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# Oxidative damage prevention in human skin and sensory neurons by a salicylic acid derivative

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ABSTRACT

*Background:* Increased protein carbonylation is a hallmark of oxidative stress, protein homeostasis dysregulation and aging in the nervous system and skin. Sensory neurons interact with skin cells and are involved in skin homeostasis. We have previously reported that the 5-octanoyl salicylic acid (C8-SA), a salicylic acid derivative, increased *C. elegans* lifespan and delayed the accumulation of carbonylated proteins, through the stimulation of autophagy.

*Objectives:* In this study we aimed to investigate if C8-SA protects human sensory neurons and human skin from extrinsic oxidative stressors as an approach to delay skin aging.

*Methods: In vitro* reconstituted human epidermis innervated with hiPSc-derived human sensory neurons, as well as *ex vivo* human organotypic full skin models were used. The fully differentiated sensory neurons were pre-treated with C8-SA before oxidative stress induction. Skin explants were maintained in culture and treated topically with C8-SA before the application of urban pollutants. Carbonylated proteins were detected using amino-oxy functionalized fluorophores and quantified. Chaperone mediated autophagy was monitored with LAMP2A immunofluorescence. Inflammation, ROS detoxification and autophagy were assessed by RT-PCR.

*Results:* C8-SA prevented the accumulation of carbonylated proteins, both in human sensory neurons and skin explants. C8-SA stimulated chaperone-mediated autophagy and modulated NRF2 antioxidant response genes, as well as catalase enzymatic activity.

*Conclusions:* C8-SA acts at two levels to protect skin against oxidative stress: 1) it prevents protein oxidation by stimulating endogenous antioxidant defense and 2) it increases the clearance of oxidized proteins by stimulating chaperone-mediated autophagy. These results suggest that C8-SA maintains skin health in urban polluted environments.

## 1. Introduction

Skin is vital for protecting the body from water loss and external aggressions. Moreover, skin harbors free nerve endings (Fig. S1), important for skin sensory functions and associated with thermoregulation, immune function, wound healing, and skin homeostasis [1]. In addition to metabolic stress associated with intrinsic aging, skin is exposed to external assaults, including Ultraviolet (UV) light and airborne pollutants. Both, induce oxidative damage to skin macromolecules, in particular proteins, leading to structural and functional dysfunction, such as decreased skin barrier, altered trans-epidermal water loss (TEWL), chronic inflammation, altered stratum corneum pH and skin microbiome dysbiosis [2]. In addition, even short-term pollutant exposure has been linked to xerosis, pruritus and exacerbation of atopic dermatitis symptoms [3]. Aerobic metabolism as well as environmental stressors lead to the production of reactive oxygen species (ROS), which stimulate cellular stress response pathways, notably, endogenous anti-oxidant mechanisms [4]. The major

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antioxidant defense pathway is regulated by the nuclear factor E2-related factor (NRF2) [5,6]. ROS are implied in cellular signaling when present at physiological levels [4]; however, elevated ROS can lead to oxidative stress that is considered as a key contributor to accelerated skin aging and age-related pathologies [7,8]. Oxidative stress is responsible for oxidative modifications and damage of DNA bases, lipids and proteins [9], and triggering inflammatory response pathways [10]. A harmful protein modification is carbonylation, impacting on protein structure and function, leading to cellular and tissular damage [11]. Increased protein carbonylation is associated with negative impacts on skin complexion [12], skin hydration [13], dermis construction and accelerated skin aging [14].

Declined proteasomal and lysosomal proteolysis with aging contribute to the accumulation of carbonylated proteins [7], which in turn could accelerate the aging process. Autophagy is an important contributor to protein homeostasis. Chaperone mediated autophagy (CMA) was shown to be induced by oxidative stress and to thereby ensure the degradation of carbonylated cytosolic proteins [15]. CMA activity decreases during aging [16], which is also associated with age-related diseases, such as neurodegenerative diseases [17]. We previously reported that 5-octanoyl salicylic acid (C8-SA, Fig. S2), a salicylic acid derivative, increased lifespan in *Caenorhabditis elegans* (*C. elegans*) by preventing protein carbonylation accumulation [18]. C8-SA activated AMP activated protein kinase (AMPK), inhibited its downstream target mTOR and stimulated autophagy in *C. elegans* and human primary keratinocytes.

In this study, we investigated if C8-SA can protect human skin sensory neurons and human skin explants upon oxidative stress. C8-SA prevented protein carbonylation in 2D and 3D cultured human sensory neurons and in skin explants. Gene expression analyses indicate that C8-SA attenuated inflammation, stimulated NRF2 and ROS detoxification and modulated CMA under basal and stressed conditions. Moreover, the compound increased the protein expression of the CMA marker LAMP2A under basal and stressed conditions in skin explants and sensory neurons. Taken together, these data suggest that C8-SA protects skin from internal and external oxidative stress.

## 2. Materials and methods

### 2.1. Sensory neurons treatment

Human sensory neurons were co-cultured with human keratinocytes from day 13. At day 16, cultures were treated with C8-SA or vehicle (0.1% end-concentration DMSO in all conditions) for 24 h and subsequently for 1 h with 100 μM $H_2O_2$ (Sigma; H1009) in the culture medium. Cells were fixed in a solution containing paraformaldehyde (2% v/v) until analyses.

### 2.2. Treatment of sensory neuron coculture with reconstituted epidermis

After 1 day of co-culture with reconstructed epidermis, neurons in culture medium were treated with C8-SA or vehicle (0.1% end-concentration DMSO in all conditions) for 10 days and subsequently for 1 h with 100 μM $H_2O_2$. Carbonylated proteins were assesed as described previously [19].

### 2.3. Human organotypic skin explant culture

Skin explants were obtained with the informed consent from abdominal surgery of a female Caucasian donor (25 years old, phototype II/III), distributed in 4 experimental groups (n = 3) and kept alive in $CO_2$-humid incubator as reported previously [20]. At day 2, a 5% hydroalcoholic solution containing C8-SA (0,05% w/v) was topically applied or not (vehicle) on skin explants and left in contact for 48 h. Then, a solution (0,375 μg/$cm^2$) containing particulate matter (ERMCZ100, Sigma-Aldrich) was topically applied and skin explants were exposed to UV-A (365 nm, 6J/cm2). The control group (basal condition, not treated) did not receive any treatment. Half of each single explant was sampled and included in OCT for cryopreservation. The other half was snap-frozen in liquid nitrogen and stored at −80 °C for molecular biology and biochemical studies.

## 3. Results

### 3.1. C8-SA prevents protein carbonylation in human sensory neurons upon acute oxidative stress

First, we assessed the effects of C8-SA on acute oxidative stress induced protein carbonylation in sensory neurons cocultured with keratinocytes. Incubation with $H_2O_2$ significantly increased the level of carbonylated proteins; however, the presence of C8-SA prevented carbonylated proteins accumulation in a dose dependent manner, with statistical significance at 1 μM C8-SA (Fig. 1A/B).

To test C8-SA in a more physiological context, an *in vitro* model of human innervated epidermis was developed. The presence of the human sensory neuron layer allowed the formation of a well-organized and thicker epidermal layer (Fig. S3A/B). This confirms the trophic effect of skin innervation and the beneficial effects on skin homeostasis. Systemic treatment of the innervated epidermis with 1 μM C8-SA significantly reduced oxidative stress induced carbonylation in the sensory neuron layer (Fig. 1C).

### 3.2. C8-SA decreases protein carbonylation in human skin explants

Next, we tested the effect of topical application of C8-SA in 5% hydroalcoholic solution in human skin explants under basal conditions and in an experimental model of urban pollution (particulate matter and subsequent UVA exposure). The treatment with oxidative stress inducers increased the level of carbonylated proteins in the *Stratum corneum (SC)*, the epidermis, and the dermis (Fig. 2A panel 1 and 3) with statistical significance in total skin and single skin compartments (Fig. 2 B–E). Pretreatment with C8-SA inhibited this accumulation (Fig. 2A panel 3 and 4) significantly in total skin (Fig. 2B), the epidermis (Fig. 2D) and the dermis (Fig. 2E). Interestingly, C8-SA treatment decreased protein carbonylation already at basal conditions (Fig. 2A panel 1 and 2) with statistical significance in total skin (Fig. 2B), the *SC* (Fig. 2C), and the dermis (Fig. 2D).

### 3.3. C8-SA modulate stress pathways response in human skin explants

To get insights into potential mechanisms of C8-SA benefits on protein carbonylation inhibition, we used the same experimental conditions as in 3.2 (topical treatment with 0.05% C8-SA in 5% hydroalcoholic solution) to assess gene expression levels of oxidative stress related pathways, including inflammation, endogenous antioxidant defense and autophagy. The expression in whole skin samples of the inflammation genes *TNF* and *IL-6* were significantly increased upon exposure to urban pollution, whereas only a slight induction of *IL-1b* was observed (Fig. 3A). Under basal conditions, C8-SA blunted the expression of all three genes, even though only the effect on *TNF* was highly significant. Under stress conditions, the increase of *TNF* was inhibited and of *IL-1B* and *IL-6* reduced by C8-SA (Fig. 3A). Together these data suggest that C8-SA decreases inflammation under basal and limits its induction under oxidative stress conditions. *NFE2L2* (encoding NRF2) and its inducible target *NQO1* and *SLC7A11* [21] were significantly increased under stress conditions, whereas the effect on *CAT* expression was not statistically significant (Fig. 3B). C8-SA did not modulate the expression of *NFE2L2*; however, it significantly induced the expression of all three target genes under basal conditions. Target gene expression under stress conditions seemed to be further boosted by C8-SA, but only the effect on *CAT* expression was statistically significant (Fig. 3B). The effect of C8-SA on catalase modulation was confirmed by measuring catalase enzymatic

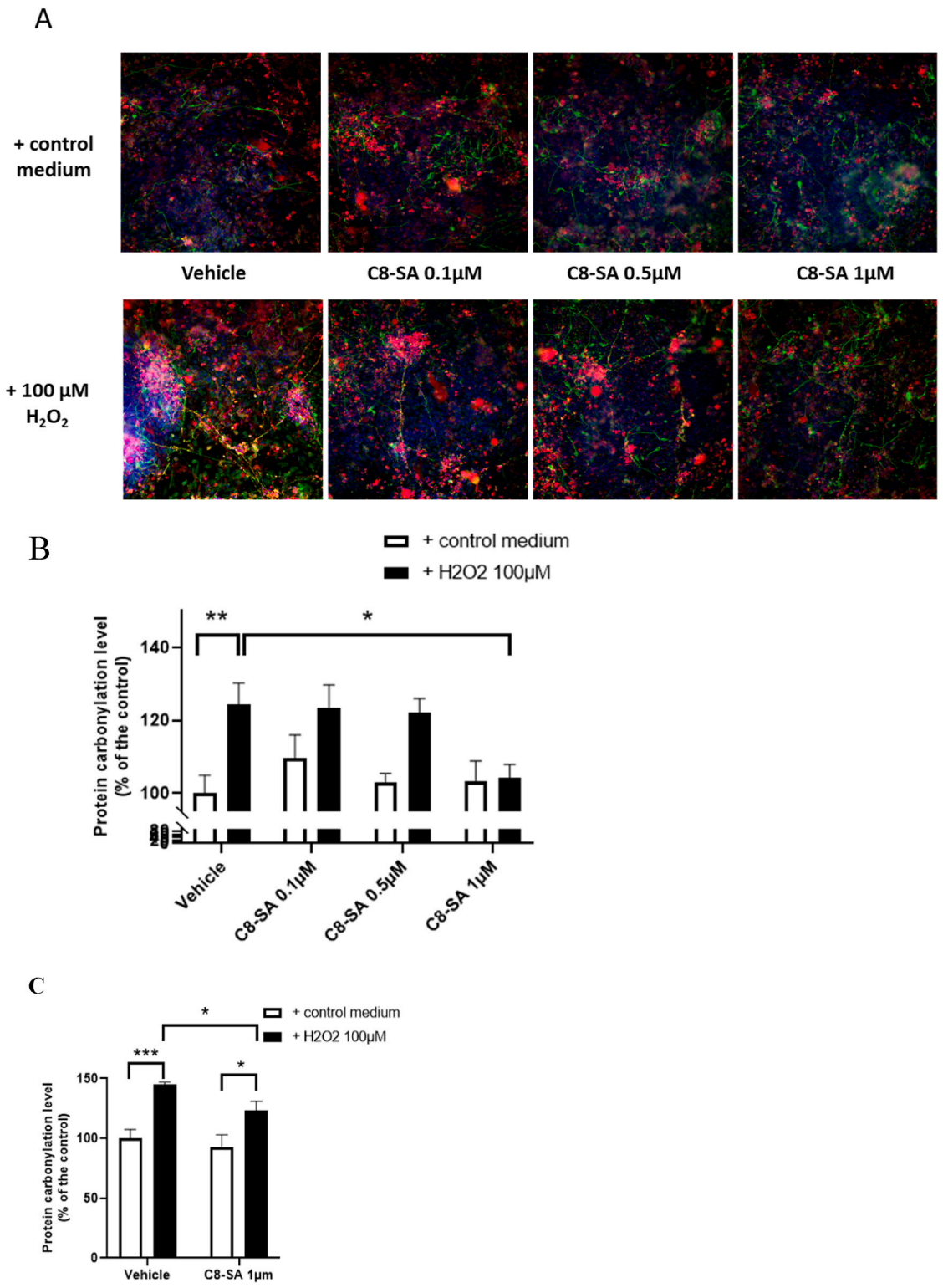


**Fig. 1. C8-SA protects human sensory neurons and innervated epidermis from $H_2O_2$ induced oxidative damage.** C8-SA was applied to cocultures of human sensory neurons and human keratinocytes for 24 h, then exposed to $H_2O_2$ (100 µM) for 1 h. Protein carbonylation level was measured immediately after $H_2O_2$ treatment by using a specific fluorophore (19). Cells were stained for β-III-tubulin (green), carbonyl groups (red) 4′,6-diamidino-2-phenylindole (DAPI; blue). Representative images (A). Images were taken with a 20x magnification. Carbonyl staining in sensory neurons (co-staining of carbonyl groups and β-III-tubulin) was expressed as a percentage of vehicle (0.1% DMSO) and shown as means ± s.e.m. (n = 6) (B). Comparative statistical analyses were obtained by variance comparisons (ANOVA) following by Dunnett's test or binary *t*-test comparisons. *p<0.05; **$p < 0.01$. C8-SA was applied to a co-culture of human sensory neurons and reconstructed epidermis for 10 days, then the culture was exposed to 100 µM $H_2O_2$ for 1 h. (C) Carbonylated proteins were detected *in situ* in the sensory neurons monolayer and revealed by epifluorescence microscopy. Quantification of carbonylated proteins, represented as a percentage of vehicle (100%) and shown as means ± S.D. (n = 3). Comparative statistical analyses were obtained by binary *t*-test comparisons. *p<0.05; ***$p < 0.00$. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

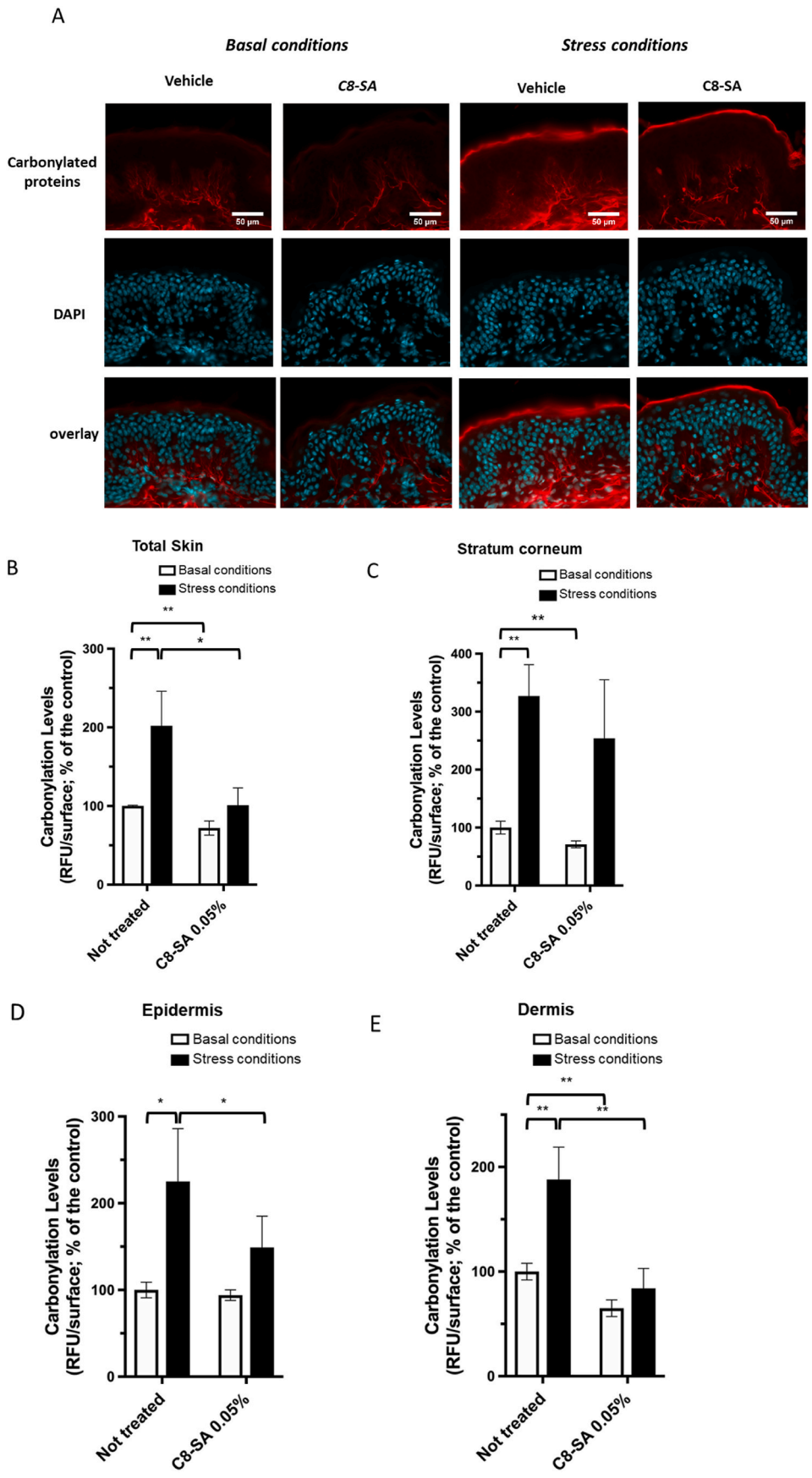


**Fig. 2. C8-SA protects human skin from pollution (PM + UVA)-induced oxidative damage**. C8-SA was applied on skin explant surface and left in contact for 2 days prior to apply particulate matter (PM) and UV-A exposure. Protein carbonylation was labeled on skin cryosections of 5 µm of thickness by using a specific fluorophore. The carbonylation levels were visualized (in red, representative images; A) and quantified by image analysis on the whole skin (B) and on the single anatomical compartments (*Stratum corneum*, C; epidermis, D; dermis, E). Carbonylation levels (RFU/surface) were expressed as a percentage of vehicle (Not treated) and reported as histograms of the mean ± SD (standard deviation). Comparative statistical analyses were obtained by binary *t*-test comparisons. $^{*}p < 0.05$, $^{**}p < 0.01$. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

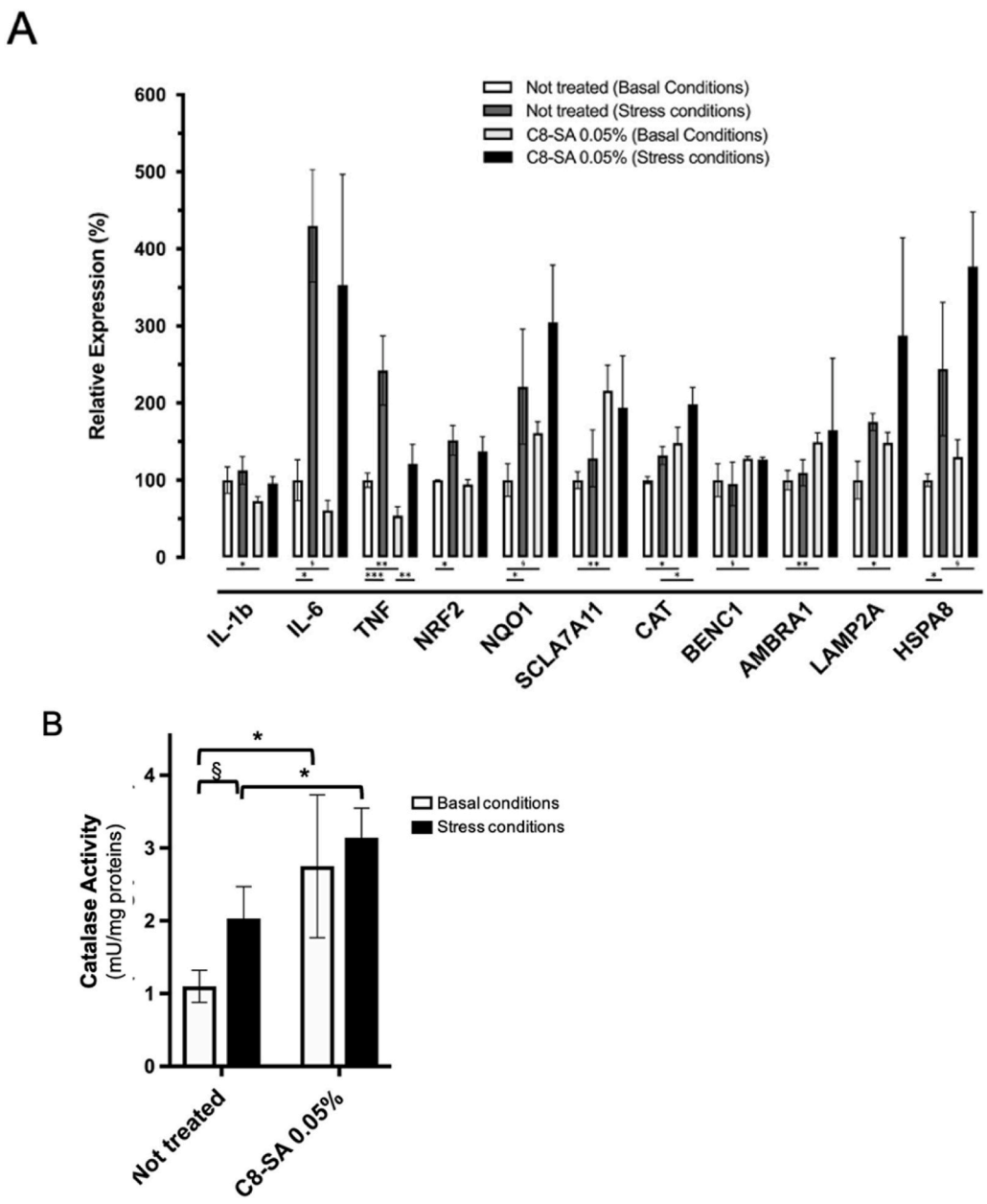


**Fig. 3. C8-SA attenuates inflammation by stimulating NRF2 regulated antioxidant response, CMA and catalase activity.** The mRNA e*xpressio*n level of different genes was measured by fluorescent real-time quantitative PCR from skin explants treated or not with C8-SA, in presence or absence of stress (PM + UVA). The mean values of relative expression ± SD are reported as histograms (A). The enzymatic activity (mU/mg proteins) of Catalase was measured by colorimetric assay and reported as average values ± SD (B). Comparative statistical analyses were obtained by binary *t*-test comparisons. $^{§}p < 0.10$, $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

activity in whole skin samples (Fig. 3C). Catalase enzymatic activity was induced by exposure to urban pollution (Fig. 3C). C8-SA stimulated catalase activity under basal conditions and increased it further under stress conditions (Fig. 3C). These results indicate that C8-SA stimulates NRF2 regulated cytoprotective response mechanisms.

To investigate autophagy, we tested gene expression of *AMBRA1*, *BENC1,* and the specific CMA genes *LAMP2* and *HSPA8* [22]. Under stress conditions, the gene expression of *LAMP2* and *HSPA8* was significantly induced, while expression levels of *AMBRA1* and *BENC1* were unaffected (Fig. 3D). Treatment with C8-SA under basal conditions stimulated the expression of all four genes, albeit non-significantly in the case of *HSPA8*. Under stress conditions, we observed a trend of boosted expression of all four genes upon C8-SA treatment. Therefore, C8-SA may act on protein quality control mechanisms by stimulating CMA.

### *3.4. C8-SA induces LAMP2A protein expression in human skin explants and human sensory neurons*

To confirm the gene expression data, LAMP2A protein expression was analyzed with immunofluorescence in human skin explants under the same experimental conditions as in 3.2 and 3.3. LAMP2A expression was significantly induced by exposure to urban pollution in the basal layer of the epidermis (Fig. 4A panel 1 and 3 and Fig. 4B). C8-SA stimulated LAMP2A expression under basal conditions (Fig. 4A panel 1 and 2) and further increased LAMP2A expression under stress conditions (Fig. 4A panel 3 and 4). Both effects were statistically significant (Fig. 4B). Surprisingly, LAMP2A was only slightly induced by application of $H_2O_2$ to human sensory neurons (Fig. 4C upper and lower left panels), which was not statistically significant (Fig. 4D). In the C8-SA treated samples, we observed a dose dependent increase of LAMP2A expression under basal and stress conditions; however, only 1 μM C8-SA in addition to $H_2O_2$ reached statistical significance, compared to the unstressed and vehicle treated condition (Fig. 4D). Hence, C8-SA induces CMA in human skin explants and to some extent in human sensory neurons.

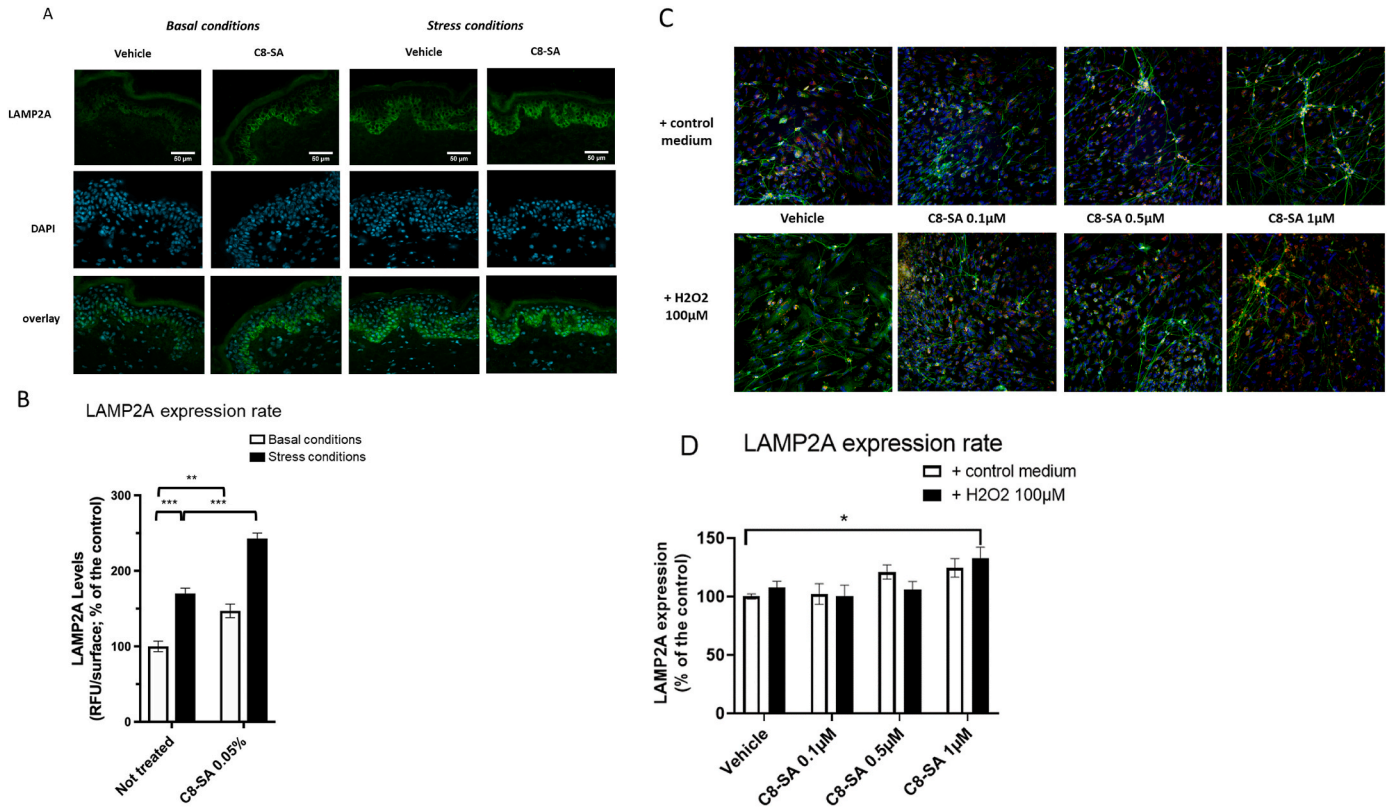


**Fig. 4. C8-SA induces LAMP2A in skin explants and sensory neurons.** (A/B) The *in-situ* visualization on skin explant cryosections of LAMP2A (in green). The specific immunofluorescence emission signal for LAMP2A is localized at the level of the basal membrane and presented alone or superposed to cellular nuclei (DAPI staining, in cyan) (A). The quantification of LAMP2A levels for each experimental group was obtained by image analysis, expressed as relative values compared to the control (vehicle) group and reported as mean ± SD (B). Comparative statistical analyses were obtained by binary *t*-test comparisons. $^{**}p < 0.01$; ***p<0.001 p-values.(C/D) C8-SA was applied to cocultures of human sensory neurons and human keratinocytes for 24 h, then exposed to $H_2O_2$ 100 µM for 1 h. LAMP2A expression was measured 24 h post incubation. Cultures were stained for β-III-tubulin (green), for LAMP2A (red), 4′,6-diamidino-2-phenylindole (DAPI, blue). Representative images (C). Images were taken with a 20x magnification. LAMP2A expression was expressed as a percentage of control (vehicle; 100%) in sensory neurons (co-staining of carbonyl groups and β-III-tubulin) and shown as means ± s.e.m. (n = 6) (D). Comparative statistical analyses were obtained by variance comparisons (ANOVA) following by Dunnett's test ordinary *t*-test comparisons. $^{*}p < 0.05$. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

## 4. Discussion

In this study, we show that a salicylate derivative (C8-SA) prevents oxidative damage on human sensory neurons and *ex vivo* skin. For skin care applications, vitamin E and other antioxidants have been traditionally used for the prevention of protein carbonylation accumulation, a hallmark of oxidative stress and skin accelerated aging [14]. Our results suggest that C8-SA action is broader than conventional antioxidants. Two main mechanisms of C8-SA activity were identified: 1) ROS detoxification by inducing the NRF2 pathway and 2) modulation of protein homeostasis by upregulating CMA. Our previous work established C8-SA as an AMPK activator [18] and when compared to the known AMPK activator salicylic acid [23], C8-SA appears to be more potent in human primary keratinocytes (unpublished data). Moreover, C8-SA induced autophagy downstream of AMPK in *C. elegans* and human primary keratinocytes [18]. Compelling evidence support the important role of autophagy in skin homeostasis [24–28]. Our present work identified CMA as an additional target of C8-SA.

Importantly, CMA is activated under oxidative stress conditions and contributes to the degradation of carbonylated proteins [15]. The CMA specific receptor LAMP2A is described as a target of NRF2 [29]. Hence, NRF2 activation by C8-SA could be associated to CMA activation. In skin explants, topical treatment of 0.05% C8-SA in a 5% hydroalcoholic solution activated the NRF2 pathway and CMA upon stress but also in basal conditions, leading to decreased protein carbonylation. These results indicate that C8-SA prevents oxidative damage in skin, but also accelerates the degradation of carbonylated proteins. The beneficial effects of C8-SA on preventing carbonylated proteins accumulation in the *SC* may be explained by the keratolytic effect of the compound [30].

The mechanism of action of C8-SA in the dermis will need further investigation. It has been discussed that oxidized proteins can be degraded by proteolytic enzymes present in the extracellular matrix or endocytosed and degraded by lysosomal proteins [9]. Further studies should address the ability of C8-SA to modulate those mechanisms.

Finally, we specifically investigated the protective effects of C8-SA on skin sensory neurons, based on previous findings that oxidative stress impairs peripheral nervous system and skin innervation in facial skin [31]. Our results show that C8-SA protects sensory neurons from protein carbonylation upon oxidative stress, leading to a neuroprotective effect. We identified the effective dose of 1 µM in 2D and 3D sensory neurons/keratinocytes co-cultures, which is compatible to the use of C8-SA in topical cosmetic formulations.

## Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.freeradbiomed.2022.01.029.

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