and lnc886 gene expression were performed as follows: after overnight fixation in AFA solution (55781-29, MM France, Brignais, France), samples were dehydrated and impregnated in paraffin using a dehydration automat (STP120 ThermoScientific™ Waltham, MA, USA). The samples were embedded using an embedding station (EC350, ThermoScientific™, Waltham, MA, USA), and the sections were mounted on Superfrost® histological glass slides. Deparaffinization and heat-induced epitope retrieval were performed simultaneously in a PT Module® (ThermoScientific™, Waltham, MA, USA). COX-2, NLRP3 and cleaved Caspase-1 immunostainings were done on paraffin sections with rabbit polyclonal anti-COX-2 (Abcam™ ab11576, Cambridge, UK), anti-NLRP3 (Abcam™ ab214185, Cambridge, UK) and anti-cleaved Caspase-1 (Invitrogen™ PA5-38099, ThermoScientific™, Waltham, MA, USA) antibodies all diluted at 1:100 in PBS-BSA 3% overnight at 4°C. Endogenous peroxidases were inhibited by

Quanto Peroxide Block (ThermoScientific™, TA-125-H_2O_2, Waltham, MA, USA) before incubation with anti-rabbit labeled polymer N-Histofine® (Nichirei Biosciences™, 414141F, MM France, Brignais, France) for 1 h. Protein expressions were revealed by DAB, a peroxidase substrate (ThermoScientific™, TA-125-QHDX, ThermoScientific™, Waltham, MA, USA). Counterstaining with Mayer's hematoxylin was performed and slides were mounted with Diamount® mounting medium (Diapath™, 0304000, Martinengo, Italy).

Staining of lnc886 was performed by Advanced Cell Diagnostics (ACBio, Hayward, USA) using the Basescope® technology on skin paraffin-embedding samples. After deparaffinization and target retrieval steps, this assay can detect short RNA targets (50 - 300 bases) using specific probes called Z pairs (i.e. RNA sequence spanning 50bp). Reagents amplified the hybridization signals *via* sequential hybridization of amplifiers and label probes. Each punctate dot signal represents a single test target RNA molecule and can be visualized with a microscope under a bright field. Stained slides were observed using a photonic Leica DM2000 microscope (Leica Microsystems™, Buffalo Grove, IL, USA). Pictures were digitized with a numeric DFC450C Leica camera and LAS X software. Quantification was done with Fiji software (National Institute of Health, USA). Two histological slides per sample were prepared and on each slide 5 pictures at the magnification x20 were performed. Image analysis was perfomed on 10 pictures per condition. Statistical analysis was performed using Student's t-test. P values <0.05 were considered significant. A single asterisk (*) denotes $0.01<p<0.05$, a double asterisk (**) $0.001<p<0.01$, triple asterisk (***) $p<0.001$.

Determination of the effect of brown seaweed *Laminaria Japonica* extract (AI) on lnc886 expression using aged skin explants

Skin explants were topically treated daily for 72 h with the active ingredient AI at 300 ppm (brown seaweed *Laminaria Japonica* extract). Histological processing and image analysis of skin samples to assess lnc886 gene expression were performed as described in the previous section. Three histological slides per sample were prepared and 5 pictures were taken on each slide at the magnification x40. Image analysis was perfomed on 15 pictures per condition.

Study related to inflammasome activation in keratinocytes under UVB and HMGB1 stress

Cell culture conditions for UVB and disulfide HMGB1 models

Human primary keratinocytes were seeded 24 h prior to treatment with either the active ingredient at 300 ppm or complete culture medium (control). The cells were cultured in EpiLife Medium (MEPICF500 GIBCO) supplemented with $CaCl_2$ (60 µM_S013 GIBCO) and HKGS (human keratinocyte growth supplement) (S0015 GIBCO). All GIBCO cell culture products were purchased from ThermoScientific™, (Waltham, MA, USA). For the UVB model, the culture medium +/- active ingredient was removed and replaced by irradiation medium (PBS only +/- treatments). UVB irradiation was done at 40mJ/cm^2. Immediately after irradiation the PBS was removed and replaced by complete culture medium. An exposure control was done in parallel. For the HMGB1 model, following the 24 h post-seeding with or without active ingredient, the disulfide-HMGB1 (HM-122, HMG Biotech, Milano, Italy) was added at a concentration of 2 µg/mL for a time depending on the experiment.

Quantification of activated caspase-1, Interleukin 18, interleukin 1β and reactive oxygen species

Activated caspase-1 was assessed after the addition of 1.5 X FAM-YVAD-FMK probes reference ab219935 (abcam, Cambridge, UK) and Hoechst 33342 (Life Technologies, California, USA) in washing buffer for 1 h at 37 °C. FAM-YVAD-FMK is a fluorescent probe that binds irreversibly to active caspase-1 in stimulated cells. In principle, the FAM-YVAD-FMK signal is proportional to the amount of active caspase-1. After incubation the cells were fixed with 4 % paraformaldehyde and then the caspase-1 and Hoechst 33342 staining were measured using the Celllinsight CX5 high content screening platform (Thermo Fisher, Waltham, MA, USA) by employing the FITC channel and DAPI filters, respectively. The nuclei were labeled with Hoechst® 33342.

The secretion of Interleukin 18 and interleukin 1β in response to activation of the stress-induced inflammatory signaling pathway was evaluated using a sandwich ELISA assay. Forty-eight hours after exposure to UVB and 6 h after exposure to HMGB1 the supernatant of cells was recovered. The samples were then treated according to the manufacturer's instructions using the IL-1β reference RAB0273D-1VL (Sigma Aldrich, Saint-Louis, MO, USA) or IL-18 antibody reference RAB0543F-1VL-KC (Sigma Aldrich, Saint-Louis, MO, USA). Interleukin production by cells was measured at 450 nm using TECAN.

Briefly, the cells were labeled after treatment according to the manufacturer's instructions using a CellROX probe. The nuclei were labeled with Hoechst®33342. The staining of cells for caspase 1 and CellRox was measured individually using the HCS Studio™ Cell Analysis Software from Cell Insight CX5 high content screening platform (Thermo Fisher, Waltham, MA, USA). The cells were visualized using Cell Insight 20X objectives and segmented using the morphology explorer software The labeling area and total intensity were evaluated individually for each cell among ~600 keratinocytes for each condition. Statistical analysis was performed using Student's t-test or a Wilcoxon test (using Excel and GraphPad Prism7 softwares). Statistical significance was considered at * $p<0.05$ and ** $p<0.005$.

Development of a full thickness skin inflammasome model suitable to study inflammasome inhibition and inflammaging

The "full thickness skin model" (T-skin, EpiSkin Laboratories, Lyon, France), reproducing dermal and epidermal compartments, was used here to set up a skin inflammasome model. We designed this model in order to determine if the active ingredient was able to mitigate inflammasome activation in skin subsequent to UV

exposure and thus mitigate inflammaging. The T-skin inflammasome model was obtained by subjecting T-skins to a standardized slight abrasion with a device inducing strokes on each tissue in order to reduce the overall stratum corneum thickness without reaching the viable epidermis followed by an irradiation with 1 MED = 0.025 J/cm^2 using a Oriel solar simulator (Oriel Newport Corporation, California, USA). The inflammasome model validated by semi-quantification of the cleaved caspase-1 by means of immunohistochemistry and quantification of the IL-1β in the culture medium of the T-skin control and the UV-irradiated T-skin. Moreover, the NfKb pathway was studied by immunofluorescence 4 h and 24 h post irradiation. At 4 hours the control skins (non-irradiated) and irradiated skins were collected for histological processing in order to quantify the nuclear translocation of NfKb.

To assess the efficacy of the active ingredient to prevent inflammasome and NfKb pathway activation, a first topical application of the product at 300 ppm was done overnight before UV irradiation. Then, a second product application at the same concentration was performed immediately after irradiation. The T-skins were collected 24 h after UV irradiation for histological analysis. The cleaved caspase-1 reference PA538099 (Invitrogen, Thermo Fischer Scientific™, Waltham, MA, USA) was assessed by immunohistochemistry using the FastRed reaction. The stained sections were visualized in a bright field on a Microscope Leica DM 2500. The images were acquired with a Leica DFC 450C Camera and LASX 3.0.1 software.

RESULTS AND DISCUSSION

Activation of the NLRP3 infammasome signaling pathway in human photo-exposed skin along with aging

According to a growing number of recent publications, activation of the inflammasome is emerging as a crucial event in inflammaging. This has been documented for several age-related diseases and also in physiological aging [4, 11]. The large variety of stimuli fueling inflammaging apparently converge in a few basic mechanisms and pathways involving the activation of the NLRP3 inflammasome pathway responsible for the production of inflammatory molecules, such as IL-1β and IL-18, after maturation of pro-caspase-1 into active caspase 1 [17]. Moreover, it was shown that NLRP3 inflammasome is also activated in several skin diseases [11]. Consequently, in our research we focused on the NLRP3 inflammasome subtype also known as NALP3, CIAS1, cryopyrin, or PYPAF1. Our results demonstrate that the NLRP3 inflammasome pathway is induced along with aging in the skin. Indeed, in ***Figure 1*** strong expression of NLRP3 (brown staining) can be observed in aged skin in comparison to younger skin. Moreover, in aged skin inflammasome activation is confirmed by the accumulation of cleaved caspase-1 in the epidermal layer. The onset of this activation seems to be around the age of 40 ***(Figure 1)***. Nethertheless, more samples would be needed to confirm the results. Although we demonstrated a strong accumulation of NLRP3 and cleaved caspase-1 along with aging in skin, we cannot exclude that part of this accumulation may be a result of UV irradiation, as the samples studied were photoexposed skin explants collected after face lift surgery. Indeed, it was demonstrated that UVB exposure triggers NLRP3 activation in human keratinocytes [18].Moreover, in contrast to NLRP1, which is expressed constitutively in the epidermis, expression of NLRP3 must be induced.

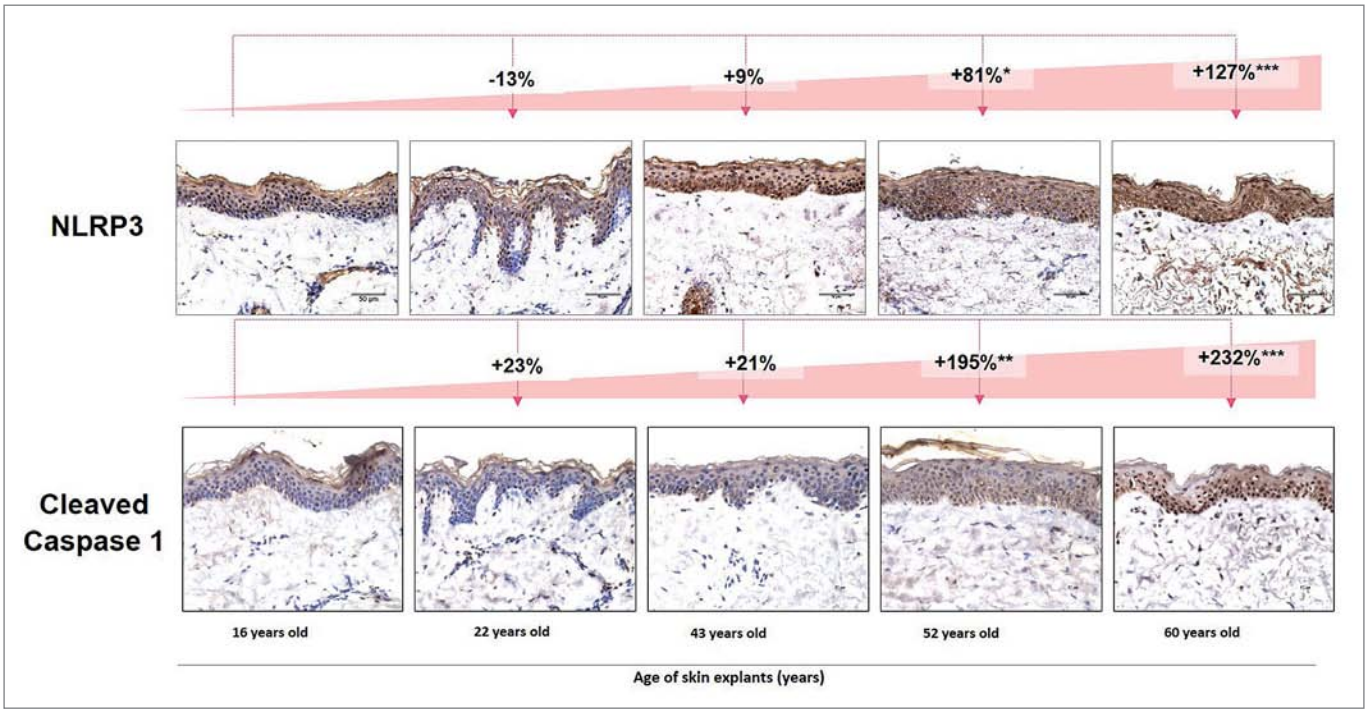

Figure 1 Expression of two inflammatory markers, NLRP3 inflammasome and cleaved caspase-1, along with aging. NLRP3 and cleaved caspase-1 expression were measured in the epidermis by immunohistochemistry (brown staining) on skin samples (facial skin).
The evolution is relative to the youngest skin (16 years old).

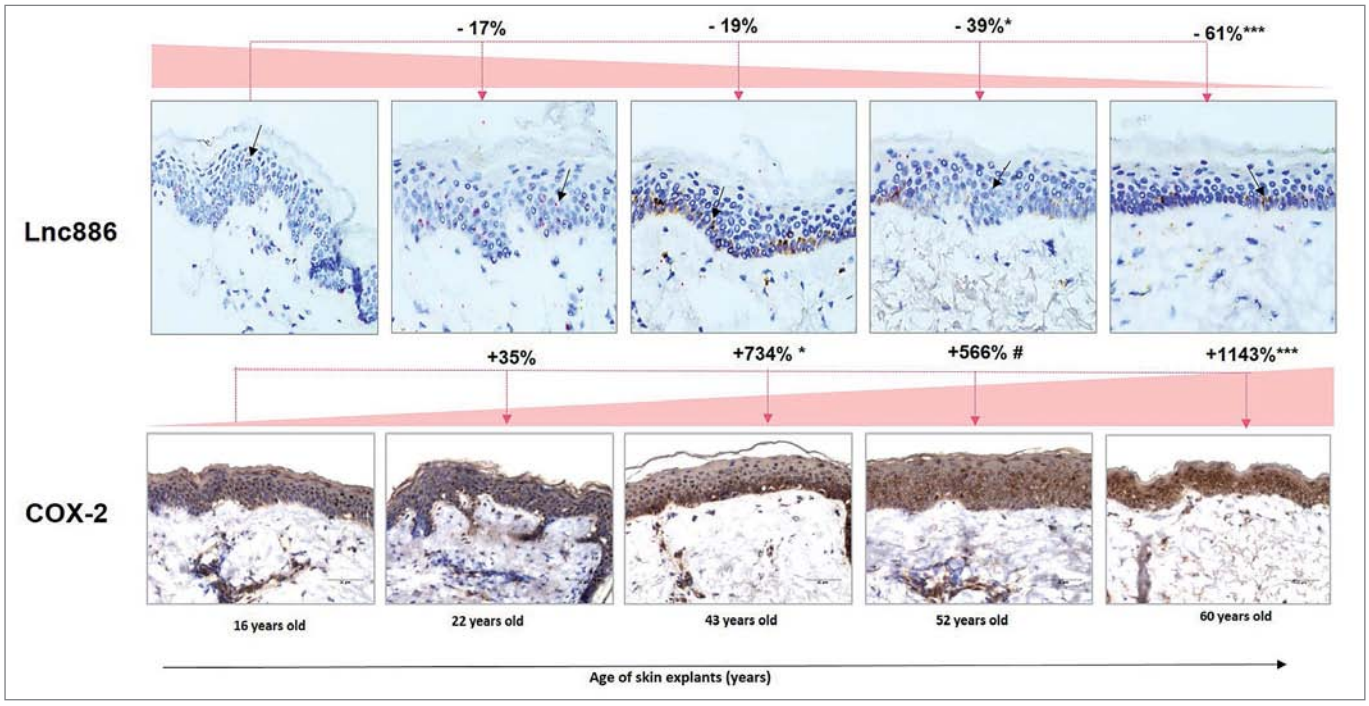

Figure 2 Expression of lnc886 and COX-2 along with aging in facial skin samples. Lnc886 expression was determined by *in situ* hybridization (red dot staining indicated by black arrows) and COX-2 expression was determined by immunohistochemistry (brown staining). Picture magnification x20. The evolution is relative to the youngest skin (16 years old). Statistical analysis was performed using a two-tailed unpaired Student's t-test (# $p<0.1$, *$p < 0.05$, **$p <0.01$, ***$p < 0.001$).

Contribution of downregulation of long noncoding RNA (lnc886) in human photoexposed skin along with aging to NLRP3 inflammasome activation is associated with an increase in COX-2 expression

We recently demonstrated in human keratinocytes that lnc886 is downregulated after UVB irradiation [9], but so far the expression profile of lnc886 along with skin aging or photoaging has not been studied. We analyzed lnc886 expression in skin explants (face lifts) taken from subject of different age using a RNA *in situ* hybridization (ISH) approach. Detection of lnc886 in tissue is possible with use of a chromogenic substrate, which produces a precipitate visible under bright-field microscopy, forming a distinct red dot. The red dots are indicated in ***Figure 2*** by black arrows. We found that the number of lnc886 copies decline along with aging ***(Figure 2)***. The onset of the decline seems to be significant around the age of 40. Interestingly, concomitantly with the decline of lnc886 the level of COX-2 is strongly increased from 40 years to 60 years, suggesting that the inflammaging process is ongoing. This result also confirms our previous work, where we observed an increase in the expression of COX-2 in the control and UVB irradiated conditions using lnc886 knockdown cells. In addition, in the same samples the content of activated caspase-1 increased in explants from the ages of 43 to 60, thus confirming inflammasome activation. Even though in ***Figure 1*** the content of NLRP3 in aged skin is significant and can explain the high content of cleaved caspase-1, we speculate that activation of the inflammasome is also a consequence of decreases in lnc886 expression. Indeed, an inverse relationship is observable, as the number of lnc886 copies decrease (along with aging and UVB irradiation9) and PKR activity increases while the phosphorylated PKR induces NLRP3 inflammasome activation. Moreover, we observed in PKR knockout cells (generated with the CRISPR/Cas9 system) in UVB irradiation conditions a reduced expression of COX-2. These results also highlight the role of PKR and lnc886 in COX-2 expression [9].

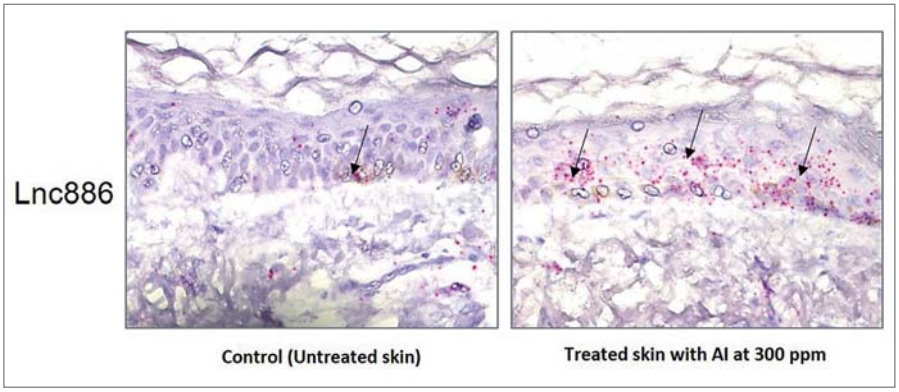

Figure 3 Visualization of lnc886 expression in aged skin treated or not treated with the active ingredient (brown seaweed *Laminaria Japonica* extract) at 300 ppm. Lnc886 expression was visualized by *in situ* hybridization (stained red dots indicated by black arrows). Picture magnification x40.

Stimulation of lnc886 expression in aged, photoexposed human skin explant by brown seaweed, *Laminaria Japonica* extract (AI)

Figure 3 shows the results obtained after 72 hours of topical application (renewed each day) of the seaweed *(Laminaria Japonica)* extract at 300 ppm to a 64-year-old skin explant. The skin explant studied here was obtained from a face lift which was considered to be photoexposed skin. The expression of lnc886 was determined using a RNA *in situ* hybridization (ISH) approach on histological slides. We found that the active ingredient at 300 ppm significantly increases lnc886 content up to +162 % in the epidermal region ***(Figure 3)***.

Effect of the active ingredient on activation of the inflammasome signaling pathway in human keratinocytes upon UVB and DAMPs stressors and in T-Skin model

In this experiment we quantified the content of active caspase-1 and its downstream targets, IL-1β and IL-18, as they are key biological markers of inflammasome activation. We observe in normal human keratinocytes a statistically significant increase in activated caspase 1 concomitantly with mature IL-1β and IL-18 release subsequent to UVB irradiation (***Table I*** and ***Figure 4***). This validates the inflammasome model. We demonstrated that the active ingredient at 300 ppm inhibits the release of IL-1β, IL-18 and accumulation of the activated caspase-1, suggesting that the active ingredient is efficient in reducing inflammasome signaling pathway activation after UVB irradiation.

In contrast to the previous *in vitro* model described above where only UVB was used to induce inflammasome activation, in the 3D full thickness *in vitro* skin model the inflammasome was activated using a slight abrasion combined with UV rays composed of UVA and UVB to better simulate reality. Indeed, photoaged skin is fragile and develops when skin is exposed to UVB and UVA [19-20]. As expected, without irradiation (NC) the amount of cleaved caspase 1 is low in the tissue but after UV irradiation (PC) the amount of cleaved caspase-1 is significantly increased in the epidermal compartment, mainly in the suprabasal layers ***(Figure 5A)***. Moreover and as expected, once irradiated the tissue produced a significantly larger amount of IL- 1β (between 4.84 and 11.8 pg/mL, ***Figure 5C***). The inflammation due to inflammasome activation is observed even after 72 h (data not shown). In the negative control ***(Figure 5B)***, IL-1β secretion was very low and below the detection limit (around 1 pg/mL). These results validate the inflammasome model. In this experiment, 300 ppm of the active ingredient limits the accumulation of activated caspase-1 (***Figure 5A*** and ***5B***), suggesting that the active ingredient is efficient to mitigate inflammasome signaling pathway activation after UV irradiation.

In this experimental assay we used the commercial disulfide-HMGB1 molecule

Table I UVB- induced accumulation of cleaved caspase-1 and release of IL-1β and IL-18 in the absence and presence of brown seaweed *Laminaria Japonica* extract AI (statistical analysis performed using the two-tailed unpaired Student's t-test, p-value < 0.05).

Conditions	Cleaved caspase-1			[IL-1 beta]			[IL-18]		
	Fluorescence count (mean +SD)	% of increase vs control / Stat. vs control (t –test)	% of decrease vs UVB condition / Stat. vs UVB condition (Wilcoxon test)	µg/µL (mean +SD)	% of increase vs control / Stat. vs control (t –test)	% of decrease vs UVB condition / Stat. vs UVB condition (t –test)	µg/µL (mean +SD)	% of increase vs control / Stat. vs control (t –test)	% of decrease vs UVB condition / Stat. vs UVB condition (t –test)
Untreated cells (control)	100 ± 30			$2.82 \times 10^{-5} \pm 1.46 \times 10^{-5}$			$1.15 \times 10^{-3} \pm 3.14 \times 10^{-4}$		
UVB treated cells (40 mJ/cm²)	4743 ± 2310	**+4643%** / P<0,0001		$1.73 \times 10^{-4} \pm 3.22 \times 10^{-5}$	**+514%** / P<0,005		$2.31 \times 10^{-3} \pm 1.29 \times 10^{-4}$	**+102%** / P<0,05	
UVB treated cells + AI at 300 ppm	3564 ± 1752		**-25%** / P<0,0001	$1.23 \times 10^{-4} \pm 4.01 \times 10^{-5}$		**-29%** / Close to significance	$1.68 \times 10^{-3} \pm 1.35 \times 10^{-4}$		**-28%** / P<0,05

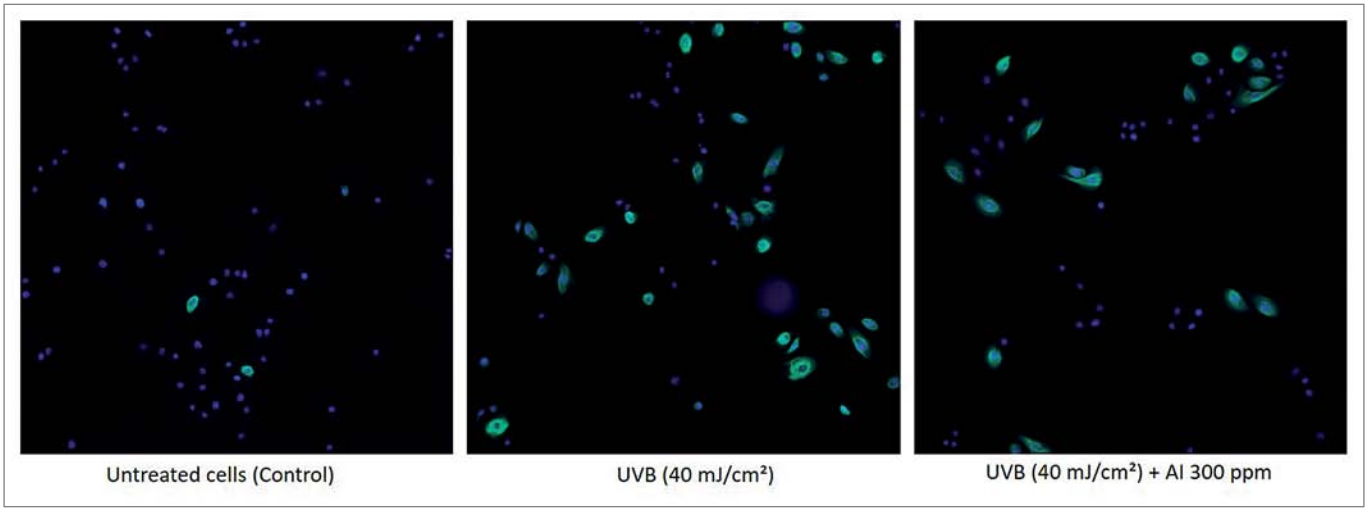

Figure 4 Visualization of cleaved caspase-1 in human keratinocytes, untreated (control) or treated with UVB (40mJ/cm^2), in the presence or absence of active ingredient (AI) at 300 ppm. UVB induces accumulation of activated caspase-1. The active ingredient at 300 ppm reduces the content of cleaved caspase-1, meaning an inhibition of inflammasome activation. The green signal represents activated caspase-1 recognized by the fluorescent probe FAM-YVAD-FMK. Pictures were acquired with the Celllinsight CX5 high content screening platform.

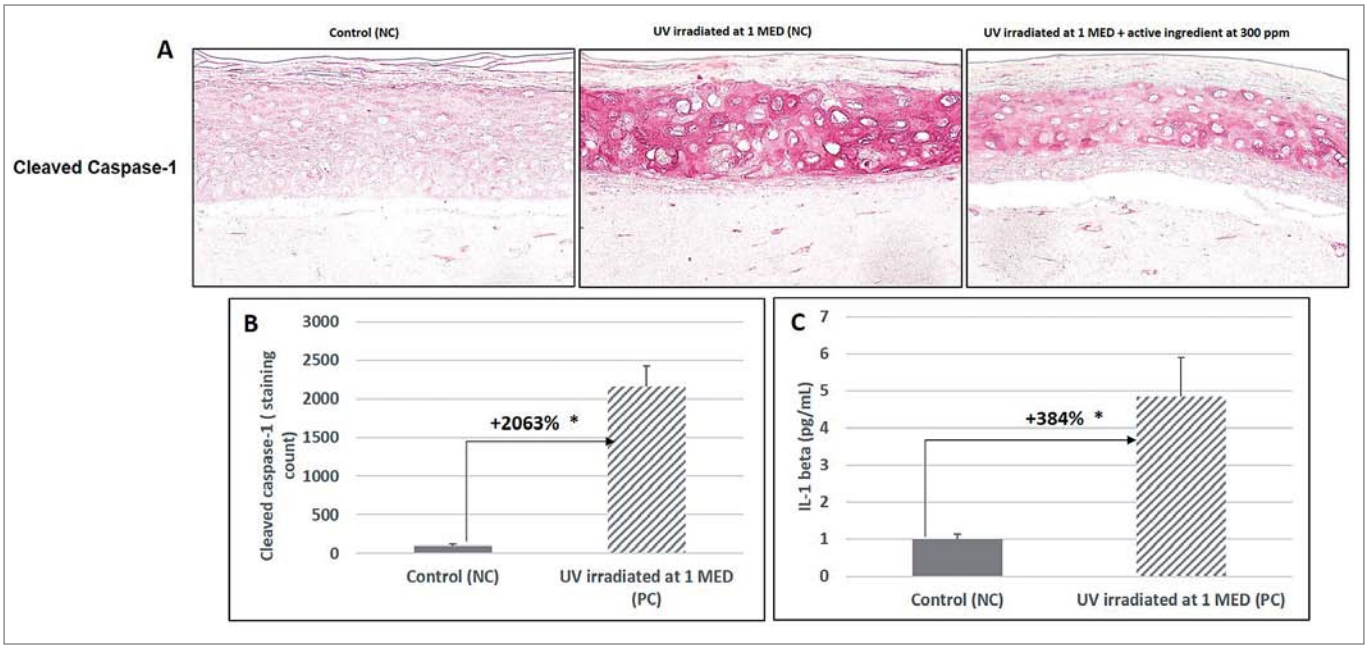

Figure 5 Quantification of cleaved caspase -1 (A and B) and IL-1β (C) 24h post UV irradiation in the full thickness reconstructed skin model. T-skin (positive controls) were irradiated with 1MED (UVA+ UVB). The negative control corresponds to nonirradiated T-skin. The quantity of cleaved caspase-1 was determined by immunohistochemistry (pink staining, picture magnification x40). The concentration of IL-1β was determined by the Elisa assay. The statistical analysis was performed using the two-tailed unpaired Student's t-test (* p-value <0.05).

as the NLRP3 inflammasome's inducer molecule. This molecule mimics the endogenous HMGB1 molecule, also known as DAMPs (damage associated molecular patterns) [21]. It has been demonstrated that the NLRP3 inflammasome is considered a general sensor of DAMPs, which may be important in the sterile inflammation observed in many human diseases and also in aging [7].

Indeed, in this experimental assay we wanted to determine if the active ingredient is able to diminish inflammasome pathway activation regardless of the stimulus involved and not only limited to the UV irradiation condition. The alerting danger signal HMGB1 occupies a crucial functional role as a signaling molecule that informs cells when damage or foreign invasion occurs. HMGB1 is an evolutionary ancient nuclear protein that exerts various biological functions depending on its location. In the nucleus HMGB1 organizes DNA and nucleosomes and regulates gene transcription. Upon cell activation or injury, nuclear HMGB1 translocates to the cytoplasm, where it is involved in inflammasome activation and pyroptosis as well as regulation of the autophagy/apoptosis balance. HMGB1 has also cytokine, che-

Table II Accumulation of cleaved caspase-1 and the release of IL-18 with HMGB1 treatment. The active ingredient at 200 ppm produces a statistically significant reduction in the content of cleaved caspase-1 and IL-18, meaning an inhibition of inflammasome activation. The table presents the mean and standard deviation of at least 3 replicates. The statistical analysis was performed using a two-tailed unpaired Student's t-test (p-value < 0.05).

Condition	Cleaved caspase-1			[IL-18]		
	Fluorescence count (mean +SD)	Stat. vs control	Stat. vs UVB condition	µg/µL (mean +SD)	Stat. vs control	Stat. vs UVB condition
Untreated cells (Control)	100 ± 38			80 ± 1.7		
Disulfide-HMGB1 treated cells (2 µ g/mL)	282 ± 84	**+182%** / P<0,05		91.5 ± 9.3	**+15%** / p=0,098	
Disulfide-HMGB1 treated cells + AI at 200 ppm	91 ± 40		**-67%** / P<0,05	72.5 ± 4		**-21%** / P<0,05

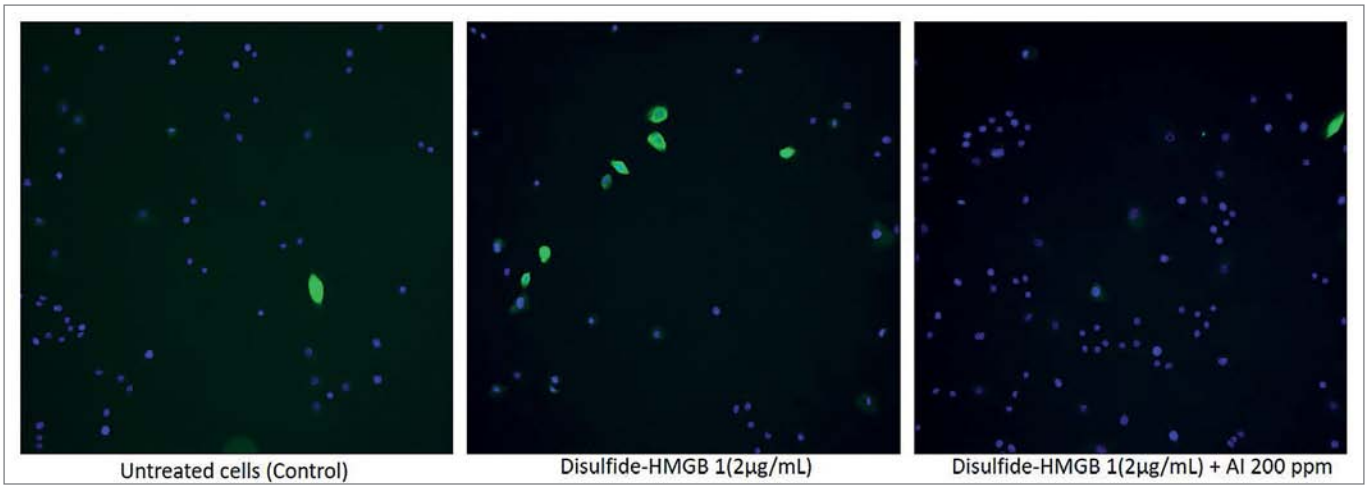

Figure 6 Visualization of cleaved caspase-1 in human keratinocytes untreated (control condition) or treated with HMGB1 (condition) in the presence or absence of active ingredient (AI) at 200 ppm . HMGB1 induces the accumulation of activated caspase-1. The active ingredient (AI) at 200 ppm reduces the content of cleaved caspase-1, meaning an inhibition of inflammasome activation. The green signal represents activated caspase-1 recognized by the fluorescent probe FAM-YVAD-FMK. Pictures were taken with the Celllinsight CX5 high content screening platform.

mokine, neuroimmune and metabolic activities when released into the extracellular medium. HMGB1 initiates and maintains immune response during infections and sterile inflammation. Moreover, in this experimental assay we demonstrated that when keratinocytes are exposed to HMGB1, IL-18 is quickly released into the cell supernatant (only after 6 h), confirming activation of the inflammasome pathway. This effect is maintained after 24 h and 72 h. On the contrary, under the same experimental conditions we did not observe IL-1β modulation by HMGB1 at any of the time points studied (6 h, 24 h, 72 h), probably due to an earlier release. Next, we demonstrated that the active ingredient inhibits the release of IL-18 and accumulation of the activated caspase-1 ***(Table II, Figure 6)***, suggesting that the active ingredient efficiently reduces activation of the inflammasome signaling pathway.

Moreover, it has been demonstrated that ROS produced by many known activators of NLRP3 inflammasomes, such as DAMPs, are a critical mechanism triggering NLRP3 inflammasome activation [22-23]. As a result, we sought to determine if the inflammasome inhibitory effect shown by the active ingredient may in part be attributable to its capability to limit ROS production following HMGB1 exposure. As expected and can be seen in ***Figure 7***,

HMGB1 stress induces ROS production in cells (+58 %). The amount of ROS generated was determined using CellROX staining. CellROX® reagent is a fluorogenic probe for measuring the cellular oxidative stress in cells. In ***Figure 7***, 200 ppm of the active ingredient produced a statistically significant reduction in ROS induction to a level similar to that of the control condition. Thus it is evident that one-way inflammasome pathway activation occurs through the increase in intracellular ROS generated either by extracellular HMGB1 or intracellular HMGB1 signals. Indeed, an accumulation of HMGB1 in the cytoplasm was observed in Hacat cells exposed to UVB. Following ROS production, HMGB1 is translocated from the nucleus to the cytoplasm [24]. The cytosolic accumulation of HMBG1 in turn contributes to continued NLRP3 inflammasome activation. On the other hand, we have shown that the inflammasome is also inducible by the PKR pathway following UVB and aging. Taken together, the data suggest that the active ingredient diminishes inflammasome activation through two mechanisms: inhibition of the PKR pathway through the increase in lnc886 transcripts and its antioxidant properties demonstrated by the reduced intracellular ROS production.

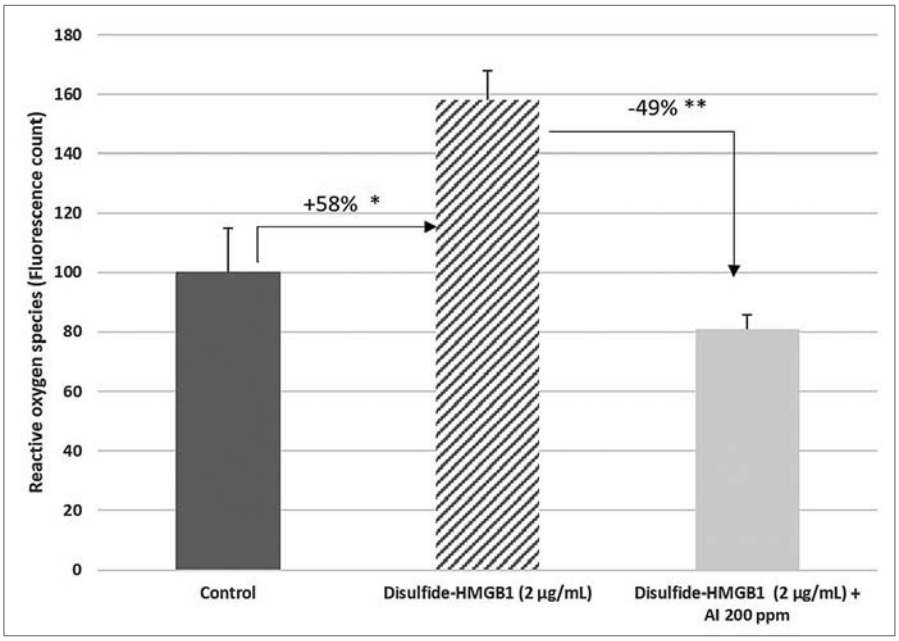

Figure 7 Determination of reactive oxygen species (ROS) levels in keratinocytes exposed or not to a DAMPs molecule (Disulfide-HMGB1) and in presence or absence of active ingredient at 200 ppm. The ROS were detected using a CellROX probe. The graph presents the mean of the induction of at least 3 replicates. The standard deviation bars are represented by black lines in each histogram. Statistical analysis was performed using a two-tailed unpaired Student t-test.
(*= vs control; p-value <0.05, ** = vs. disulfide-HMGB1, p-value <0.005)

NfKb signaling pathway activation in a full-thickness reconstructed skin model mitigated by the active ingredient

NfKb plays a major role as a mediator of inflammatory response [25]. We could demonstrate that the NfKb pathway is activated after UV irradiation of full thickness skin. We noticed a strong expression of NfKb in cell cytoplasm after UV irradiation and the nuclear translocation of this transcription factor is clearly visible ***(Figure 8)***. This result validates the model. Moreover, when the skin was treated with 300 ppm of the active ingredient, we could observe a strong decrease in NfKb expression in the cytoplasm 24 h after stress ***(Figure 9)***. This effect highlights the anti-inflammatory property of the active ingredient.

Interconnection of the PKR, NfKb and inflammasome pathways

We recently showed that exposure of human keratinocytes to UVB induces p38 and SAPK/JNK signaling, which in turn leads to PKR phosphorylation [9]. Phosphorylated PKR through the NfKb pathway controls the production of proinflammatory cytokines, such as IL-1β, IL-8, and TNF-α, and also the gene expression of the NLRP3 inflammasome [26]. These molecules are involved in the inflammaging process. In addition, PKR was shown to contribute to the activation of the NLRP3 inflammasome through direct interaction with NLRs [27]. Indeed, it was demonstrated that a loss of PKR results in attenuated IL-1β and IL-18 cleavage and HMGB1 release in response to various stimuli [6]. The increased release of HMGB1 under UVB irradiation has also been described [28]. Under our experimental conditions we could show that the PKR ligand, lnc886, inhibits the upregulation of UVB-mediated PKR sig-

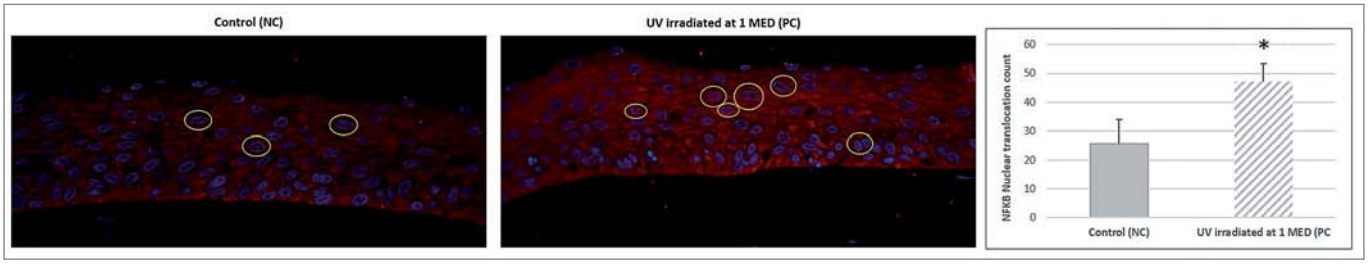

Figure 8 Visualization and quantification of NfKb nuclear translocation 4 hours post UV irradiation in a full thickness skin reconstructed model. The T-skin (positive control PC) were irradiated with 1MED (UVA+ UVB). The negative control corresponds to nonirradiated T-skin. NfKb was detected by immunofluorescence (red staining); in the pictures the yellow circles show the nuclear positioning of NfKb. Statistical analysis was performed using a two-tailed unpaired Student's t-test (*p-value <0.05).

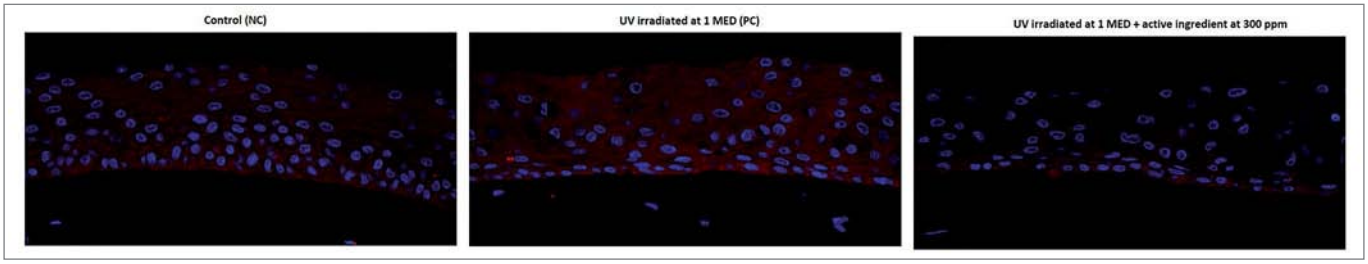

Figure 9 Visualization and quantification of NfKb 24 hours post UV irradiation in a full thickness skin reconstructed model.
The T-skin (positive control PC) were irradiated with 1MED (UVA+ UVB). The negative control (NC) corresponds to non-irradiated T-skin.
NfKb was detected by immunofluorescence (red staining) in the pictures and NfKb staining is localized in cytoplasm.
Statistical analysis was performed using a two-tailed unpaired Student's t-test (*p-value <0.05).

naling, as confirmed by the decrease in activation of NfKb and NLRP3. Moreover, we found in human aging skin a strong decrease in lnc886 transcripts associated with a strong increase of caspase-1 and COX-2 expression, which means that the NfKb and inflammasome pathways are stimulated. Indeed, with a lack of lnc886, inhibition of the PKR pathway does not occur and the vicious inflammatory cycle involving NfKb and NLRP3 pathways is maintained. We also could demonstrate in normal human keratinocytes that the disulfide HMGB1 (DAMP molecule mimic) induces NLRP3 inflammasome activation almost in part *via* ROS production. After an extensive screening of plant extracts, we discovered that the *Laminaria Japonica* extract can stimulate expression of lnc886 in human keratinocytes exposed to UVB stress ***(Figure 10)*** [9] and also in aged skin.

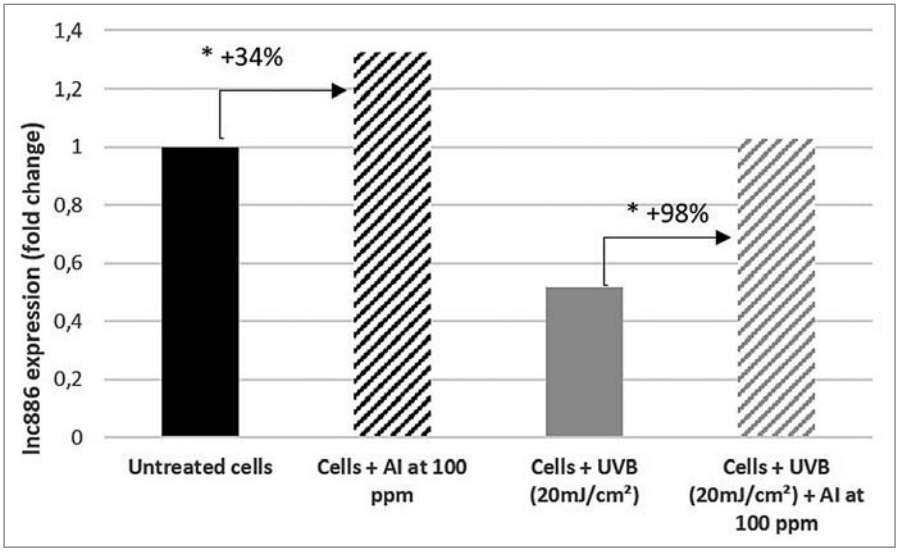

Figure 10 Quantification of nc886 expression in human keratinocytes under basal or UVB stressed conditions and exposed or not to the active ingredient laminaria Japonica extract at 100 ppm.
Quantification by qRT-PCR. Statistical analysis: Student's t test * p <0.01.

This active can mitigate the inflammaging process by targeting the inactivation of the three pathways, PKR, NfKb and NLRP3. ***Figure 11*** sums up the mechanism of action of the three interconnected pathways and the biological targets of the active ingredient *Laminaria Japonica* extract. The interconnection of the three pathways is shown in ***Figure 11A***. Under UV conditions or along with aging, PKR activation occurs through its phosphorylation. Indeed, under UVB and along with aging the number of copies of the direct inhibitor of PKR activation, lnc886, is decreased. As a consequence of PKR activation, the downstream inflammatory targets NfKb and NLRP3 are induced. The nuclear translocation of NfKb permits the transcription of IL-1β, COX-2 and NLRP3. NLRP3 activation leads to the release of the active caspase 1 responsible for the maturation of pro IL-1β and pro IL-18 into IL-1β and IL-18, respectively, but also to the accumulation of intracellular HMGB1. The release of IL-1β and IL-18 maintains the vicious inflammatory circle by stimulating also the NfKb pathway. In parallel, UV irradiation induces the activation of the NLRP3 inflammasome *via* ROS generation and contributes also to the cytoplasmic accumulation of HMGB1 after its translocation from nucleus to cytoplasm. When HMGB1 is present at the extracellular level following a damage associated molecular patterns stress (DAMPs) signal, the NLRP3 inflammasome is activated either through a ROS mechanism or indirectly (unknown mechanism), leading again to the release of IL-1β, IL-18 and intracellular HMGB1. Presented in ***Figure 11B*** is a schematic representation of the mechanism of action of the active ingredient (AI) *Laminaria Japonica* extract involved in the mitigation of inflammaging. The AI stimulates the expression of lnc886, which in turn limits PKR activation. As a consequence, the downstream NfKb and NLRP3 inflammasome pathways are less activated. The AI inhibits the production of ROS, reducing again NLRP3 inflammasome pathway activation. The AI is also able to directly inhibit NLRP3 activation and cleaved caspase 1 activity as well as to inhibit NfKb and COX-2 expression.

CONCLUSION

Currently, the contribution of inflammaging to the skin aging process is well known. Inflammaging is defined as chronic and low-grade inflammation triggered by the malfunction of the innate immune system following exposure to stressors (UV, DAMPs, pathogens, etc.). The innate im-

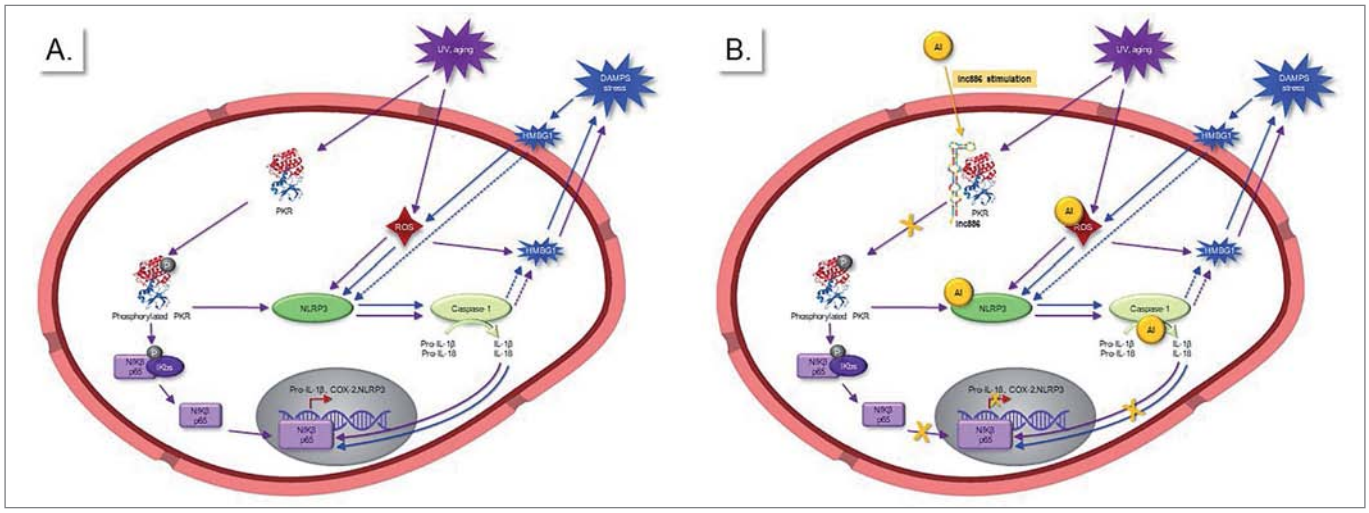

Figure 11 Schematic representation of the activation of PKR, NfKb and NLRP3 inflammasome pathways under UV, aging and damage associated molecular patterns (DAMPs). (A) Interconnection of the three inflammasome pathways. (B) Schematic representation of the mechanism of action of the active ingredient (AI) Laminaria Japonica extract involved in the mitigation of inflammaging.

mune system mobilized in the inflammatory process involves two different inflammatory pathways: the inflammasomes and NfKb. Moreover, as the activation of these two pathways are under the control of PKR and PKR is known to be regulated by an epigenetic mechanism involving the long noncoding RNA lnc886, we propose here an anti-inflammaging strategy based on modulation of the expression of this long noncoding RNA. Indeed, we propose here a *Laminaria Japonica* extract as a cosmetic active ingredient capable of stimulating the expression of lnc886. We show that along with aging and UVB exposure, an inverse relationship exists in the skin between inflammation and the expression of lnc886. We demonstrate here that the activation of these pathways is dependent on an epigenetic mechanism. Moreover, we show using several models including the inhibition of PKR activation, the NLRP3 inflammasome and the NfKb pathways that an epigenetic mechanism is efficient in mitigating skin inflammaging.

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