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Ubiquitination as a key regulatory mechanism for O_3-induced cutaneous redox inflammasome activation

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ABSTRACT

NLRP1 is one of the major inflammasomes modulating the cutaneous inflammatory responses and therefore linked to a variety of cutaneous conditions. Although NLRP1 has been the first inflammasome to be discovered, only in the past years a significant progress was achieved in understanding the molecular mechanism and the stimuli behind its activation. In the past decades a crescent number of studies have highlighted the role of air pollutants as Particulate Matter (PM), Cigarette Smoke (CS) and Ozone (O_3) as trigger stimuli for inflammasomes activation, especially via Reactive Oxygen Species (ROS) mediators. However, whether NLRP1 can be modulated by air pollutants via oxidative stress and the mechanism behind its activation is still poorly understood. Here we report for the first time that O_3, one of the most toxic pollutants, activates the NLRP1 inflammasome in human keratinocytes via oxidative stress mediators as hydrogen peroxide (H_2O_2) and 4-hydroxy-nonenal (4HNE). Our data suggest that NLRP1 represents a target protein for 4HNE adduction that possibly leads to its proteasomal degradation and activation via the possible involvement of E3 ubiquitin ligase UBR2. Of note, Catalase (Cat) treatment prevented inflammasome assemble and inflammatory cytokines release as well as NLRP1 ubiquitination in human keratinocytes upon O_3 exposure.

The present work is a mechanistic study that follows our previous work where we have showed the ability of O_3 to induce cutaneous inflammasome activation in humans exposed to this pollutant. In conclusion, our results suggest that O_3 triggers the cutaneous NLRP1 inflammasome activation by ubiquitination and redox mechanism.

1. Introduction

The Immune system is the first host defense against harmful stimuli involved in maintaining the normal tissue homeostasis, protecting us from infections and diseases and providing an immediate response. The innate immune system relies on pattern recognition receptors (PRRs) that recognize components deriving from invading pathogen called pathogen-associated molecular patterns (PAMPs), and Danger-associated molecular patterns (DAMPs), normally released from host cells during cell damage or death [1]. Activation of PRRs leads to downstream signaling cascades, resulting in the activation of pro-inflammatory pathways and the production of type 1 Interferons (INF-ɒ, INF-β) as well as pro-inflammatory cytokines. In 2002, *Martinon et al*, identified for the first time a new cytosol multiprotein oligomers complex of the innate immune system, called Inflammasome [2]. Inflammasomes are normally expressed in immune cells such as monocytes, macrophages, dendritic cells, neutrophils as well as other cells type, such as keratinocytes, and they are involved in the inflammatory response against several stimuli [3]. Aberrant activation of inflammasomes has been correlated to inflammatory reactions and to the exacerbation of different inflammatory pathologies including skin diseases [4]. Inflammasomes can be divided in several families among which the nucleotide-binding oligomerization domain-like receptors (NOD-like receptors, NLRs) family is one of the main studied and characterized. So far 22 NLRs have been identified in humans and, of those, NLRP3, NLRC4 and NLRP1, represent the most studied inflammasomes. Once

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activated, Inflammasomes subsequently recruit the protein adaptor ASC for scaffold assemble, caspase 1 activation and IL-1β/IL-18 release, based on the interaction between CARD-CARD or PYD-PYD domains of the different inflammasomes co-receptors [3,5–11]. In the past decades a crescent number of studies have highlighted the role of air pollutants as Particulate Matter (PM), Cigarette Smoke (CS), Ozone (O_3) and also UV radiation as trigger stimuli able to modulate inflammasomes activation, promoting the onset of several skin conditions [12–15]. Indeed, the continuous exposure of the cutaneous tissue to the noxious environmental stimuli eventually leads to a positive feedback between oxidative stress and inflammatory reactions (OxInflammation), with the exacerbation of many cutaneous pathologies [16]. In particular, reactive oxygen species (ROS) associated to pollutants exposure, have been identified as crucial figures in triggering the activation of certain types of inflammasomes [17–20]. NLRP1 that is the most present inflammasome within the human skin has been reported to exacerbate the inflammatory status displayed in many skin pathologies such as vitiligo, atopic dermatitis, psoriasis, acne, carcinogenesis, melanoma [4,21–23] and also be particularly susceptible to environmental stimuli such as UV radiation. However, despite other well-known and characterized inflammasomes, very few is known about the mechanism behind NLRP1 activation in humans. Recent evidences have demonstrated that NLRP1 inflammasome activation relies on the proteasomal degradation of its N-terminus via ubiquitination, a mechanism called "functional degradation" [24,25], that can occur in response to determined stimuli among which the *Bacillus Anthracis* Lethal factor toxin (LF) is the most recognized [26–33]. Regarding humans, a similar mechanism can be ascribed to the inhibition of specific dipeptidyl peptidases, such as DPP8 and 9, that usually stabilize NLRP1 [34–36]. Whether the proteasome could be involved in the pollutants induced NLRP1 activation in human skin is still an unexplored field. Our group previously demonstrated that O_3 can activate the cutaneous NLRP1 inflammasome via a redox regulated mechanism, mainly mediated by O_3 -oxidative stress mediators as Hydrogen peroxide (H_2O_2) and 4-hydroxynonenal (4HNE) [37]. Indeed, even though O_3 is not able to penetrate the skin, it can oxidize components of the cell membrane, such as lipids and proteins, resulting in the production of H_2O_2 and reactive aldehydes (4HNE), that are able to propagate the oxidative damage throughout the cutaneous tissue eventually activating the cutaneous inflammatory response [38]. Skin exposure to high levels of O_3 has been associated with the depletion of the levels of antioxidants, activation of pro-inflammatory pathways, modulation of factors involved in wound healing process, as well as skin aging and cancer [39–41]. Considering that O_3 exposure leads to post translational modifications (PTMs) of its target proteins, as phosphorylation, ubiquitination, oxidation etc., eventually inducing protein misfolding and their degradation, *we wondered whether the cutaneous NLRP1 activation induced by O_3 exposure could be mediated by the ubiquitin-proteasome system via an oxidative related mechanism.* Our data show that inflammasome NLRP1 is in great part involved in the cutaneous inflammatory status promoted by O_3 since human keratinocytes silenced for NLRP1 displayed decreased NLRP1-ASC scaffold assemble as well as Caspase 1 and IL-1β release. Moreover, we found that Catalase (Cat) abrogated NLRP1 inflammasome activation as well as its ubiquitination in response to O_3, by preventing the production of oxidative stress mediators induced by the pollutant. Interesting the N-end rule E3 ubiquitin ligase UBR2 seemed to be involved in NLRP1 proteasomal degradation upon O_3 exposure. Taken together our results show that O_3-induced NLRP1 inflammasome activation is a redox regulated process that involves the ubiquitin-proteasome system most likely following the N-end rule pathway.

2. Materials and methods

2.1. O_3 generator

O_3 was generated via electrical corona arc discharge from O_2 and combined with ambient air to flow into a plexiglass box (ECO3 model CUV-01, Model 306 O_3 Calibration Source, 2B Technologies, O_3 Solution, Torino, Italy), as previously described [42]. The concentration of O_3 in the chamber was adjusted to 0.4 ppm and continuously monitored by an O_3 detector.

2.2. Human keratinocytes treatments and O_3 exposure

HaCaT cells were cultured in high glucose Dulbecco's Modified Eagle's Medium (Corning, Glendale, AZ, USA) supplemented with 10% FBS (Sigma-Aldrich, St. Louis, MO, USA), 100 U/ml penicillin and 100 μg/ml streptomycin (Gibco, ThermoFisher Scientific, Waltham, MA, USA). Human primary keratinocytes from foreskin were purchased from Lonza (Lonza, Walkersville, MD, USA) and cultured in Epilife medium (cat. MEPI500CA Gibco, ThermoFisher Scientific, Waltham, MA, USA) supplemented with Human Keratinocytes Growth Supplements (HKGS) (cat. S0015 Gibco, ThermoFisher Scientific, Waltham, MA, USA, cat S0015)(ref. Mechanisms of Ageing and Development, 2018, 172, pp. 86–95). Cell cultures were performed at 37°C in 5% CO_2 and 95% air. For the experiments, keratinocytes were grown in 6 cm^2 petri dishes (Corning, Glendale, AZ, USA) at a density of 1.5×10^6 cells in 3 ml of media. HaCaT cells were pre-treated either with 20 or 50 μM of 4HNE (CAS 75899-68-2 Chem Cruz, Santa Cruz Biotechnology Inc., Dallas, TX, USA), 20 or 50 μM of H_2O_2 (CAS 7722-84-1 Sigma-Aldrich, Burlington, VT, USA) and placed in the incubator at 37°C and 5% CO2. After 1 h of pre-treatment, the untreated cells petri dishes were placed in the plexiglass box connected to the O_3 generator, where they were exposed for 1 h to 0.4 ppm of O_3. For the catalase experiments, HaCaT cells were pre-treated with 1000 U/ml of catalase (Cat. C4963, Sigma-Aldrich, Burlington, VT, USA) for 2 h and then exposed to 0.4 ppm of O_3 for 1 h. MG-132 (Cat. 474787, Merck Millipore, Burlington, MA, USA) was used as proteasome inhibitor, at a concentration of 20 μM for 2 h. The Caspase 1 inhibitor Z-YVAD- fmk (Cat. 1012, BioVision inc. Milpitas, CA, USA) was used at a concentration of 2 μM for 1 h. After the different treatments/exposure, RNA, proteins, or media samples were collected at the indicated timepoints for the subsequent analysis.

2.3. EpiDerm 3D skin models O_3 exposure and treatments

EpiDerm skin model samples were obtained from MatTek corporation (EPI-200, EpiDerm) as previously described [43]. Briefly, under sterile conditions and using sterile forceps, 24 inserts containing tissues were transferred into 6-well plates pre-filled with 1 ml of MatTek Assay medium, according to the manufacturer's instructions. The plates were placed in the incubator overnight (5% CO2, 37 °C) for recovery. After the overnight recovery, 1 ml of fresh new media was added to each well of the 24 wells plate containing the EpiDerm 3D skin models and to the 6 wells plates containing the Human skin biopsies. The tissues were exposed to 0.4ppm of O_3 for 4 h into the plexiglass box connected to the O_3 generator. For the catalase experiments, RHE were pre-treated with 2000 U/ml of Catalase (Cat. C4963, Sigma-Aldrich, Burlington, VT, USA) for 2 h and then exposed to 0.4 ppm of O_3 for 4 h. At the end of O_3 exposure, the tissues were placed into the incubator and protein, RNA, histological samples, and media were collected at the indicated timepoints for the subsequent analysis.

2.4. Human keratinocytes transfection for NLRP1

HaCaT Cells and human 1° KC were transfected with siRNA against human *NLRP1 by using the NLRP1* (NLRP1) Human siRNA Oligo Duplex, Locus ID 22861, sequence SR307826B (Cat. SR307826, Origene™ Technologies, Rockville, MD, USA) or the negative control Scrambled (Scrbl) siRNA using the Lipofectamine RNAiMAX transfection reagent (Cat. 13778030, Invitrogen, ThermoFisher Scientific, Waltham, MA, USA), according to the datasheet. Briefly, HaCaT cells and human 1° KC were seeded in 12 wells plates (1.2×10^5/well) and in 1 ml of EpiLife

medium containing 100 ul of transfection mix, consisting of RNAiMAX transfection reagent (6 μl/well) and scrambled or *NLRP1* siRNA (final concentration 10 nM). After 24 and 48 h cells were collected for subsequent NLRP1 gene expression and protein expression analysis. Upon 24 h from transfection, cells were exposed to O_3 0.4 ppm for 1 h and collected at the indicated timepoints for immunocytochemistry, western blotting, and ELISA assays.

2.5. Immunocytochemistry

HaCaT cells or 1° KC were grown on 10 mm^2 coverslips at a density of 70'000 cells/coverslip in 24 wells plates. Cells were fixed in 4% paraformaldehyde (PFA) in PBS for 10 min at RT, permeabilized with 0.25% of Triton X-100 in PBS for 10 min and then blocked in PBS containing 1% BSA at room temperature for 30 min as previously described [44]. Coverslips were incubated with primary antibody **ASC** (Cat. NBP1-78977 Novus Biological, Littleton, CO, USA) 1:100, **NLRP1** (sc-166368 Santa Cruz Biotechnology Inc., Dallas, TX, USA) 1:50, **4HNE** (ab46545, Abcam, Cambridge, UK) 1:400, **DPP9** (ab42080, Abcam, Cambridge, UK)1:400, **UBR2** (18852-1-AP, Proteintech Group Inc., Rosemont, IL, USA) 1: 150 in 0.25% BSA/PBS overnight at 4°C. The Alexa Fluor Fluorochrome-conjugated secondary antibodies (A11004 Alexa Fluor 568, A11008 Alexa Fluor 488 Invitrogen, ThermoFisher Scientific, USA) were used at a dilution of 1:1000 in PBS-BSA 0.25% for 1 h at RT. Nuclei were stained with DAPI (D1306 Invitrogen ThermoFisher Scientific, Waltham, MA, USA) after removal of secondary antibody. Coverslips were mounted onto glass slides using PermaFluor Aqueous Mounting Medium (TA-006-FM, ThermoFisher Scientific, Waltham, MA, USA), and examined using a Zeiss Z1 AxioObserver LSM10 confocal microscope equipped at 40x and 60x magnification. Images were quantified using ImageJ software.

2.6. 3D skin models collection and paraffin embedding

3D Epiderm tissues were collected for each timepoints by rinsing them in fresh PBS for 3 times. The samples RNA extraction were frozen in nitrogen for few seconds and then stored at -80°C for future extractions, whereas the histology samples were placed in cassettes and fixed in formalin for 48 h at 4°C. After 2 days, the samples were dehydrated in a series of alchohol gradients and after xylene, they were left in paraffin overnight. The day after the tissues were embedded in paraffin and the paraffin blocks were stored at 4°C. 4 μm paraffin sections of human skin biopsies were cut using a microtome (Leica Microsystem, Wetzlar, DE) and left drying for 24 h at RT before proceeding with the histological analysis.

2.7. Protein extraction and western blotting

Cell lysates were extracted in ice-cold buffer containing 50 mM Tris (pH 7.5), 150 mM NaCl, 10% glycerol, 1% Nonidet P-40, 1 mM EDTA, 0.1% SDS, 5 mM N-ethylmaleamide (Sigma-Aldrich, Burlington, VT, USA) and protease and phosphatase inhibitor cocktails (Sigma-Aldrich, Burlington, VT, USA). Lysates were cleared by centrifugation (12700 rpm) for 30 min at 4°C, and protein concentration was measured by Bradford method (Bio-Rad Laboratories, Inc., Hercules, CA, USA) (Ref. Exp Dermatol. 2015 Jun; 24(6):449-54. https://doi.org/10.1111/exd.12696). Equivalent amounts of proteins were loaded onto 4–12% polyacrylamide SDS gels and separated by molecular size. Gels were electroblotted onto nitrocellulose membranes and then were blocked for 1 h in Tris-buffered saline, pH 7.5, containing 0.5% Tween 20 and 5% milk. Membranes were incubated overnight at 4°C with primary antibodies **ASC** (Cat. NBP1-78977 NovusBio, USA) 1:1000, **Caspase 1** (2225S Cell Signaling Technology, Danvers, MA, USA) 1:1000, **NLRP1** (AB-84361, SIC, Rome, Italy) 1:1000, **Ubiquitin** (ab7780, Abcam, USA) 1:2000, **DPP9** (ab42080, Abcam, USA) 1:1000, **4HNE** (ab46545, Abcam, USA), **UBR2 (**18852-1-AP Proteintech, USA**)** in TBS-T with 1% non-fat milk (Bio-Rad Laboratories, Inc., USA). The membranes were then incubated with horseradish peroxidase conjugated secondary antibodies (Immunological Sciences, Rome, Italy) 1:5'000 in TBS-T with 1% non-fat milk for 90 min at RT. **β-actin** (A3854 Sigma-Aldrich, Burlington, VT, USA) was used as loading control at 1:50'000 dilution in TBS-T with 1% of non-fat milk. Images of the bands were digitized, and densitometry analysis was performed using Image J software.

2.8. Immunoprecipitation

HaCaT cells were seeded in 10 cm petri dishes and once reached the 70% of confluence, cells were treated with catalase 1000 U/ml for 2 h and exposed to O_3 0.4 ppm for 1 h. Samples were collected right after O_3 exposure by washing them twice in PBS and centrifuged 250 *g* for 5 min. Proteins were extracted as previously described in general methods and the concentration was measured by Bradford assy. The immunoprecipitation assay for NLRP1 was performed using the Dynabeads^{TM} Protein G (Cat. 10003D, Invitrogen, ThermoFisher Scientific, USA) as following: 4 ug of **NLRP1** antibody (sc-166368 Santa Cruz Biotechnology Inc., USA) was diluted in 200 μL PBS-T and incubated with 50 ul of Dynabeads Protein G for 3.5 h at RT using a rotator. After washing the antibody-protein G complex with PBS-T 3 times, the antibody was crosslinked to the beads by using the crosslinker Disuccinimidyl Suberate, DSS (CAS 68528-80-3 Alfa Aesar, Thermo Fisher scientific, USA) at a concentration of 5 mM. The beads were incubated with 250 ul of a 5 mM DSS Conjugation Buffer solution (20 mM Sodium Phosphate, 0.15 M NaCl (pH 7–9) for 1 h at RT on the rotator. After quenching the crosslinking reaction by adding 12.5 μL of Quenching Buffer (1 M Tris HCl (pH 7.5) for 30 min at RT, the cross- linked Dynabeads were washed three times with 200 μL PBST and then incubated with 250 ug of sample proteins overnight at 4°C using a rotator. The magnetic bead-Ab- Ag complex was washed in PBS-t for 3 times and then 30 ul of Laemmli buffer 1x containing 10% beta-mercaptoethanol were added. The samples were denatured at 95 °C for 5 min and Western Blot was performed as previously described. Membranes for immunoprecipitated samples were incubated for **4HNE** (ab46545, Abcam, USA), or **Ubiquitin** (ab7780, Abcam, USA) and input samples for **NLRP1** (sc-166368 Santa Cruz Biotechnology Inc., USA).

2.9. Amplex Red assay

H_2O_2 production rate was evaluated using the Amplex Red–horseradish peroxidase (HRP) method, as previously described [45] in media of HaCaT cells o, 1 and 3 h after 1 h exposure to 0.4 ppm of O_3. Briefly, 10 μl of media for each sample were added to the reaction mixture where and, in presence of horseradish peroxidase (HRP), H_2O_2 reacted with the Amplex® Red reagent (A12222, ThermoFisher Scientific, USA) resulting in the formation of the red fluorescent resorufin product. The content of H_2O_2 was determined through a 4P-logistic regression curve by comparing the fluorescence at Ex/Em 531/595 nm with that of H_2O_2 standard curve. Readings were performed by the microplate reader Victor3 (PerkinElmer, Inc., Waltham, MA, USA). The calibration curve was assessed using H_2O_2 solutions as a standard, and the H_2O_2 production was expressed in μM.

2.10. RNA extraction and quantitative real time PCR

HaCaT cells were seeded in 6 wells plates at a density of 0.8×10^6 cells/wells. After 24 h cells were exposed to O_3 0.4 ppm for 1 h and then collected at the indicated timepoints. RNA was extracted by using the Ribospin (304-150, GeneAll Biotechnology CO., Seoul, Korea) and Riboclear plus (313-150, GeneAll Biotechnology CO., Korea) kits, according to the manufacture's protocol. For 3D skin models, total RNA was extracted using the Aurum Total RNA Mini Kit with DNase digestion (Cat. 7326820, Bio-Rad Laboratories, Inc., Hercules, CA, USA), according to the manufacturer's recommended procedure. Briefly, after adding

700 μl of Lysis buffer provided by the Kit, the tissues were homogenized using the Precellys tissue homogenizer using 9 cycles of 30 s with a 30 s break at 8000 rpm at 4°C. RNA concentration was measured using Shimadzu BioSpec-nano spectrophotometer (Shimadzu Biotech, Duisburg, DE). cDNA was generated from 1 μg of total RNA, using the IScript reverse Transcription Supermix for RT-qPCR kit (Cat. 1708841, Bio-Rad, USA). The mRNA levels of **NLRP1, ASC and IL-1β** genes were analyzed by quantitative Realtime PCR by using the Sso Advanced Universal SYBR® Green Supermix (Cat. 1725271 Bio-Rad Laboratories, Inc., USA) on a CFX Connect Real-Time PCR System (Bio-Rad Laboratories, Inc., USA), according to the manufacturer's protocol. **β-actin** was employed as the reference gene and the samples were compared using the relative cycle threshold (CT). After normalization, the fold change was determined using the 2^- ΔΔCT method, as previously described [46].

The primers used are listed in the table below:

Gene	Forward sequence	Reverse sequence
NLRP1	5'-CCCTCTTAACTCCGGGACA-3'	5'-GAGTGCGCTTTATTGGCGAG-3'
ASC	5'-ATGCGCTGGAGAACCTGA-3'	5'-TCTCCAGGTAGAAGCTGACCA-3'
IL-1β	5'-CACGATGCACCTGTACGATCA-3'	5'-GTTGCTCCATATCCTGTCCCT-3'
β Actin	5'-ATTGCCGACAGGATGCAGA-3'	5'-AGTACTTGCGCTCAGGAGGA-3'

2.11. Detection of caspase 1 and IL-1β using ELISA assays

Caspase 1 levels were measured both in HaCaT cells exposed to O_3 and pre-treated with Z-YVAD-fmk and in 3D skin models exposed to O_3 for 4 h and treated with Catalase. Briefly, Media was collected at the indicated timepoints, and Caspase 1 levels were measured by Human Caspase 1/ICE Quantikine ELISA Kit (cat. DCA100, R&D System, Inc., Minneapolis, MN, USA), according to the manufacture's protocol. IL-1β levels were measured in media of HaCaT cells, human 1° KC and RHE tissues by using the IL-1β ELISA kit (Cat. DY201-05, Novus Biologicals, USA), according to the manufacturer's instructions. Caspase 1 and IL-1β levels were adjusted for media (pg/ml). Gen5 software (BioTek, Agilent, Santa Clara, CA, USA) was used for the detection.

2.12. Statistical analysis

GraphPad Prism 6 was employed to perform all the statistical analyses. For each of the variables tested, analysis of variance (1-way or 2-way ANOVA), followed by Tukey's post-hoc test, was used. Statistical significance was considered at $p<0.05$. Data are expressed as mean ± SD of duplicate determinations obtained in three independent experiments.

3. Results

3.1. NLRP1 mediates the inflammatory response in human keratinocytes via caspase 1

Our previous work demonstrated that O_3 could trigger inflammasome NLRP1 activation in several skin models including human skin explants, promoting ASC recruitment, Caspase 1 activation and eventually leading to increased inflammatory cytokines IL-1β and IL-18 release [37]. To prove that NLRP1 might represent one of the main sensors involved in the O_3-induced cutaneous inflammatory response and to better study the molecular mechanism behind its activation, we silenced human keratinocytes for NLRP1 for 24 or 48 h. The gene expression analysis revealed a 60% of NLRP1 silencing compared to Scrbl cells at T24h and a less but still significant silencing 48 h after (T48), accompanied to a significant decrease in NLRP1 protein expression levels at the selected timepoints (Fig. S1a). We then exposed NLRP1 silenced cells to O_3 to evaluate the inflammatory response against the pollutant. Silenced keratinocytes displayed lower expression levels of both inflammasome components NLRP1 and ASC upon O_3 exposure and a significant resistance in NLRP1-ASC colocalization compared to exposed control cells at all the timepoints investigated, as showed in the double immunofluorescence staining (Fig. 1a and S1b). As consequence of the non-assembly of NLRP1 and ASC, we found decreased levels of active p20 Caspase 1 upon O_3 exposure in HaCaT cells silenced for NLRP1 (Fig. 1b). In addition, HaCaT cells released less mature IL-1β 12 and 24 h after O_3 insult (T12, T24) when silenced for NLRP1 (Fig. 1c). To validate the use of HaCaT cells as a good model to study the mechanism behind NLRP1 involvement in O_3-induced skin inflammatory damage, we decided to adopt human primary keratinocytes (1° KC). As depicted in Figs. S1c–f, we found a similar response upon NLRP1 silencing and O_3 exposure as 1° KC lacking NLRP1 did show a significant decrease in NLRP1-ASC assemble as well as IL-1β release at the selected timepoints. Since Caspase 1 is usually recruited upon inflammasomes activation to promote maturation of IL-1β, we inhibited Caspase 1 to investigate its involvement in O_3- induced cutaneous inflammatory damage. Inhibition of Caspase 1 through the inhibitor Z-YVAD-fmk led to a significant decrease in active p20 Caspase 1 protein expression levels (Fig. 1d) as well as a reduction in mRNA expression levels of IL-1β in keratinocytes exposed to O_3 at all timepoints (Fig. 1e). In addition, we found that IL-1β release upon O_3 exposure was significantly affected when the cells were treated with Z-YVAD-fmk 12 and 24 h after O_3 insult (T12, T24) (Fig. 1f).

3.2. Catalase treatment inhibits the cutaneous O_3-induced inflammasome activation

The deleterious effect of O_3 is mainly due to its ability to promote the formation of oxidative stress mediators such as H_2O_2 and 4HNE able to perpetuate the oxidative and inflammatory damage within the cutaneous tissue. Previously we showed that inflammasome NLRP1 can be triggered by O_3 via a redox-regulated mechanism that involves H_2O_2 and 4HNE [37]. To prove this, we treated HaCaT cells with Catalase (Cat), to prevent the cutaneous oxidative stress response induced by O_3 exposure. First, we measured the levels of H_2O_2 within HaCaT cells in response to O_3 to validate our model. As shown in Fig. 2a, HaCaT cells displayed increased levels of H_2O_2, and Cat pre-treatment could in part prevent the damage. We then measured the expression levels of the main inflammasomes components in O_3 exposed HaCaT cells pre-treated with Cat. We found that Cat decreased transcription levels of NLRP1 and ASC in response to O_3 insult, respectively 0 and 3 h post-O_3 exposure (T0, T3) (Fig. 2b). In addition, Cat prevented NLRP1 and ASC increased protein expression levels as well as their colocalization at all timepoints, as shown in the immunofluorescence staining (Fig. 2c and S2a). We also found reduced levels of Caspase 1 released in the media 6,12 and 24 h post-exposure (T6, T12, T24) (Fig. 2d). In addition, HaCaT cells treated with Cat displayed reduced IL-1β transcription levels, a final mediator of inflammasome activation, already 0 and 3 h post-O_3 exposure (T0, T3) and more evidently at T6 (Fig. 2e), as well as reduced IL-1β release in media, at all timepoints (Fig. 2f). To confirm the results obtained in HaCaT cells, we decided to adopt a more complex skin model as the 3D Reconstructed Human Epidermis (RHE). RHE were treated with Cat for 2 h, exposed to 0.4 ppm O_3 for 4 h and subsequently collected 6 h after the end of O_3 exposure (T6) to analyze the expression levels of inflammasomes components. We found that Cat pre-treatment significantly reduced the increased mRNA expression levels of NLRP1, ASC and IL-1β (Fig. S2b) after O_3 exposure. Moreover, whereas O_3 exposure induced increased released levels of Caspase 1 and IL-1β in media of RHE samples, Cat pre-treatment completely abrogated the inflammasome activation by inhibiting the release of both markers at the selected timepoint (Fig. S2c).

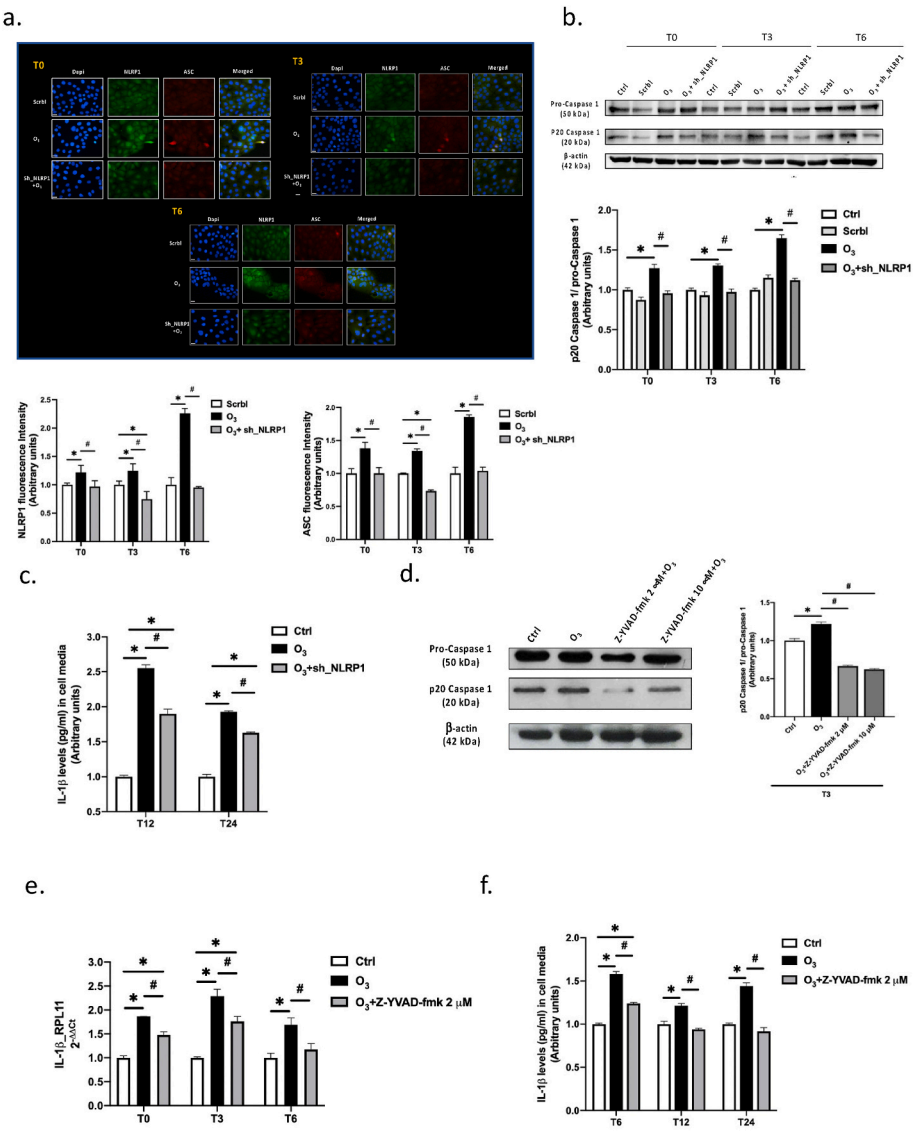

Fig. 1. O_3 activated NLRP1 inflammasome via Caspase I (a) Immunofluorescence staining for NLRP1 (green) and ASC (red) in HaCaT cells silenced for NLRP1 10 nM for 24 h and exposed to O_3. The blue staining (DAPI) represents nuclei. Images were taken at 40 × magnification (scale bar = 40 μm) and the fluorescent signal was quantified using ImageJ software. **(b)** Protein expression levels and relative quantification graph of p20 Caspase 1 over pro-Caspase 1 in HaCaT cells silenced for NLRP1 10 nM and then exposed to O_3. **(c)** IL-1β released levels in media of HaCaT cells silenced for NLRP1 10 nM for 24 h and then exposed to O_3. **(d)** Protein expression levels of p20 Caspase 1 over pro-Caspase 1 in HaCaT cells pre-treated with Caspase 1 inhibitor Z-YVAD-fmk 2 and 10 μM for 1 h and then exposed to O_3. IL-1β mRNA expression levels (**e**) and released levels of IL-1β (**f**) in HaCaT cells pre-treated with Caspase 1 inhibitor Z-YVAD-fmk 2 μM for 1 h and then exposed to O_3. For all the experiments HaCaT cells were exposed to 0.4 ppm O_3 for 1 h and then collected at the indicated timepoints post 1-h O_3 exposure. For all western blotting the protein expression level was quantified using ImageJ software and β-actin was used as internal control. Data are the results of the averages of at least three different experiments, $^{*}p < 0.05$ and $^{\#}p < 0.05$ by 2-way ANOVA followed by Tukey's post-hoc comparison test. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

3.3. NLRP1 is a target protein of 4HNE after O_3 exposure

4HNE is a very reactive aldehyde able to form adducts with its target proteins, compromising their function and functionality. Therefore, we wondered whether NLRP1 could represent a target protein for 4HNE, marking it for degradation. As shown in Fig. 3a, HaCaT cells exposed to crescent doses of O_3 displayed increased 4HNE protein adducts expression levels in a dose- dependent manner. O_3 and its mediator H_2O_2 could induce an increase in 4HNE-NLRP1 protein adducts expression levels (Fig. 3b and S3a), indicating that NLRP1 is actually a target protein of 4HNE via an oxidative stress related mechanism. The 4HNE-NLRP1 protein adducts formation was ulteriorly confirmed by the marked colocalization occurred between NLRP1 and 4HNE upon O_3 insult at all timepoints, as depicted in the immunofluorescence staining (Fig. 3c and S3b). 4HNE protein adducts can be recognized and marked for degradation via different mechanisms, including the ubiquitin proteasome pathway [47]. Recently several studies showed that when activated, NLRP1 N-terminal fragment is marked for ubiquitination and degraded via the proteasome, leading to its activation. Since O_3 exposure is known to lead to protein ubiquitination, we wondered whether O_3 could trigger the NLRP1 inflammasome activation by marking it for ubiquitination and therefore targeting it for proteasomal degradation. First, we confirmed the ability of O_3 in induce higher levels of ubiquitinated proteins in HaCaT cells 0, 3 and 6 h post- O_3 exposure (T0, T3, T6) (Fig. 3d and S3c). Of note, we found that also O_3 oxidative stress mediators H_2O_2 and 4HNE happened to be involved in the protein ubiquitination process (Figs. S3d–e). As shown in Fig. 3e, NLRP1 turned out to be marked for ubiquitination right after O_3 exposure and this process was prevented by Cat pre-treatment, confirming that NLRP1 ubiquitination occurred upon O_3 exposure via oxidative stress reactions.

3.4. Inflammasome activation induced by O_3 is modulated via the ubiquitin-proteasome pathway in a redox dependent manner

To prove that inflammasome activation upon O_3 exposure occur via the ubiquitin proteasome system, we decided to treat HaCaT cells with proteasome inhibitor MG-132 and investigate the NLRP1 inflammasome activation. As shown in Fig. 4a MG-132 treatment reduced NLRP1 protein expression levels especially 0- and 3-h post-exposure (T0, T3). Moreover, we found decreased colocalization levels between NLRP1 and ASC upon MG-132 treatment and O_3 exposure compared to O_3 exposed cells without MG-132 pre-treatment (Fig. 4b and 4Sa). The lack of NLRP1 and ASC assemble by proteasome inhibition was ulteriorly confirmed by the abolished Caspase 1 activation found in HaCaT cells treated with MG-132 and exposed to O_3 (Fig. 4c). Moreover MG-132 treatment seemed not to affect Caspase 1 activation compared to

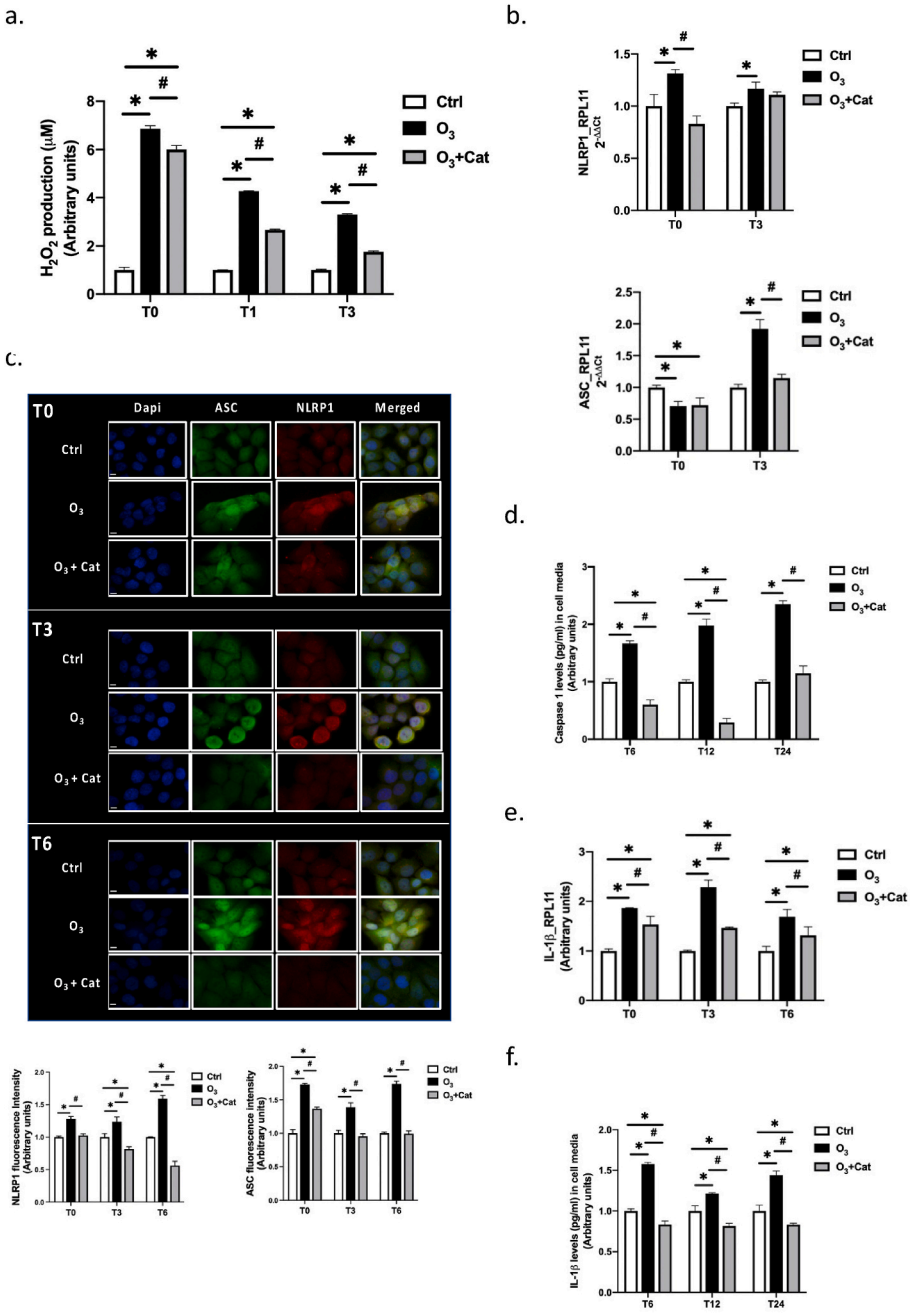

Fig. 2. NLRP1 inflammasome activation by H_2O_2
(a) H_2O_2 levels production in media of HaCaT cells pre-treated with 1000 U/ml of Catalase (Cat) for 2 h and then exposed to O_3 assessed by AmplexRed assay. **(b)** mRNA expression levels of NLRP1 (upper panel) and ASC (bottom panel) in HaCaT cells pre-treated with 1000 U/ml of Cat for 2 h and then exposed to O_3. **(c)** Double Immunofluorescence staining for NLRP1 (red) and ASC (green) in HaCaT cells pre-treated with Cat 1000 U/ml for 2 h and then exposed to O_3. Blue staining (DAPI) represents nuclei. Images were taken at 100 × magnification (scale bar = 2.5 μm); the fluorescent levels were quantified using ImageJ software. **(d)** Caspase 1 released levels in media of HaCaT cells pre-treated with Cat 1000 U/ml for 2 h and then exposed to O_3. **(e)** IL-1β mRNA expression levels in HaCaT cells pre-treated with Cat 1000 U/ml for 2 h and then exposed to O_3. **(f)** Released levels of IL-1β in media of HaCaT cells pre-treated with Cat 1000 U/ml for 2 h and then exposed to O_3. For all the experiments HaCaT cells were exposed to 0.4 ppm O_3 for 1 h. Samples were collected at the indicated timepoints post 1-h O_3 exposure. For all western blotting the protein expression level was quantified using ImageJ software and β-actin was used as internal control. Data are the results of the averages of at least three different experiments, $^{*}p < 0.05$ and $^{\#}p < 0.05$ by 2-way ANOVA followed by Tukey's post-hoc comparison test. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

untreated cells (Fig. 4Sb), suggesting that the selected proteasome inhibitor and the indicated experimental conditions were not able to modulate the activation of other inflammasomes. Human NLRP1 inflammasome activation has been correlated to the inhibition of DPP9, a dipeptidyl dipeptidase protein able to stabilize NLRP1 and prevent its degradation via the proteasome [34,48]. Thus, we evaluated whether DPP9 could be involved in O_3 induced NLRP1 inflammasome activation. As shown in Fig. 4Sc, O_3 slight modulated DPP9 protein expression levels in HaCaT cells as confirmed also in the double immunofluorescence staining (Fig. 4Sd-e). Indeed, even though DPP9 seemed to be initially modulated by O_3 exposure, we did not detect any significant changes in DPP9 expression levels at later timepoints (T3 and T6). Of note, although MG-132 treatment did not affect DPP9 expression, inhibition of the proteasome prevented NLRP1 overexpression upon O_3 exposure, indicating the involvement of the proteasome in its activation. UBR2 has been demonstrated to be a major E3 ubiquitin ligase targeting NLRP1 N-terminus for proteasomal degradation via the N-end rule pathway. Interesting we found that O_3 exposure modulated UBR2 protein expression levels and that proteasome inhibition could prevent UBR2 overexpression upon O_3 exposure (Fig. 4d). This data was ulteriorly confirmed by the Co-IF staining for NLRP1 and UBR2 where O_3 exposure modulated either NLRP1 and UBR2 expression levels, increasing their colocalization at all selected timepoints (Fig. 4e and 4Sf) and MG-132 treatment was able to prevent these phenomena especially at the early timepoints (T0 and T3).

4. Discussion

NLRP1 is one of the major inflammasomes modulating the inflammatory response of the skin and so correlated to a variety of cutaneous condition [23]. Although NLRP1 has been the first inflammasome to be discovered, multiple questions regarding its activation are still

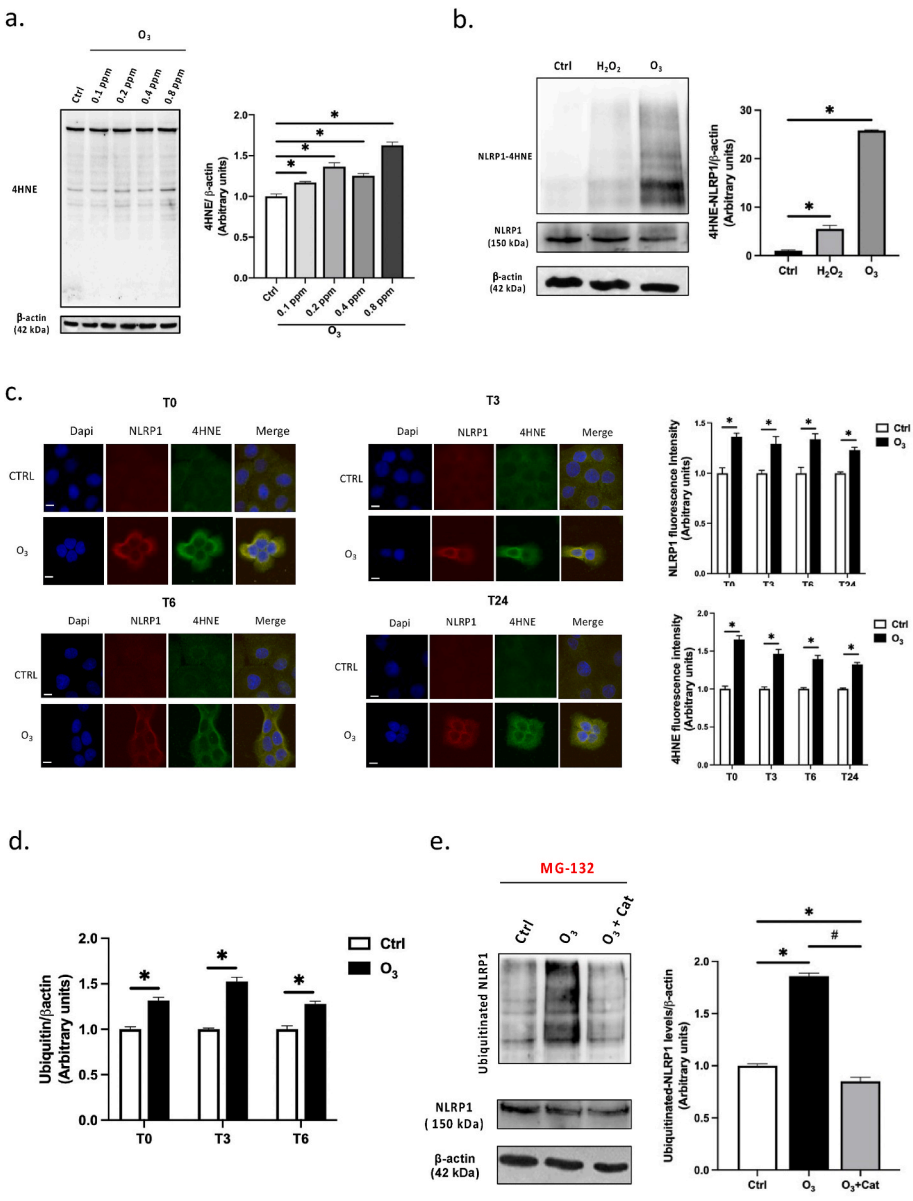

Fig. 3. Involvement of 4HNE protein adducts in NLRP1 inflammasome activation (a) 4HNE protein expression levels in HaCaT cells exposed at the indicated doses of O_3 for 1 h and collected right after the end of O_3 exposure. **(b)** Immunoprecipitation assay for NLRP1 probed with 4HNE in HaCaT cells pre-treated with proteasome inhibitor MG-132 20 μM for 2 h and exposed to O_3 or H_2O_2 50 μM for 1 h. Samples were collected right after the end of 1-h O_3/H_2O_2 exposures. **(c)** Double Immunofluorescence staining for NLRP1 (red) and 4HNE (green) in HaCaT cells after O_3 exposure. The blue staining (DAPI) represents nuclei. Images were taken at 60 × magnification (scale bar = 10 μm) and the fluorescent levels were quantified using ImageJ software. **(d)** Quantification of protein expression levels of ubiquitinated proteins in HaCaT cells exposed to O_3. **(e)** Protein expression levels of ubiquitinated-NLRP1 after Immunoprecipitation for NLRP1 in HaCaT cells pre-treated with proteasome inhibitor MG-132 20 μM for 2 h and Cat 1000 U/ml for 2 h and exposed to O_3 right after the end of exposure. For all the experiments HaCaT cells were exposed to 0.4 ppm O_3 for 1 h and then collected at the indicated timepoints post 1-h O_3 exposure. For all western blotting the protein expression level was quantified using ImageJ software and β-actin was used as internal control. Data are the results of the averages of at least three different experiments, $^{*}p < 0.05$ and $^{\#}p < 0.05$ by 2-way ANOVA followed by Tukey's post-hoc comparison test. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

unanswered. As a matter of fact, in the past years a significant progress was achieved in understanding the molecular mechanism and the stimuli behind NLRP1 activation. In particular, the ubiquitination and proteasomal degradation of NLRP1 N-terminus, termed functional degradation, has been found to represent a common event shared between different NLRP1 triggering stimuli as Lethal factor toxin, DPP8/9 inhibitors, long dsRNA and enteroviral 3C proteases (3Cpros) of human rhinovirus (HRV) [49–51]. However, the proteasomal degradation of NLRP1 N-terminal fragment can follow different pathways depending on the stimuli. Environmental pollutants have been represented an important field of investigation in the past few decades due to their involvement in the onset of a variety of inflammatory pathologies including skin conditions. Interesting inflammasomes turned out to be particularly susceptible to ROS produced by pollutants, modulating the inflammatory response of several tissues. For instance UVB radiation can activate NLRP1 inflammasomes in human keratinocytes in a redox sensitive fashion [52–54]. However, the fine mechanism regulating inflammasomes activation in response to air pollutants is still a great area of investigation. Our previous study demonstrated that cutaneous inflammasome NLRP1 is a sensor of O_3 and that its activation upon exposure to the pollutant occurs via a redox regulated mechanism involving H_2O_2 and 4HNE [37]. As follow up of the previous study we demonstrated that NLRP1 might represent the major inflammasome in mediating the O_3 induced cutaneous inflammatory response as NLRP1 silencing in human keratinocytes reduced ASC expression and even the scaffold assemble in response to O_3 (Fig. 1a and S1b). This data suggest that ASC is required from NLRP1 to promote the inflammatory response either in HaCaT cells and primary keratinocytes exposed to O_3. Indeed, the recruitment of ASC in promoting NLRP1 related Caspase 1 maturation has been long discussed, due to the structural conformation of NLRP1 consisting of 2 additional domains at its C-Terminus: FIIND domain (function to FIIND domain) and CARD. The presence of a CARD domain suggested the possibility that ASC wasn't necessary for the maturation of pro-Caspase 1 and that NLRP1 was able to recruit pro-Caspase 1 via a CARD-CARD domain interaction [55–57]. Although other inflammasomes such as NLRC4 or CARD8 can directly interact with pro-Caspase 1 without recruiting ASC [34,58,59], the role of ASC in NLRP1 inflammasome activation is still controversial [2,29,35,60,61]. Our data demonstrated not only that ASC is recruited by NLRP1 upon O_3 exposure but also that NLRP1 activation is involved in Caspase 1

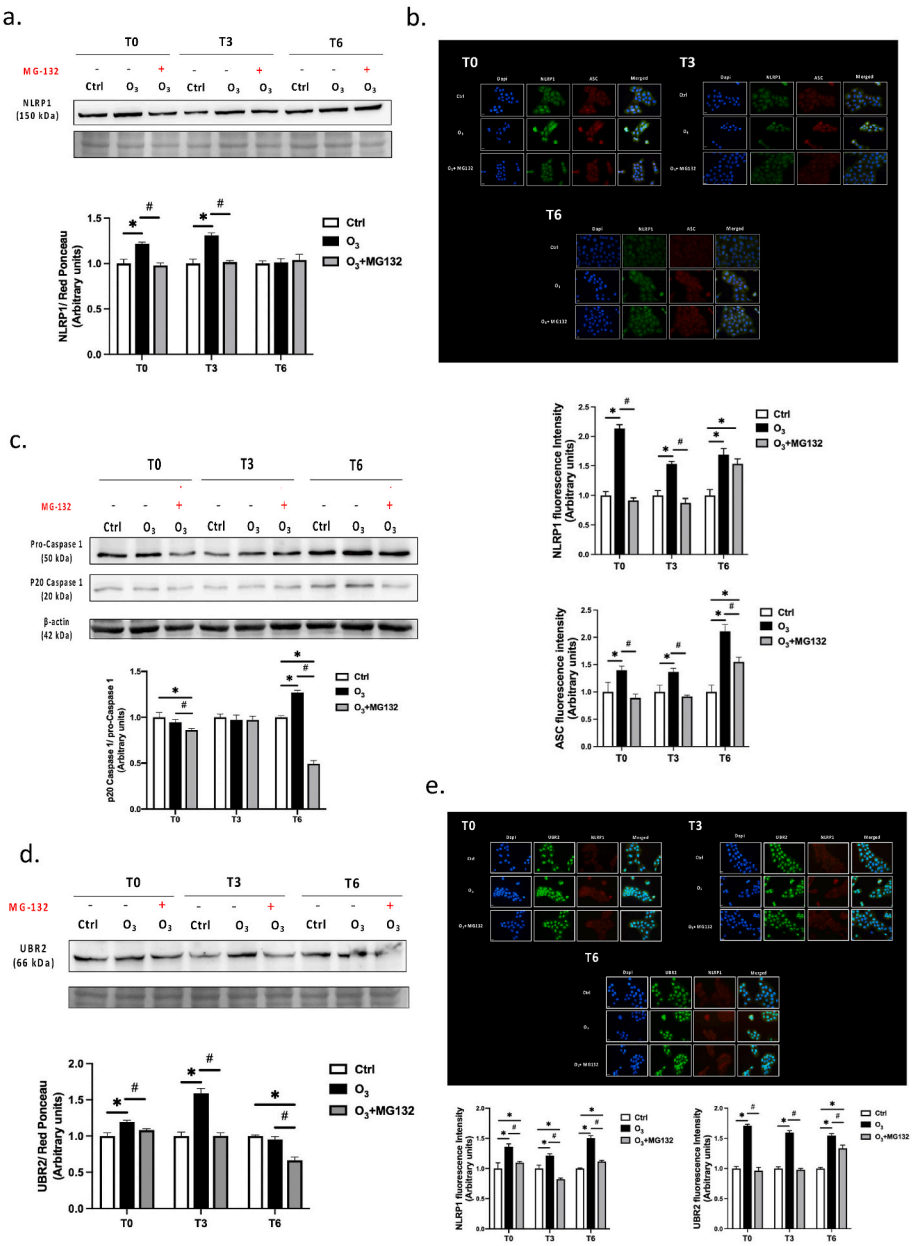

Fig. 4. NLRP1 activation is mediated by its ubiquitination. Protein expression levels of NLRP1 **(a)** in HaCaT cells pre-treated or not with the proteasome inhibitor MG-132 and then exposed to O_3. **(b)** Double immunofluorescence staining for NLRP1 (green) and ASC (red), in HaCaT cells pre-treated with MG-132 and then exposed to O_3. Blue staining (DAPI) represents nuclei. Images were taken at 40 × magnification (scale bar = 20 μm). The fluorescent levels were quantified using ImageJ software. Protein expression levels of p20 Caspase 1 over Pro-Caspase 1 (**c**), and UBR2 (**d**) in HaCaT cells pre-treated with MG-132 and exposed to O_3. **(e)** Double IF staining for NLRP1 (red) and UBR2 (green) in HaCaT cells pre-treated with MG-132 and exposed to O_3. Blue staining (DAPI) represents nuclei. Images were taken at 40 × magnification (scale bar = 20 μm). For all the experiments HaCaT cells were pre-treated or not with the proteasome inhibitor MG-132 20 μM for 2 h and then exposed to O_3 0.4 ppm for 1 h. Samples were collected at the indicated timepoints post 1-h O_3 exposure. For all western blotting the protein expression level was quantified using ImageJ software and β-actin or Red ponceau were used as internal control. Data are the results of the averages of at least three different experiments, $^{*}p < 0.05$ and $^{\#}p < 0.05$ by 2-way ANOVA followed by Tukey's post-hoc comparison test. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

maturation in human keratinocytes (Fig. 1b). Interesting we found that HaCaT cells lacking NLRP1 just partially failed in release IL-1β in response to O_3 exposure (Fig. 1c), whereas Caspase 1 inhibition significantly prevented IL-1β release (Fig. 1e–f). These data suggest that either other caspases could be involved in the inflammatory response mediated by NLRP1 or that other inflammasomes can be activated by O3. For instance several evidences have reported that NLRP1 is able to recruit other types of caspases in spite of Caspase 1, such as Caspase 5, to mediate the release of inflammatory cytokines [2,15]. It is worth to mention that although the modulation of other inflammasomes as NLRP3 and AIM2 within the skin in response to O_3 exposure is still a completely unexplored field, inflammasomes can be simultaneously activated by other environmental stressors [46,62] and being differently modulated depending on the pollutant stimuli and the tissues [46,63]. This evidence suggests that even in the context of O_3 exposure, multiple inflammasomes might be activated and promote the inflammatory response within the cutaneous tissue. Indeed, considering that Caspase 1 activation and cytokines release are mutual events shared between many types of inflammasomes, it is not unexpected that its inhibition could completely abrogate the cutaneous inflammatory response induced by O_3, whereas the silencing of NLRP1 could just partially prevent this event. However, since the decrease of IL-1β release in NLRP1 silenced keratinocytes was evident when compared to cells just exposed to O_3, we suggest that NLRP1 might represents one of the main cutaneous inflammasomes involved upon O_3 exposure, most likely via Caspase 1. Whether other caspases could be activated in response to O_3 challenge or other inflammasomes could take part in the cutaneous damage induced by O_3 exposure is currently under investigation in our laboratory. Since we previously showed that H_2O_2 and 4HNE are important mediators of NLRP1 inflammasome activation, we wanted to investigate the mechanism behind this regulation. So far several studies supported the idea that ROS production can modulate inflammasomes, although the role of ROS as effectors or triggers factors in inflammasomes activation is still under investigation [64]. Air pollutants and UV radiations have been shown to modulate certain types of inflammasomes, in particular NLRP3, mediating an oxidative stress mechanism. For instance PM and O_3, can trigger NLRP3 inflammasome via ROS production in several cardiovascular and lung/pulmonary diseases such

as Malignant mesothelioma, fibrosis, lung cancer, asthma and chronic obstructive pulmonary diseases (COPD) [17–20,65–68]. Although very few is known whether the oxidative insult of air pollutants can activate NLRP1, in this work we were able to show that NLRP1 inflammasome can be redox modulated as the treatment with Catalase (Cat) was able to prevent not only NLRP1 and ASC transcription and their assemble, but also Caspase 1 activation and IL-1β release in human keratinocytes (Fig. 2b–f) and RHE model (Figs. S2b–c). Of note we found that O_3 exposure is able to promote the formation of 4HNE protein adducts with NLRP1 (Fig. 3b–c), event that can affect proteins activity and eventually their degradation via different pathway including the ubiquitin proteasome system [38,69].

Based on our previous findings we hypothesize that O_3 exposure, through the formation of ROS and/or 4HNE, could induce post-translational modifications (PTMs) in NLRP1 and therefore modulate the inflammasome activation. Indeed, inflammasomes, have being found to be regulated at different levels by PTMs, as phosphorylation and ubiquitination, playing a key role in maintaining the homeostatic balance of its activation [70–73]. In addition, ROS and 4HNE have been reported to induce several modifications in their molecular targets [74,75] mainly due to phosphorylation and ubiquitination mechanisms, leading to an altered skin homeostasis [69]. As a matter of fact, higher levels of ubiquitinated proteins were found in HaCaT cells exposed to O_3 (Fig. 3d) and NLRP1 turned out to be targeted for ubiquitination upon O_3 exposure, event that was reverted by Cat pre-treatment (Fig. 3e), which confirmed our hypothesis. Recently, ubiquitination has been shown to be an essential PTM in NLRP1 activation. Several studies have demonstrated that the mouse variant NLRP1b is activated by the *Bacillus Antracis* Lethal factor toxin (LF), that induce the cleavage of the N-terminus within the FIIND domain of both rat and mouse NLRP1 promoting its degradation through the N-end rule pathway [26–30], an important event of the ubiquitin-proteasome system necessary for proteins turnover or to activate particular circuits [32,33]. This degradation would allow the release of the active C-terminal fragment containing the CARD domain which can contribute to the assemble of the scaffold necessary for inflammasome activation [25,61]. [28], demonstrated that the cleavage of NLRP1b is not only necessary but also sufficient for inflammasome activation by LF and that this cleavage occurs even in the absence of LF, suggesting that NLRP1b acts just as a sensor of protease activity and therefore would be able to detect non only *B. anthracis* but also other pathogens and stimuli. For instance, in the last few years it has been reported that chemical inhibitors of dipeptidyl peptidases DPP8 and 9 are able to activate both the murine Nlrp1b inflammasome and the human NLRP1 [34,35]. Indeed, DPP9 and 8 seem to display an important role in regulate the inflammasome activation involved in human autoinflammatory diseases, stabilizing NLRP1 by chemical interacting with NLRP1 FIIND domain, preventing its autoproteolysis and spontaneous activation [76]. However, the mechanism is still unclear. Indeed, even though DPP9 inhibitors can activate NLRP1 inducing proteasomal degradation of the Nlrp1b N-terminus, this event does not follow the N-end rule pathway as DPP9 inhibitor ValaBoroPro (VbP) does not promote the direct N-terminal fragments cleavage [24,36]. Our data show that DPP9 was not strongly modulated by O_3 exposure, nor by proteasome inhibition (Figs. S4b–c). The early DPP9 overexpression in response to O_3 and its decrease upon MG132 treatment, could be just due to an initial accumulation of full length NLRP1 related to proteasome inhibition, event that was confirmed by a major increase of NLRP1 expression levels at T0 compared to the other timepoints (Fig. 4a–b). Indeed, DPP9 has been found to form a ternary structure with full length NLRP1 and C-terminus NLRP1 sequestering the latter to prevent NLRP1 activation [36]. Moreover, it is worth to mention that both DPP9 binding and enzymatic activity are responsible for the activation of certain type of inflammasomes, as in the case of CARD8 [77]. Since we found a slight increased colocalization of DPP9 with NLRP1 upon O_3 exposure while NLRP1 was activated (Fig. S4d), whether the enzymatic activity of DPP9 rather than the chemical interaction with NLRP1 is important in O_3-induced inflammasome activation is currently under investigation in our laboratory.

Although DPP9 did not turn out to be particularly susceptible to O_3 exposure, we did demonstrate that O_3-induced NLRP1 inflammasome activation is a proteasome related event since MG132 treatment prevented NLRP1 assemble and activation (Fig. 4a–c and S4a). The E3 Ubiquitin ligase UBR2 has been shown to mark NLRP1 N-terminus for proteasomal degradation via the N-end rule pathway [78,79] that rely on the recognition of degradation signals called N-degron present in the N-terminal residue of proteins. These residues can be post-translationally modified via arginilation (Arg/N-end rule), or acetylation (Ac/N-end rule) and be targeted for proteasomal degradation by a recognition component (N-recognin) as the E3 ubiquitin ligases of the UBRs family [31,80]. Our results, although very preliminary, showed that O_3 exposure modulates UBR2 expression levels in human keratinocytes and that NLRP1 is a target protein of UBR2 for proteasomal degradation (Fig. 4d–e, Fig. S4e). Due to the strong reactivity of O_3, we speculate that O_3 might promote NLRP1 ubiquitination by inducing oxidative post-translational modification its N-terminal fragment. Moreover, it has been shown that cysteine residues are particularly susceptible to oxidation through non enzymatic reactions that requires Nitric Oxide (NO) and Oxygen. Oxidized cysteine can then be targeted for arginilation and be degraded via the Arg/N-end rule pathway, that it is therefore considered as a NO/O_2 sensor pathway involved in the elimination of misfolded proteins [81]. In addition, other PTMs events have been reported to modulate NLRP1 since MAPK kinases ZAKα, p38 and c-Jun N-terminal kinase (JNK) could induce NLRP1 activation via phosphorylation, particularly in response to UV radiations [82–84]. This evidence may suggest a similar mechanism for O_3 that has been found to modulate these kinases especially in the airway inflammatory response [85–87]. Considering that O_3 is one of the most oxidative agents among environmental pollutants, well known to oxidize several biomolecular targets including, DNA, lipids, and proteins, it might promote PTMs and oxidation of NLRP1 N-terminus residues via ROS production marking it for proteasomal degradation. Taken together these evidences suggest that PTMs may represent essential modulating events in O_3-induced NLRP1 inflammasome activation in human skin.

NLRP1 although the most present in skin is not the only one linked to skin conditions. For instance, a fairly studies have shown that both the overexpression and constitutive activation of NLRP3 could contribute to the development and progression of various cancers including malignant melanoma [88], highlighting the interested also to NLRP3 for the development of skin conditions. This is not so surprising since both NLRP1 and NLRP3 have very similar structure with the only difference that NLRP1 contains a function-to-find domain (FIIND) and CARD but in both cases the succession of events is the same, with the activation of CASP 1 that then will cleave the pro- IL-1beta and IL-18 and their release in the systemic system. Therefore, it is possible that the activation of NLRP1 or NLRP3 in the skin lead to similar effect. Whether similar stimulus can activate and lead to the same cutaneous effects is not clear.

5. Conclusion

In conclusion, the present work suggests that human NLRP1 is activated by O_3 exposure via ubiquitination and that this mechanism is regulated by oxidative stress events involving O_3-mediators as H_2O_2 and 4HNE. We suggest that O_3 might induce PTMs modification of NLRP1 N-terminus via oxidative stress events, leading to its ubiquitination and proteasomal degradation, promoting NLRP1 activation. Considering that the majority of skin issues related to pollution exposure are inflammatory conditions, it could be worth it to develop new technologies with NLRP1 inhibitors to prevent pollution induced skin damage. Finally, it is possible that other cutaneous inflammasomes are activated by ozone exposure such as NLRP3, NLRC4, AIM2 etc and further studies need to clarify this, although, NLRP1 has been shown to be the main inflammasome present in the skin.

Contributions

Conceptualization, F.F, V.C., A.P, F.C and G.V.; methodology, F.F., V. C., A.V., A.G, M.B, E.P, and G.V.; validation, A.P., and G.V.; formal analysis, F.F; investigation, F.F., V.C., A.P..; resources, F.F, A.P. and G. V.; writing—original draft preparation, F.F; writing—review and editing, G.V.; visualization, F.F; supervision, G.V. and A.P.; funding acquisition G.V. All authors have read and agreed to the published version of the manuscript.

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Declaration of competing interest

The authors declare no competing interests.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.redox.2022.102440.

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