Tropospheric ozone affects SRB1 levels via oxidative post-translational modifications in lung cells

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Abstract

Exposure to air pollution is associated with increased respiratory morbidities and susceptibility to lung dysfunction. Ozone (O_3) is commonly recognized as one of the most noxious air pollutant and has been associated with several lung pathologies.

It has been demonstrated that decreased lung disorder severity and incidence are connected with the consumption of a diet rich in fruits and vegetables, suggesting that higher intake of dietary micronutrients and phytoactive compounds can be beneficial. However, dietary supplementation - i.e. vitamin E (α-tocopherol) or vitamin A - has not always been effective in improving pulmonary function.

Recently, research on the role of nutritional antioxidants on human health has focused more on studying their uptake at the cellular level rather than their effective ability to scavenge reactvive oxygen species (ROS).

The Scavenger Receptor B1 (SRB1) has been shown to play a prominent role in the uptake, delivery and regulation of vitamin E in the lung. Given the importance of SRB1 in maintaining lung tissue in a healthy condition, we hypothesize that its expression could be modulated by pollution exposure, which thus could indirectly affect the uptake and/or delivery of lipophilic substances, such as vitamin E.

To characterize the molecular mechanism involved in the redox modulation of SRB1, its cellular levels were assessed in human alveolar epithelial cells after O_3 exposure. The results demonstrated that O_3 induced the loss of SRB1 protein levels. This decline seems to be driven by hydrogen peroxide (H_2O_2) as a consequence of an increased activation of cellular NADPH oxidase (NOX), as demonstrated by the use of NOX inhibitors or catalase that reversed this effect.

Furthermore, O_3 caused the formation of SRB1-aldheyde adducts (4-hydroxy-2-nonenal) and the consequent increase of its ubiquitination, a mechanism that could account for SRB1 protein loss.

Introduction

Ozone (O_3) is a gas that is naturally present in our atmosphere and about 90% is found in the stratosphere where it protects life on earth by shielding UV radiation, while the remaining 10% is found at the ground level (troposphere).

The tropospheric O_3 formation occurs when nitrogen oxides (NOs), carbon monoxide (CO) and volatile organic compounds (VOCs) react with sunlight present in the atmosphere. The major anthropogenic sources of these chemicals are represented by motor vehicle exhaust, industrial emissions, and chemical solvents (Sillman, 199). As the concentrations of these pollutants increase with industrialization and vehicle traffic, O_3 levels rise at the ground level where living organisms are exposed to this pollutant (Health Effects Institute B). In the last few decades, the attention of researchers on O_3 impact on human health has been largely investigated and now it is clear that exposure to O_3 can dramatically affect lung functions (Uysal & Schapira, 2003).

Indeed, numerous studies have demonstrated a tight correlation between the exposure to oxidant air pollutants such as O_3 and increased respiratory morbidities and susceptibility to lung dysfunction (Cross et al., 1994; van der Vliet et al., 2000).

The first lung barrier encountered by inhaled environmental gases is the respiratory tract lining fluid (RTLF). RTLF contains a network of antioxidants that provide protection against potential oxidants. Among them, Vitamin E is considered one of the major inhibitors of lipid peroxidation in human tissues, including the airways (van der Vliet et al., 1999; Mudway et al., 2001; Halliwell & Gutteridge, 2007), thus exerting an important function in maintaining cell membrane integrity (Cross et al., 2006; Halliwell & Gutteridge, 2007). Experimental data have indeed demonstrated that Vitamin E deficiency potentiates O_3-induced respiratory tract toxicity (Sato et al., 1980).

Although α-tocopherol (αT), the main isoform of vitamin E in mammalian tissues, is thought to be the main inhibitor for lipid radicals formation and has a major role in preventing tissue inflammation, clinical trials have not clearly shown the efficacy of αT (or vitamin A) supplementation in preventing diseases or in improving COPD related lung disease endpoints (pulmonary function) or inflammation (Daga et al., 2003; Nadeem et al., 2008).

To date we do not know why, in some cases, supplements are ineffective and recent research on the role of antioxidants on human health has been mainly focused on studying their uptake at the cellular level rather than in establishing their effective ROS quencher properties.

In this regard, circulating lipoproteins, and particularly HDL, play a major role in αT delivery to lung cells and this process is facilitated by scavenger receptor SRB1 (Kolleck et al., 1999).

Almost a decade ago, Krieger et al. identified SRB1 as an HDL receptor (Acton et al., 1996) and noticed that $SRB1^{-/-}$ mice had 64% less lung vitamin E (Mardones et al., 2002) and since then, the role of SRB1 in lipid soluble vitamin lung tissue uptake has been well characterized (Kolleck et al., 2002; Valacchi et al., 2015).

Following our previous *in vivo* study on whole lung homogenate, where we showed that O_3 exposure induced the loss of SRB1 (Valacchi et al., 2007), in the present study we aimed to better understand the mechanism involved in O_3-mediated lung SRB1 decrease. Our data suggest that the drop in SRB1 levels after O_3 exposure is mainly caused by oxidative post-translational modifications due to the formation of SRB1-4HNE adducts that lead to SRB1 ubiquitination and that H_2O_2, generated by cellular NOX, seems to be the key molecule that triggers the loss of SRB1 protein.

Materials and methods

Cell culture and treatments

A549 cells (ATCC; Manassas, VA) were grown in HAM-F12 (Lonza, Milan, Italy), supplemented with 10% FCS and 1% penicillin/streptomycin and 2 mM L-glutamine as previously described (Cervellati et al., 2014). Cell suspension containing 10 or 1×10^5 viable cells/ml were used. Cells were incubated at 37°C for 24 h in 95% air/5% CO_2 until 80% confluency.

A549 cells were treated with 4-hydroxy-2-nonenal (HNE) (Calbiochem, La Jolla, CA) or glucose oxidase (GO; type II from Aspergillus niger, 15.5 U/g; Calbiochem, La Jolla, CA) or pre-treated (2 h) with PEG-catalase (PEG-CAT) or diphenyleneiodonium chloride (DPI) before O_3 exposure, and then resuspended in medium supplemented with 10% FBS. After the treatments, the cells were collected by centrifugation for the assays described below.

O_3 exposure

O_3 was generated from O_2 by electrical corona arc discharge (ECO_3 model CUV-01, Torino, Italy). The O_2–O_3 mixture (95% O2, 5% O_3) was combined with ambient air and allowed to flow into a Teflon-lined exposure chamber, with the O_3 concentration in the chamber adjusted to varying ppm outputs and continuously monitored by an O_3 detector. Exposure to filtered air was carried out in similar exposure chambers except that filtered airflow was released into the chamber at flow rates similar to the O_3 output. Cells (1×10^6 cells/well in 1.2 ml of media in 6 cm Petri dishes) were exposed to filtered air or 0.5 ppm O_3 for 1 h. Subsequently, the medium was replaced with fresh medium + 10% FBS. The O_3 dose and the exposure time were determined by the current literature on O_3 pollution levels. Temperature and humidity were monitored during exposures (37°C and 45–55%, respectively) (Canella et al., 2017).

Cellular viability

Viability studies were performed by measurement of LDH release and cytofluorimetric assay as previous described (Sticozzi et al., 2013). The LDH levels in the supernatant were calculated based on the kit instructions (EuroClone Milan, Italy). All tests were performed in triplicate and assays were repeated five times independently with average results reported.

Cytofluorimetric assay was performed using Muse Count & Viability Kit (Millipore, Corporation, Billerica, MA, USA). Briefly, cells were suspended in PBS. Then, 380 µl of Muse Count & Viability working solution was added to the cells, and 20 µl of this cell suspension was incubated for 5 minutes at room temperature in the dark. Cells viability was determined by Muse Cell Analyzer.

H_2O_2 production

H_2O_2 levels in the media and intracellular H_2O_2 production rate were evaluated by the Amplex Red-Horseradish Peroxidase (HRP) method (Chen et al., 2003). Resorfurin formation due to Amplex Red (25 µM) oxidation by HRP (0.5 U/mL) bound to H_2O_2 was measured in Synergy H1 Hybrid Multi-Mode Reader (BioTek Instruments, Inc., Winooski, VT, US) at 530 nm (excitation) and 590 nm (emission). After an initial stabilization period, 10 µl of maintenance medium or 30 µl of freshly cells suspension were added to the reaction mixture. A calibration curve was performed using H_2O_2 solutions as standard and its production rate was expressed as pmol/min (Magnani et al., 2016). Controls in the absence of sample or HRP indicate that nonspecific probe oxidation was minimum (< 1%). Results were expressed as nmol/min mg protein (Chen et al., 2003).

4-hydroxynonenal (4-HNE) assay

Lipid peroxidation of A549 exposed to O_3 was evaluated by measuring 4-hydroxynonenal (4-HNE) levels according to manufacturer's instructions of commercially available kit (BioSource, Milan, Italy). The measured amount of 4-HNE protein adduct was normalized with protein concentration measured with the Bradford method. A calibration curve was performed using 4-HNE standard. Results are expressed as µg 4-HNE/mg protein.

Immunocytochemistry

A549 cells were grown on coverslips at a density of 1×10^5 cell/ml, and after O_3 exposure fixed in 4% paraformaldehyde in PBS for 20 min at room temperature (RT) as previously described by Sticozzi et al. (2012). Briefly, after permeabilization, cells were blocked in PBS containing 5% BSA, at RT for 1 hr and then incubated for 1 hr with primary antibody, followed by secondary antibodies incubation. Nuclei were stained with 1 µg/ml DAPI (Molecular Probes) for 1 min after

removal of secondary antibodies. Coverslips were mounted onto glass slides using anti-fade mounting medium 1,4 diazabicyclooctane in glycerine (DABCO) and examined with a Leica microscope equipped with epifluorescence at 20× magnification. Negative controls for the immunostaining experiments were performed by omitting primary antibodies. Images were acquired and analyzed by Leica software.

Western blot analysis

Total cell lysates were extracted in RIPA buffer containing protease and phosphatase inhibitor cocktails (Sigma–Aldrich Corp.) as described before (Sticozzi et al., 2012).

After proteins concentration determination by the method of Bradford (Bio-Rad Protein assay, Milan, Italy), samples were loaded onto 10% sodium dodecyl sulphate–polyacrylamide electrophoresis gels. The gels were then electro-blotted onto nitrocellulose membranes which were then blocked for 1 hr in Tris-buffered saline, pH 7.5, containing 0.5% Tween 20 and 5% milk. Membranes were incubated overnight at 4°C with primary antibody, anti-SR-B1 (Novus Biologicals, Inc.; Littleton, CO, USA) or β-actin (Cell Signalling; Celbio, Milan, Italy). The membranes were then incubated with horseradish peroxidase-conjugated secondary antibody for 1 hr, and the bound antibodies were detected using chemiluminescence (Bio-Rad). The blots were then stripped and re-probed with β-actin (1:1000) as the loading control. Images of the bands were digitized and the densitometry of the bands were performed using Image-J software.

Quantitative real-time PCR

Quantitative real-time PCR was carried out as described in detail previously (Pecorelli et al., 2016). Briefly, total RNA was extracted, using an AURUM total RNA Mini Kit with DNase digestion (Bio-Rad), from A549 for each experimental condition, according to the manufacturer's recommended procedure. First-strand cDNA was generated from 1 µg of total RNA using the iScript cDNA Synthesis Kit (Bio-Rad). The primer pairs (Table 1) capable of hybridization with unique regions of the appropriate gene sequence were obtained from the Real-Time PCR GenBank Primer and Probe Database Primer Bank, RTPrimerDB. Quantitative real-time PCR (qPCR) was performed using SYBR green on the CFX Multicolor real-time PCR detection system (Bio-Rad). The final reaction mixture contained 300 nM each primer, 1 µl of cDNA, and 7 µl of iQ SYBR

Green Supermix (Bio-Rad), with RNase-free water being used to bring the reaction mixture volume to 15 µl. All reactions were run in triplicate.

Preparation of cell lysate for enzymatic assay

After centrifugation, pellet was re-suspended in cold lysis buffer and the suspension was then incubated at 4°C for 30 minutes and centrifuged at 10 000g for 30 minutes (Cervellati et al., 2015). After centrifugation, the protein concentration of the supernatant was determined by the Bradford method.

NADPH oxidase (NOX) activity assay

The cell pellet was re-suspended in cold Krebs Buffer with 1mM PMSF and protease inhibitor cocktail (Sigma-Aldrich) as previously described by Cervellati et al. (2015). Membranes suspension (10 µg) was evaluated for NOX-enzyme activity in 200 µL of Krebs buffer with 50 µM lucigenin, 100 µM NADPH. The chemiluminescence was measured immediately and at 30 seconds intervals for 15 minutes. In order to confirm the assay specificity, NOX inhibitor VAS 2870 was used to identify chemiluminescence that was not NOX related. NOX activity was expressed as RLU/min/mg protein.

Proteasome activity assay

A549 pellets were re-suspended in proteasome lysis buffer as previously described by Cervellati et al. (2015). After centrifugation, supernatant was used for proteasome assay using fluorescent-AMC peptides. Samples were pre-incubated with or without proteasome inhibitor (50µM MG132 from R&D) for 45 minutes at 37°C. Chymotrypsin-like, trypsin-like and caspase-like activity were measured in separate wells by adding 50µl of substrate at each well; respectively 200 µM Suc-LLVY-AMC; 200µM Boc-LRR-AMC; 400µM Z-LLE-AMC. Fluorescence were read for 60 minutes at 10 minutes intervals at λecc=360; λemm=465, gain 60. Results were expressed as RFU/min/mg protein using angular coefficient of interpolate line.

Statistical analysis

For each of the variables tested, two-way analysis of variance (ANOVA) was used. A significant effect was indicated by a P-value <0.05. Data are expressed as mean ± S.D. of triplicate measurements obtained in 5 separate independent experiments.

Results

O_3 effect on cells viability

Cytotoxicity of 0.5 ppm O_3 exposure was evaluated in A549 cells at different times (1, 6, 12 and 24 h) by means of LDH release (Fig. 1A) and cytofluorimetric assay (Fig. 1B). As shown in Fig. 1A, under our experimental conditions, O_3 exposure did not affect LDH release nor cell viability (Fig. 2A) at the different time points analysed.

O_3 exposure decreased SRB1 expression

As shown in Fig. 2A, SRB1 protein level markedly decreased upon O_3 exposure starting at 6 h (2-fold) and reaching almost 4-fold decrease 24 h after the exposure compared to the air-exposed cells. To verify whether O_3-induced SRB1 protein loss occurred at the transcriptional level, SRB1 gene expression was determined by Real time PCR. The Fig. 2B shows that O_3 exposure significantly induced a time-dependent increase of SRB1 mRNA levels reaching circa 3-fold increase at 24hrs, revealing a discrepancy between gene expression and matching protein.

O_3 exposure increased oxidative stress markers in lung cells

The ability of O_3 to induce oxidative stress has been well characterized, therefore we have evaluated the levels of ROS in A549 cells exposed to O_3 at different time points from 0 to 60 minutes. As shown in Fig. 3A, H_2O_2 production by A549 cells significantly increased in a time dependent manner after O_3 exposure with a 2-fold increase at 60 minutes. In addition, as depicted in Fig. 3B, the levels of H_2O_2 in the culture medium increased approximately 4-fold after 30 min and a slight decline at 60 min. Increased oxidative stress levels were further confirmed by the the evaluation of HNE protein adducts levels. Also in this case, the increase was evident immediately after the exposure to O_3 (1 h) and, although declined over time, it was still significant at 6 hrs (Fig. 3C).

HNE and H_2O_2 mimic O_3 effect on SRB1 expression in A549 cells.

The dogma that the interaction between O_3 and cell membrane leads to the formation of aldehydes and H_2O_2 has been suggested more than 20 years ago (Pryor & Church, 1991). Therefore, to understand whether the effect of O_3 on SRB1 loss was mediated by these molecules, A549 cells were treated with either HNE or glucose oxidase (GO), an enzyme that continuously generates

H_2O_2 in the cells. As shown in Fig. 4A, 60 μM of HNE treatment was able to affect SR-B1 expression at the later time points while lower doses did not have any effect (data not shown). Similar results were observed when the cells were treated with 0.5 U/ml GO but, as it can be noted in Fig. 4B, H2O2 mimics the O_3 effect on SRB1 expression in a more consistently than HNE treatment.

Activation of NADPH oxidase after O_3 exposure

Since NADPH oxidase (NOX) is one of the possible cellular source of H_2O_2, we determined whether O_3 exposure induced activation of this enzyme. As shown in Fig. 5A, the protein levels of both the NOX subunits $p47^{phox}$ and $p67^{phox}$ were significantly increased after O_3 exposure, as measured by immunocytochemistry analysis. These data were further confirmed by luminescent assay, that showed a clear and significant dose-dependent increase of NOX activity in A549 cells after O_3 exposure (Fig. 5B).

The decrease in SRB1 levels is rescued by catalase and DPI

To confirm that the decreased level of SRB1 after O_3 exposure was mainly driven by the production of H_2O_2, A549 cells were pre-treated with PEG-catalase (PEG-CAT) or with DPI (a general inhibitor of flavoproteins including NOX) before O_3 exposure. As shown in Fig. 6A, both PEG-CAT and DPI were able to prevent SRB1 decrease induced by O_3 exposure.

O_3 exposure induced HNE-SRB1 adducts in A549 cells

Next, we evaluated whether the formation of HNE protein adducts in cells exposed to O_3 (Fig. 3) could form covalent protein adduct with SRB1. As shown in Fig. 7, after O_3 exposure, the levels of SRB1 decreased dramatically (green signal) with an evident increase of HNE (red signal). The co-localization (yellow) showed the presence of HNE-SRB1 adducts.

O_3 exposure induced SRB1 ubiquitination and proteasome activation.

As it is shown in Fig. 8A, the activation of the proteasome in cells exposed to O_3 showed a trend toward an increased enzymatic activity, when compared to controls. In particular, chymotrypsin-, trypsin- and caspase-like activities exhibited an increase of 5, 1.5 and 2 fold, respectively. These data are consistent with the double-immunofluorescence analysis depicted in Fig. 8B that shows

increased levels of Ubiquitin (red signal) with a concomitant decrease of SRB1 protein expression (green signal). The co-localization (yellow) showed the presence of Ubiquitin adducts on SRB1.

Discussion

O_3 is a highly reactive oxidant and a common urban air pollutant that exerts detrimental effects on bronchial and alveolar epithelial cells, leading to airway inflammation and respiratory symptoms. The high toxicity of this pollutant accounts for the significant epidemiological association between O_3 exposure and increased respiratory hospitalizations and mortality (Jerrett et al., 2009; Turner et al., 2016; Raza et al., 2018).

It has for long time been suggested that O_3, although it is not a radical species *per se*, is able to oxidize components of the cell membranes, mainly lipids, generating classical radical species. Indeed, the interaction between a molecule of O_3 and PUFA is able to generate H_2O_2 and aldehydes making both, aldehydes and H_2O_2 the main oxidative mediators produced by the interaction of this pollutant with biological systems (Pryor, 1994).

This mechanism was confirmed also in our study where both H_2O_2 and 4HNE were significantly increased after O_3 exposure and this is consistent also with our previous finding in other cell models (Sticozzi et al., 2012).

The widely held hypothesis that oxidative imbalance due to external stressors, e.g. O_3 and cigarette smoke, is an important pathogenic component of lung diseases, such as asthma, lung cancer and COPD, has led to calls for trials of dietary interventions based on the use of food antioxidants, in particular vitamin A and E. Despite repeated efforts and some sparse promising results, these studies grossly failed to provide epidemiological/clinical evidence in support of a preventive effect of dietary intervention on respiratory disorders (Shaheen et al., 2001; Berthon & Wood, 2015). In our view, this failure does not preclude the possibility that this type of intervention might be beneficial for lung health, but rather suggests that diet manipulation may be not the unique prerequisites to guarantee the effectiveness of these molecules. Indeed, what needs to be taken into consideration is not just the kind and the concentration of the dietary supplements but also the ability of the target tissues to take them up.

Fairly recently, SRB1 has been shown to be a key player in the tissue uptake of lipophilic vitamins including vitamin E and carotenoids (Witt et al., 2000; Reboul et al., 2006). In our earlier *in vivo* work, we reported that lung SRB1 levels decline, not only with age, but also after environmental challenges such as cigarette smoke and O_3, a finding that was in line with the observation that the noxious effects of O_3 are more evident in the elderly (Servais et al., 2005).

In the current study, we showed that exposure to O_3 significantly decreases the level of SRB1 protein in epithelial lung cells, concomitantly with the increased levels of H_2O_2 and 4HNE (α,β-unsaturated aldehyde produced by lipid peroxidation). Since a previous *in vivo* investigation also demonstrated that SRB1 expression might be redox-regulated (Guo et al., 2011), in the current study we have evaluated whether the observed increase in oxidative stress in our system is the cause of O_3-induced SRB1 loss.

The treatment of the cells with increasing concentrations of 4HNE and H_2O_2 showed that both molecules, although with different trends and at different degrees, mimic O_3 in altering SRB1 protein levels. Between 4HNE and H_2O_2, the latter emerged as the most influential determinant for SRB1 down-regulation, thus we explored more deeply this possible interaction. First, we sought to confirm the inhibitory effect of H_2O_2 on SRB1 by measuring the level of the receptor in the presence of PEG-CAT, and found that this peroxide scavenger prevents SRB1 decrease driven by O_3 exposure. Second, we investigated the possible endogenous source of the observed H_2O_2 increase by focusing on one of the most proficient endogenous ROS generators, NADPH oxidase (NOX). This enzyme appeared to be more expressed and more catalytically active in response to O_3, as well as to strongly contribute to SRB1 loss, as demonstrated by experiments with the NOX inhibitor.

Starting from the concept that 4HNE is able to form adducts with most cellular proteins, inducing structure modification and function corruption (Poli et al., 2008), we investigated the presence of this post-translational modification on SRB1 following O_3 exposure. Noticeably, we detected a clear time-dependent increase in 4HNE-SRB1 adducts which paralleled with 4HNE increase and SRB1 progressive loss suggesting the ability of O_3 to affect SRB1 post-translational structure.

4HNE-covalent modification in proteins is irreversible and, thus, to prevent the risk of a massive modified protein accumulation, their degradation is required. 20S proteasome proteolytic activities seem to be the main cause responsible of the degradation of 4HNE-targeted proteins (Castro et al., 2017). Although several proteolytic pathways exist for the degradation of HNE-adducted proteins, the observed increase in SRB1 ubiquitination induced by O_3 suggests that the ubiquitin-proteasome degradation pathway could take place for SRB1 (Botzen & Grune, 2007).

Our hypothesis that the increase in 20S degrading activity could result from the attempt of cells to eliminate HNE-proteins adducts, might seem in contrast with the postulated inhibitory effect of oxidative stress on proteasome activity. However, as elegantly reviewed by Castro et al. (2017),

the impairment of proteasome machinery occurs only at very high concentrations of oxidants, which most probably were not achieved in our experimental conditions.

Overall our study suggests that O_3 induces the loss of SRB1 in lung cells via an oxidative post translational modification as a consequence of HNE-SRB1 binding that likely leads to its degradation via the proteasome machinery. This effect was also observed in a previous study on keratinocytes where the involvement of the proteasome in SRB1 loss was also confirmed by the use of the proteasome inhibitor MG132 (Sticozzi et al., 2012).

Furthermore, SRB1 degradation could help explaining the discrepancy observed between SRB1 mRNA and matching protein level, i.e. increase in SRB1 gene expression and decrease in protein levels, which might be ascribed to an ineffective attempt to counteract the protein loss.

However, further studies will be necessary to also evaluate the impact of oxidative stress on SRB1 transcriptional/translational reprogramming (Grant et al., 2011).

Whatever the mechanism(s), the current results suggest that upon lung exposure to O_3 and the attendant oxidative stress, a positive feedback loop is induced that increases lipid soluble antioxidant consumption and, at the same time, decreases the expression of SRB1, a key means of antioxidant delivery. These combined effects result in reduced lung antioxidant content, thereby rendering it more vulnerable to further insults.

Legend

Fig. 1. Exposure to O_3 did not affect A549 viability. Cells were exposed to 0.5 ppm of O_3 for 1 h and then harvested at different time points (1 to 24 hrs). Cell viability was determined by LDH release (A) and cytofluorimetric (B) assay (averages ± SEM of five independent experiments).

Fig. 2. Exposure to 0.5 ppm of O_3 decreased SRB1 protein levels in A549 cells. (A) Cells were exposed to 0.5 ppm of O_3 for 1 h and then harvested at different time points (1 to 24 hrs) and SRB1 protein expression was measured by Western blot analysis. Quantification of the SRB1 bands is shown at the bottom panel (averages ± SEM of five independent experiments, *$p< 0.05$. (B) SRB1 mRNA expression at different time points (from 1 to 24 hrs) was determined by real time PCR (averages ± SEM of five independent experiments, *$p< 0.05$.

Fig. 3. Exposure to O_3 (0.5 ppm) increased the levels of H_2O_2 in A549 cells. Cells were exposed to O_3 for 15, 30 and 60 min, then intracellular H_2O_2 production (A) and release in the culture medium (B) were determined by Amplex Red assay. (C) Exposure to O_3 increased the levels of 4HNE protein adducts in A549 cells. Cells were exposed to 0.5 ppm of O_3 for 1 h and then harvested at different time points (1 to 24 hrs) and the formation of 4HNE protein adducts was determined by OxiSelect™ 4HNE-His Adduct ELISA Kit (averages ± SEM of five independent experiments).

Fig. 4. 4HNE (A) or GO (B) treatments decrease SRB1 levels. Cells were exposed to different treatments for 1 h and cells were harvested at different time points (1 to 24 hrs). Quantification of the bands is shown at the bottom panel (averages ± SEM of five independent experiments, *$p < 0.05$).

Fig. 5. Exposure to O_3 (0.5 ppm) increased the expression and activity of NADPH oxidase in A549 cells. (A) The expression of NADPH oxidase was determined by ICC of p67 and p47 subunits of enzyme, and (B) by using luminescent assay. Data are expressed in RLU/min/mg protein (averages ± SEM of five independent experiments, *$p< 0.05$).

Fig. 6. The decreased levels of SRB1 after O_3 (0.5 ppm) exposure is reversed by catalase (CAT) and diphenyleneiodonium chloride (DPI) pre-treatment in A549. Cells pre-treated with CAT or DPI were exposed to O_3 for 1 h and harvested at different time points (1 to 24 hrs).

Fig. 7. O_3 (0.5 ppm) exposure induces the formation of 4HNE-SRB1 adducts. Immunocytochemistry of A549 cells showing localization of 4HNE-adducts (red colour), SRB1 (green colour) and 4HNE-SRB1 adducts (yellow colour). Images are merged in the bottom panel and the yellow colour indicates overlap of the staining

Fig. 8. O_3 (0.5 ppm) exposure induces the increase of proteasome activation as well as the formation of Ubiquitin-SRB1 adducts. A549 cells were exposed to O_3 and then processed for fluorescence assay (A) and ICC (B) (averages ± SEM of five independent experiments, *$p < 0.05$).

Table 1

Gene	Primer sequence	T_a °C	Product length (bp)	QPCR Amplification Efficiency* (%)	n° of cycles	Ref. Primer Bank
SRB1	F: 5'-gaattcgcctttcgtccccg -3' R: 5'-ttgaaggacaggctactggg 3'	60.1	236	96.2	39	GenBank Accession BC112037.1
RPL13A	F: 5'-cctaagatgagcgcaagttgaa- 3' R: 5'-ccacaggactagaacacctgctaa-3'	60.2	203	97.3	39	Pattyn *et al.* 2006
RPL11A	F: 5'-tgcgggaacttcgcatccgc-3' R: 5'-gggtctgccctgtgagctgc-3'	60.1	108	96.5	39	GenBank Accession NM 000975.2
GAPDH	F: 5'- tgacgctggggctggcattg -3' R: 5'- ggctggtggtccaggggtct -3'	60	134	94.6	39	GenBank Accession NM 002046.3

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