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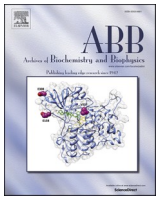

The role of potassium current in the pulmonary response to environmental oxidative stress

Rita Canella [a,*], Mascia Benedusi [a], Andrea Vallese [b], Alessandra Pecorelli [b], Anna Guiotto [b], Francesca Ferrara [c], Giorgio Rispoli [a], Franco Cervellati [a], Giuseppe Valacchi [b,d,e,**]

[a] Department of Neuroscience and Rehabilitation, University of Ferrara, Via L. Borsari, 46, Ferrara, Italy
[b] Department of Environmental Sciences and Prevention, University of Ferrara, Via L. Borsari, 46, Ferrara, Italy
[c] Department of Chemical, Pharmaceutical and Agricultural Sciences, University of Ferrara, Via L. Borsari, 46, Ferrara, Italy
[d] NC State University, Plants for Human Health Institute, Animal Science Dept. NC Research Campus 600 Laureate Way, Kannapolis, NC, 28081, USA
[e] Department of Food and Nutrition, Kyung Hee University, Seoul, South Korea

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ABSTRACT

Exposure of human lung epithelial cells (A549 cell line) to the oxidant pollutant ozone (O_3) alters cell membrane currents inducing its decrease, when the cell undergoes to a voltage-clamp protocol ranging from −90 to +70mV. The membrane potential of these cells is mainly maintained by the interplay of potassium and chloride currents. Our previous studies indicated the ability of O_3 to activate ORCC (Outward Rectifier Chloride Channel) and consequently increases the chloride current.

In this paper our aim was to understand the response of potassium current to oxidative stress challenge and to identify the kind potassium channel involved in O_3 induced current changes.

After measuring the total membrane current using an intracellular solution with or without potassium ions, we obtained the contribution of potassium to the overall membrane current in control condition by a mathematical approach. Repeating these experiments after O_3 treatment we observed a significant decrease of $I_{potassium}$. Treatment of the cells with Iberiotoxin (IbTx), a specific inhibitor of BK channel, we were able to verify the presence and the functionality of BK channels. In addition, the administration of 4-Aminopyridine (an inhibitor of voltage dependent K channels but not BK channels) and Tetraethylammonium (TEA) before and after O_3 treatment we observed the formation of BK oxidative post-translation modifications.

Our data suggest that O_3 is able to inhibit potassium current by targeting BK channel. Further studies are needed to better clarify the role of this BK channel and its interplay with the other membrane channels under oxidative stress conditions. These findings can contribute to identify the biomolecular pathway induced by O_3 allowing a possible pharmacological intervention against oxidative stress damage in lung tissue.

1. Introduction

Air pollution is an environmental phenomenon due to the introduction into the air of foreign elements of natural and/or anthropogenic origin able to alter its normal composition, causing harmful effects to living organisms.

It consists of a set of solid and gaseous particles dispersed in the air such as atmospheric particulate matter, cigarette smoke and ozone (O_3) [1].

Inhalation of high concentrations of pollutants results in a wide range of respiratory symptoms including decreased lung function and increased respiratory tract hyperactivity in 10–20% of cases in the healthy population [2].

Chronic exposure to pollution is also linked to an increased risk of cardiovascular and pulmonary diseases and it is associated with millions of premature deaths per year worldwide [3].

One of the main target of pollution exposure is the airway epithelium. Because of the extensive conducting airways and large alveolar surface area (about 120m^2) [4],[5], it is particularly susceptible to oxidative stress-induced damage. The presence in the inspired air of

* Corresponding author.
** Corresponding author. Department of Environmental Sciences and Prevention; University of Ferrara, Via L. Borsari, 46, Ferrara, Italy.
E-mail addresses: rita.canella@unife.it (R. Canella), gvalacc@ncsu.edu (G. Valacchi).

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contaminants such as particulate matter from exhaust emissions, cigarette smoke, ozone, nitrogen dioxide, and various allergens, contribute to the production of reactive nitrogen species and reactive oxygen species, in the lungs [6],[7],[8],[9].

Among the several environmental stressors to which we are daily exposed, O_3 is one of the most reactive and toxic 10,18[10]. It irritates the lungs at concentrations that are fairly common in urban settings, particularly in summer months [11]. Its toxicity is mainly due to its ability to oxidize cellular components such as unsaturated and polyunsaturated lipids and thiol groups of proteins present in the cell membrane [12], leading to the formation of reactive biomolecules among which the aldehyde 4-hydroxynonenal (4HNE) has been shown to be one of the most reactive [13].

Airway epithelium has developed various strategies to defend itself against inhaled pollutants: for example, its capacity to produce pro- and anti-inflammatory cytokines makes it involved in inflammatory response [14],[15] and the ability to regenerate an intact epithelium after damage protects it against injuries [16–18]; moreover mucociliary clearance of ciliated cells drains secretory products and particulate matter out of the sinuses [19,20].

Alveolar surface is formed by two types of epithelial cells: pneumocytes I and pneumocytes II. Type I pneumocytes are flat and cover more than 95% of the gas exchange surface [21], whereas type II pneumocytes are cuboidal and contribute to the healthy functioning of the epithelium in many ways [22]: participating in clearance and in repair by proliferating and migrating to damage areas, differentiating into type I cells, and participating in defence responses by expressing various receptors. Moreover, they are involved in the lung cytokine/chemokine network by secreting and responding to an array of cytokines and chemokines [23,24].

The main function of type II cells is the secretion of the Epithelial Lining Fluid (ELF) that lowers the surface tension in alveoli, preventing their collapse and controlling the ionic composition and volume of fluids bathing the alveolar cells [25]. Modifications of ELF composition can lead to serious pathologies, such as cystic fibrosis (CF), due to the alteration of the CFTR chloride channel [26]

In our previous studies, we analyzed the action of ozone on type II pneumocytes cells model (A549 cells 27, and considered the possible alterations of the chloride current induced by exposure to O_3. We found that, the hydrogen peroxide (H_2O_2) that derives from the interaction between ozone and the cells, is able to affect the chloride current [28]. In the specific, H_2O_2 causes an increase in chloride current mainly due to the activation of the Outward Rectifier Chloride Channel (ORCC) [29].

In the present study we analyze the effect of O_3 on the flow of potassium, another fundamental actor in the control of both, membrane potential and ionic exchanges through type II alveolar membrane cells [30,31].

About 40 types of K^+ channels transcripts have been found in the airways, but it is difficult to say if all of them are functional. Bardou et al. [30] described four types of K^+ channels in A549 cells: Kir3.x, Kv9.3, KCa3.1 and BK channels. In our previous paper [32] we also demonstrated the presence and functionality of TREK-1 potassium channel on the A549 membrane. In physiological conditions this channel contributes to the leakage current of the cell, but it can be activated by swelling and cell stretching, by phosphorylation, polyunsaturated fatty acids, volatile anaesthetics, and acid pH [33].

Under physiological conditions, Kir channels generate a large K^+ conductance at potentials negative to E_K but permit less current flow at potentials positive to E_K. Cells that express a large Kir conductance are expected to show the resting membrane potential (E_{res}) close to E_K and no spontaneous electrical activity [34,35].

Kv9.3 is an electronically silent K_V α-subunit that does not form electronically functional channels when expressed as a homomultimer [36,37] while KCa3.1 channel is involved in aggressive behaviour of non-small cell lung cancer (NSCLC) cells, and it can serve as a prognostic marker in NSCLC [38].

BK is a calcium-dependent potassium channel of high conductance (up to 300pS) ubiquitously expressed in our tissues, activated also by very strong depolarization (up to 200 mV), [39,40]. At the cellular level, BK channels can be found on the plasma membrane (pmBK), mitochondrial membrane (mitoBK) and nuclear membrane (nBK). mitoBK is sensitive to multiple stimuli regarding mitochondrial function (i.e., Ca^{2+} and O_2 concentration, membrane potential); it also regulates the electron transport chain, reactive oxygen species (ROS) production, and apoptosis. Although it has been shown that nBK plays a role in nuclear Ca^{2+} signalling and gene expression modulation, its role in intracellular signalling pathways is still not fully understood [41,42].

pmBK, in addition to being a regulator of membrane potential, can integrate changes in intracellular calcium level and membrane potential. Therefore, as calcium influx regulator, the channel is involved in many important physiological processes such as neuronal excitability, regulation of smooth muscle tone and neurotransmitter release [43]. In non-excitable cells, such as in airway epithelia, the hyperpolarizing action of BK facilitates Ca^{2+} influx via ligand gated Ca^{2+} channels [44]. It was suggested that BK channel openers may be considered in the treatment of various diseases [45] such as stroke, epilepsy, psychoses, bladder overactivity, erectile dysfunction, asthma, arterial hypertension, ischemic heart disease, and gastric hypermotility [46].

In agreement with the model of [31]; K_{Ca} channels are present on apical and basolateral membrane of II type pneumocyte. The presence of BK channels at the apical membrane of airway epithelial cells indicates their important role in creating the electrochemical gradient necessary for Cl^- secretion, at least through CaCC (Ca-dependent Chloride Channel) and maybe even through CFTR channels. The inhibition of BK channels with blockers, leads to airway surface dehydration and periciliary fluid collapse, as revealed by low ciliary beat frequency. Thus, BK channels seem critical for adequate ELF volume maintenance and mucociliary clearance [47,48].

BK has also been identified as O_2 pressure sensor in the airways as early as 2002 [49,50] and it also regulates the release of CCL-2 [51].

Therefore, the present work aimed to evaluate the effect of O_3 exposure on the potassium current by mean of an electrophysiological approach. We also try to identify the main channel target of ozone exposure, addressing our research towards BK channels, despite the presence of other potassium channels on the cell model used. Indeed Hermann et al. [40], in their review, indicated that BK channels can be modulated by ROS as a consequence of the interaction with cysteine and methionine residues of the protein and [52] explained the mechanism by which oxidation of methionine residues increases the BK activity, while oxidation of cysteine inhibits it. For these reasons we considered it an excellent candidate for our investigation.

2. Materials and Methods

2.1. Cell culture and treatments

The A549 (A549: ATCC, Manassas, VA, human alveolar epithelial cells) was cultured in Dulbecco's modified Eagle's medium high glucose (DMEM, Lonza®, Milan, Italy) supplemented with 10% fetal bovine serum (FBS, EuroClone, Milan, Italy), L-glutamine at 1%(Lonza) and antibiotics penicillin (100 U/ml) and streptomycin (100 μg/ml) at 1% (Lonza). The cells were then kept in an incubator at a temperature of 37 °C with 95% air and 5% CO2 until the ideal confluence was reached as previously described [53].

2.2. Immunocytochemistry

A549 cells were grown on coverslips in a 12-well plate at a density of 1 × 105 cells/ml, and after 24 h they were fixed in 4% paraformaldehyde for 30 min at room temperature (RT) as previously described [13]. Cells were then permeabilized for 5 min at RT with phosphate-buffered saline (PBS) containing 0.2% Triton X-100, then the

coverslips were blocked in PBS containing 1% BSA at RT for 1 h. Coverslips were then incubated with the appropriate primary antibodies (KCNMA1 CSB-PA614255LAO1HU, CUSABIO dilution 1:500; 4HNE AB5605, Merck dilution 1:500) in PBS 1X, 0.5% BSA at 4 °C overnight. After 3 wash with PBS 1X, the coverslips were incubated with the appropriate secondary antibody (goat anti-rabbit IgG (ab150116 Alexa Fluor 594, Abcam, Cambridge, MA) (1:100) for 1 h at RT. Nuclei were stained with 1 μg/ml 4′,6-diamidino-2-phenylindole (DAPI; Sigma-Aldrich) for 1 min. Coverslips were mounted onto glass slides using PermaFluor® Aqueous Mounting Medium (TA-006-FM, Thermo Fisher Scientific, Waltham, MA), and examined using a fluorescent microscope (Nikon Microphot FXA microscope; Nikon Instruments, Amsterdam, NL).

2.3. Protein extraction and western blotting

Total cell lysates were extracted in ice-cold RIPA buffer 1X 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*-ethylmaleimide, and 10% protease inhibitor cocktail (100 μL for a total volume of 1 ml) (SIGMA-FAST™ Protease Inhibitor Tablets, Merck), β-glycerol phosphate (10 μL for a total volume of 1 ml), and orthovanadate (5 μL for a total volume of 1 ml) [54]. Lysates were cleared by centrifugation (13,000 rpm) for 15 min at 4 °C, and protein concentration was quantified using the Bradford protein assay with absorbance measurement at 595 nm (Bio-Rad, Protein Assay; Bio-Rad Laboratories, Inc., Milan, Italy). Equivalent amounts of proteins (40 μg) were loaded onto 10% polyacrylamide SDS gels and separated by molecular size. Gels were electroblotted onto nitrocellulose membranes and then were blocked for 1 h in PBS, pH 7.5, containing 0.5% Tween 20 and 5% milk. Membranes were incubated overnight at 4 °C under gentle rocking with the appropriate primary antibodies diluted in 1% non-fat milk PBS-T. (KCNMA1 antibody, CSB-PA003200, CUSABIO 1:1000). The membranes were then incubated with horseradish peroxidase conjugated secondary antibodies for 2 h; anti-rabbit (cat. AB6721, Abcam) and the bound antibodies were detected by chemiluminescence using the ECL kit reagents WESTAR ETAC ULTRA 2.0 (cat. XLS075, 0100, CYANAGEN, Bologna, Italy) and Bio-Rad ChemiDoc™ imaging system. β-Actin (cat. A3854, Merck) was used as loading control. Images of the bands were digitized, and densitometry analysis was performed using ImageJ software.

2.4. RNA extraction and quantitative real-time PCR (q-rtPCR)

For A549 cells, total RNA extraction was performed via the Aurum Total RNA Mini Kit with DNase digestion (Bio-Rad, Milan, Italy), according to the manufacturer's instructions. The amount and quality of isolated RNA were analyzed by BioSpec-nano (Shimadzu, Kyoto, Japan). cDNA was then generated from 1 μg of total RNA, using the iScript cDNA Synthesis Kit (Bio-Rad). Evaluation of the mRNA levels of KCNMA1 genes was assessed via quantitative real-time PCR using SYBR® Green Master Mix (Bio-Rad) on a CFX Real-Time PCR Detection System (Bio-Rad), according to the manufacturer's protocol. After amplification, a melting curve analysis to confirm the specificity of the amplicons was performed. β-actin was employed as the reference gene, and the samples were compared using the relative cycle threshold (CT). Then the fold change was determined using the 2-ΔΔCT method [55].

The primers used are listed here:

β-actin forward ATTGCCGACAGGATGCAGA/reverse AGTACTTGC GCTCAGGAGGA (Ref. [56]

KCNMA1forwardTATCTCTCCAGTGCCTTCGTGG/reverse CTCTCTCGGTTGGCAGACTTGT [57]

2.5. Exposure to ozone

O_3 was generated from O_2 by electrical corona arc discharge (ECO3 model CUV-01, Torino, Italy). The O_2–O_3 mixture (95% O_2, 5% O_3) was combined with ambient air and allowed to flow into a Teflon-lined exposure chamber, with the O_3 concentration in 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 to ozone 0.1 ppm, for 30 min. Subsequently, the medium was replaced with fresh medium +10% FBS (3 ml). The O_3 dose and the exposure time were determined by the current literature on O_3 pollution levels and on our recent publications [58]. Temperature and humidity were monitored during exposures (37 °C and 45–55%, respectively).

2.6. Patch clamp technique

Patch pipettes were pulled from Drummond 50 μl glass capillaries (Cat. No. 2-000-050) with 1.0 mm outer diameter using a micropipette puller (NARISHIGE Instruments, Japan, mod PP-830), fire-polished (tip resistance between 2 and 5 MOhm) and filled with an intracellular solution.

In order to characterize the overall membrane current response, the following solutions were employed:

Intracellular solution containing (in mM): 145 KCl, 1 $MgCl_2$, 10 HEPES, and 5 EGTA; the pH was adjusted with KOH up to the value of about 7.3.

To isolate chloride current, we substituted intracellular KCl with CsCl (130 mM) and pH was adjusted with TEAOH up to the value of about 7.3.

Extracellular solution containing (in mM) 145 NaCl, 1.8 $CaCl_2$, 1 $MgCl_2$, 5.4 KCl, 10 glucose, and 10 HEPES; pH was adjusted with NaOH until the value of about 7.35.

If necessary, osmolality was adjusted with sucrose to obtain values between 300 and 310 mOsm/Kg.

Iberiotoxin (IbTx; Sigma–Aldrich), Tetraethylammonium-chloride (TEA-Cl; Sigma-Aldrich) and 4-aminopyridine (4-AP; Sigma–Aldrich) were dissolved in water and added to the perfusion solution to achieve a final concentration of 100 nM, 10 mM and 5 mM respectively [59–61].

Cells were viewed through a TV monitor connected to a contrast enhanced video camera (T.I.L.L. Photonics, Planegg, Germany). The camera was coupled to an inverted microscope (Olympus IMT-2, Tokyo, Japan) equipped with a 40 × Hoffman-modulation contrast objective.

Whole cell currents were elicited by voltage-clamp pulses (1400 ms duration) between −90 and + 70 mV in 20 mV steps from a beginning holding potential of −30 mV.

The voltage protocol and data acquisition were performed with Digidata card 1322A and pClamp package (version 9.2). The currents were recorded with a commercial patch clamp amplifier (EPC-7; Consumer E-List, Darmstadt, Germany); the recordings were filtered at 5 kHz and acquired at 10 kHz and stored on disk.

2.7. Data analysis and statistical procedures

Data are reported in the text and figures as mean ± SEM. The control-treated comparisons were made with GraphPad Prism v.8, and the significance of p values were reported in the text and figures (Tukey's test and Pearson's correlation were applied; significant differences for $P < 0.05$).

3. Results

By patch clamp technique, we analyzed the movement of ions through the membrane of type II pneumocytes, before and after O_3 exposure.

Fig. 1 shows representative current traces obtained from control cell (panel A), ozone-exposed cell (panel B), IbTx treated cell (panel C) and 4-AP treated cell (panel D). The recordings were obtained under voltage-

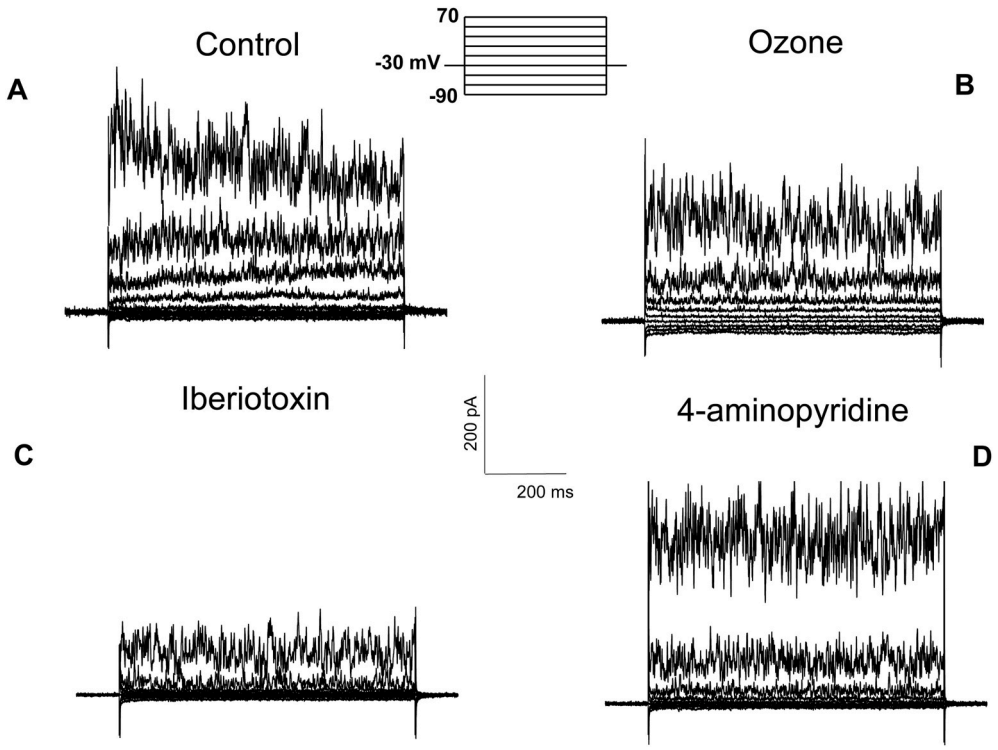


Fig. 1. Representative families of total recording currents in control condition (A), after ozone exposure (B), after IbTx (C) and 4-AP (D) treatments. Dashed line indicates zero-current level.

clamp condition: the cells were kept at a holding potential of −30 mV and then brought from −90 mV to +70 mV, with steps of 20 mV as shown in the insert.

As it is shown in Fig. 1, it is evident that O_3 exposure induces a decrease of the total membrane current and that also IbTx treatment lowers the total current; on the other hand, it seems that 4-AP does not have any effect on the membrane current. The effect of ozone was confirmed by applying the protocol to 23 control cells and 7 O_3 treated cells, obtaining the current-voltage (I–V) curves shown in Fig. 2, panel A. Tukey's test indicated that O_3-exposure significantly decreases the total membrane current ($p < 0.01$) particularly at very positive potentials (+50 and + 70 mV).

In our experimental conditions, we assumed that I_{tot} is due to the contribution of potassium current ($I_{potassium}$), chloride current (I_{Cl}) and leak current (I_{leak}) that include the current flowing through the other non-voltage dependent channels (i.e. ENaC, CFTR, CNG, GABA etc. channels) and that we have shown to be insensitive to the action of ozone [29,62], as described in equation [1]:

$$I_{tot} = I_{potassium} + I_{Cl} + I_{leak} \quad [1]$$

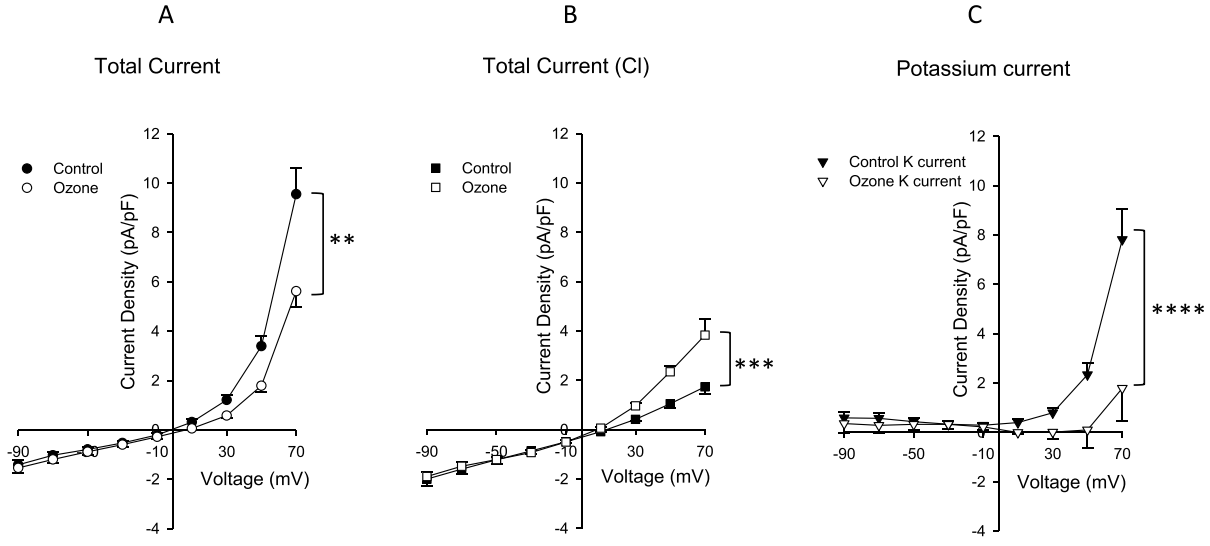


Fig. 2. Panel A: Current-Voltage relationship of control (n = 23) and ozone exposed (n = 7) cells. Panel B: Current- Voltage relationship of Cl-Control (n = 21) and Cl-ozone exposed cells (n = 8). Panel C: Current-Voltage curves mathematically obtained as indicated in Results: contribution to Itot due to Ipotassium before and after ozone exposure. The results were expressed as mean ± SEM. Comparisons between curves were performed by (Tukey's test ** = $p < 0.01$; *** = $p < 0.001$; **** = $p < 0.0001$).

In order to dissect the potassium current ($I_{potassium}$) in control conditions and after O_3 exposure, we substituted the KCl with CsCl in the intracellular solution as described in the Methods and we applied the protocol described above to the cells. The I–V curves obtained before and after ozone are depicted in Fig. 2B. As already demonstrate in our previous studies [62], with a 0-K^+ intracellular solution, O_3 exposure significantly increases the membrane current activating Cl^- channels without affecting I_{leak} (Tukey's test; $p < 0.001$).

In this condition we can assume that:

$$I_{tot(Cl)} = I_{Cl} + I_{leak} \quad [2]$$

To estimate the contribution of $I_{potassium}$ to the total control current, we can subtract [2] to [1]:

$$I_{potassium} = I_{tot} - I_{tot(Cl)} \quad [3]$$

We can calculate $I_{potassium(O3)}$ in the same way:

$$I_{potassium(O3)} = I_{tot(O3)} - I_{tot(Cl-O3)} \quad [4]$$

Fig. 2C shows $I_{potassium}$ and $I_{potassium(O3)}$ obtained from the previously described subtraction. K channels do not contribute to the inward part of the total current in both conditions because of the very negative E_{rev} of potassium current and O_3 treatment significantly decreases $I_{potassium}$ in its outward component (Tukey's test; $p < 0.0001$).

Literature indicated the presence of BK channels in A549 cells and their sensitivity to oxidative stress [47,52] therefore we want to verify the presence and functionality of BK channel. The channel is formed by four pore-forming subunits that are encoded by a single KCNMA1 gene [63].

The expression of KCNMA1 in the A549 cell line was demonstrated by immunocytochemical approach. Fig. 3A shows a positive staining for KCNMA1 and the immunoreactivity was localized in both nuclear and cytoplasmic compartments. Nuclei were visualized by staining with DAPI: the merged image of the two signals confirmed the nuclear staining for KCNMA1.

Treatment with IbTx, a specific inhibitor of BK channels, demonstrated its functionality, as depicted in Fig. 3B. The comparison between the control curve and the treated curve was highly significant (Tukey's test; $p < 0.0001$); IbTx cancels almost all the K^+ current, indicating that BK channels are the main way for K^+ flow. To confirm this result, we dissolved TEA-Cl, a non-specific inhibitor of K^+ current [60], in the intracellular solution and repeated the experiment. The I–V curve in presence of IbTx and the I–V curve in presence of TEA-Cl are not significantly different (Tukey's test; n.s.) indicating BK channel as the main active K^+ channel in these cells.

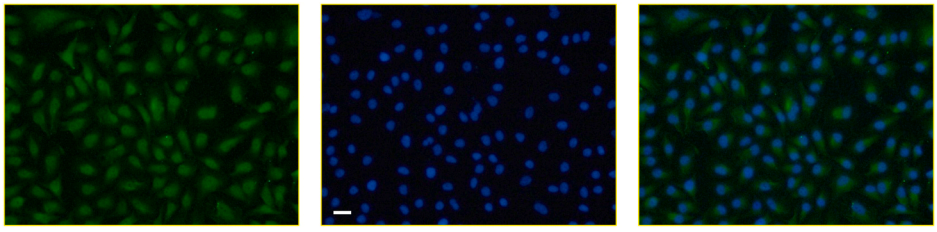
The expression of KCNMA1 in the A549 cell line after exposure to 0.1 ppm O_3 was analyzed by Western blot. As depicted in Fig. 4A, no differences were observed between A549 cells exposed to the pollutant and the control ones for all the analyzed time points (T0, T10′, T20′, T30′, T45′ and T60'), suggesting that the observed current decrease was not due to altered expression level of this potassium channel.

Concerning the qRT-PCR analysis of KCNMA1 gene expression (Fig. 4B), although at 60′ time point we observed a statistical difference between exposed and un-exposed cells, the statistical analyses indicated that the overall difference between the two experimental conditions is not statistically relevant (Tukey's test p = ns). Maybe the effect of ozone on gene expression needs more time to occur and this can be an interesting data to analyze in the future.

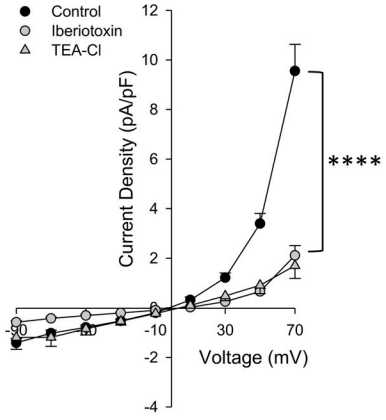
Therefore, to investigate whether the decreases in BK channel activity was due to post-translational modification of the channel induced by ozone exposure, we performed a co-localization analysis for KCNMA1 and 4HNE (one of the most reactive compounds generated by the interaction of ozone with cell membrane). As shown in Fig. 5, we observed a higher co-localization coefficient in A549 cells exposed to O_3 when compared to the control at all time points, suggesting an increased 4HNE-KCNMA1 protein adducts formation after pollutant exposure. This results can suggest that the formation of KCNMA1-4HNE adducts could be a possible mechanism by which ozone affects BK channel functionality.

Concerning the effect of ozone on the functionality of BK channel, we treated the cells with 4-aminopyridine, an inhibitor of voltage dependent potassium channels that does not affect BK channels efficiency.

In Fig. 6A, we compared the I–V curve obtained in this experimental condition with the control curve. Statistical analysis indicated that there was not significant difference between control and treated cells (Tukey's test; n.s.) demonstrating that the potassium current in control cells is



A



B

Fig. 3. Panel A: Representative image of immunocytochemistry for BK channel in A549 cell line: (left) FITC staining, (middle) DAPI staining of the same cells, (right) merge of the two previous images. Images are shown at 20X magnification. Scale bar = 50 μm. Panel B: Current-voltage relationship of control (n = 23), treated with IbTx (n = 6) cells and treated with TEA-Cl cells (n = 6). The results were expressed as mean ± SEM. Comparisons between curves were performed by Tukey's test (**** = $p < 0.0001$).

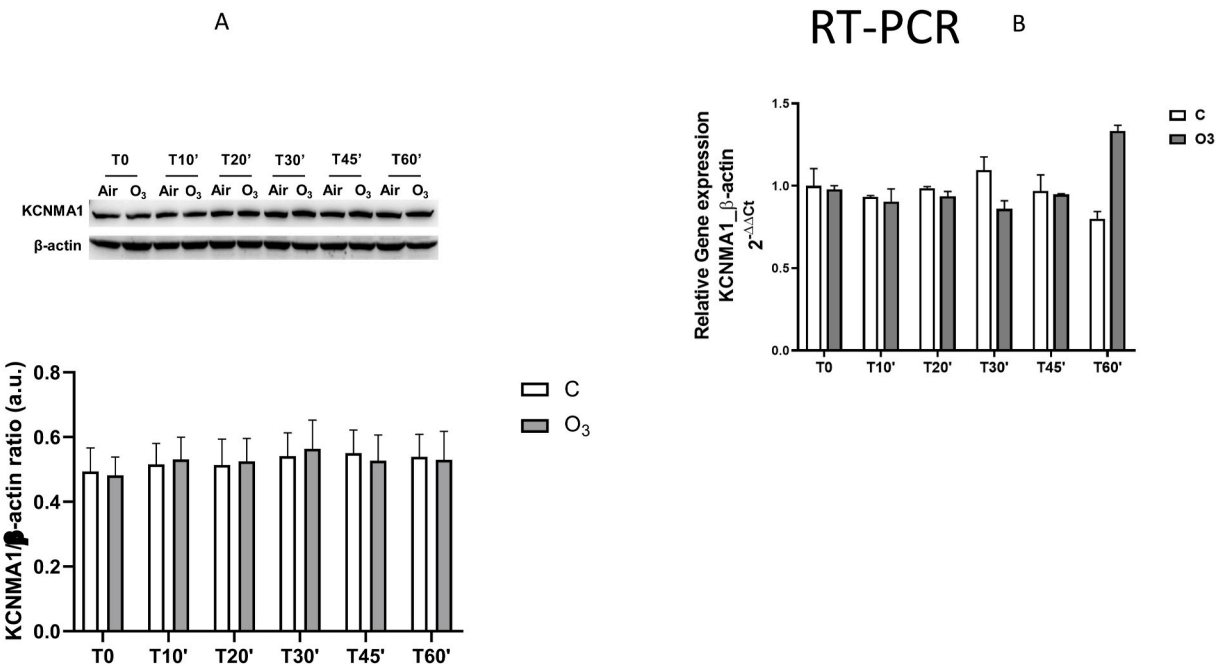


Fig. 4. Panel A: Expression level of BK channel in A549 cells at different time points after 0.1 ppm O_3 exposure. All the results were expressed as mean ± SEM. Different conditions were tested by Tukey's ($p = ns$). Panel B: Relative expression of BK channel mRNA in in A549 cells after 0.1 ppm O_3 exposure was determined using qRT-PCR. For all genes, the constitutively expressed β-actin rRNA was used to normalization. For each time point, the mean ± SEM of three independent experiments is shown. Different conditions were tested by Tukey's test ($p = ns$).

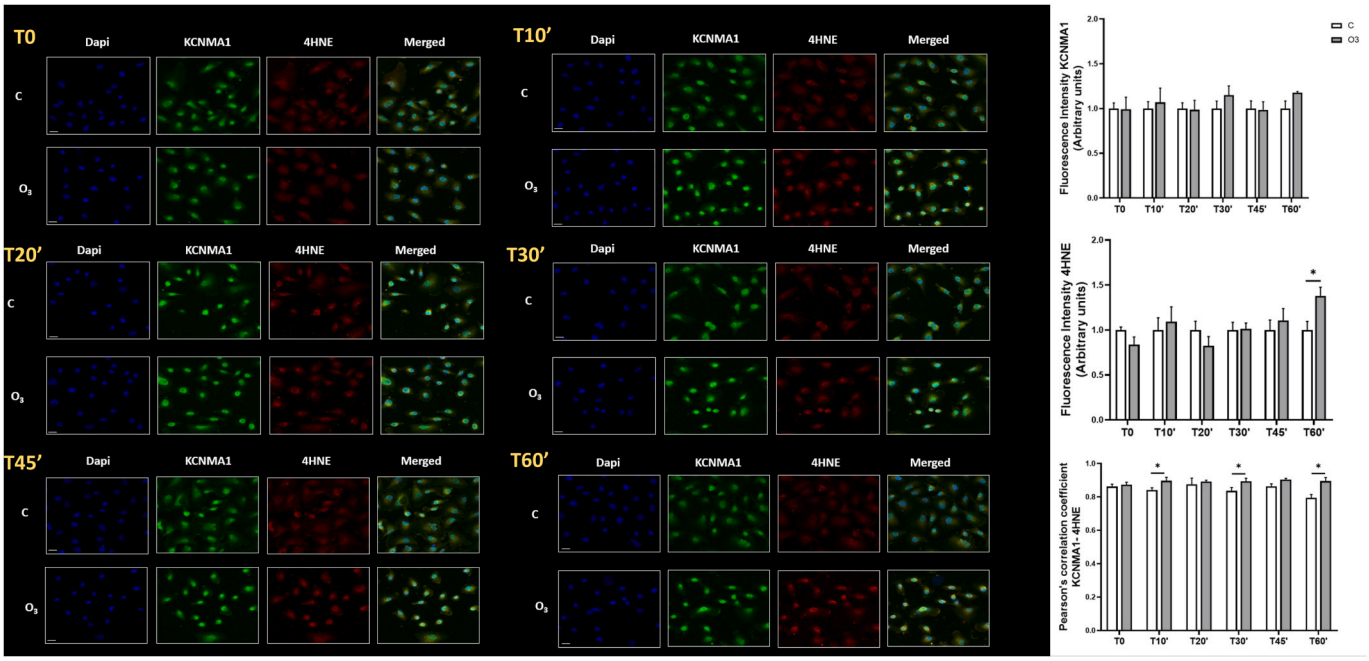


Fig. 5. Left panel: Immunofluorescence staining for KCNMA1 (green) and 4HNE (red) in A549 cells at different time points (0, 10', 20', 30', 45' and 1 hour) after 0.1 ppm O_3 exposure. The blue staining (DAPI) represents nuclei. Images were taken at 40X magnification (scale bar = 40 μm). Right panels: Analysis of fluorescence intensity of KCNMA1 and 4HNE signals, and Pearson's correlation values for co-localization of the two compaunds (* = $p < 0.05$). All the results were expressed as mean ± SEM.

mainly due to BK channels.

Panel 6B compared the I–V curve of cells treated with 4-AP with that of cells treated with both O_3 and 4-AP. It is evident that the ozone significantly lowered the membrane current (Tukey's test; $p < 0.05$) and we can infer that BK channel activity is its main target.

4. Discussion and conclusion

This work investigated the capability of ozone, at the concentrations present in the city troposphere (0.1 ppm [58]), to modulate the potassium current in lung epithelial cell line. This pollutant is a strong oxidant that in the lung, reacts rapidly and completely within the epithelial

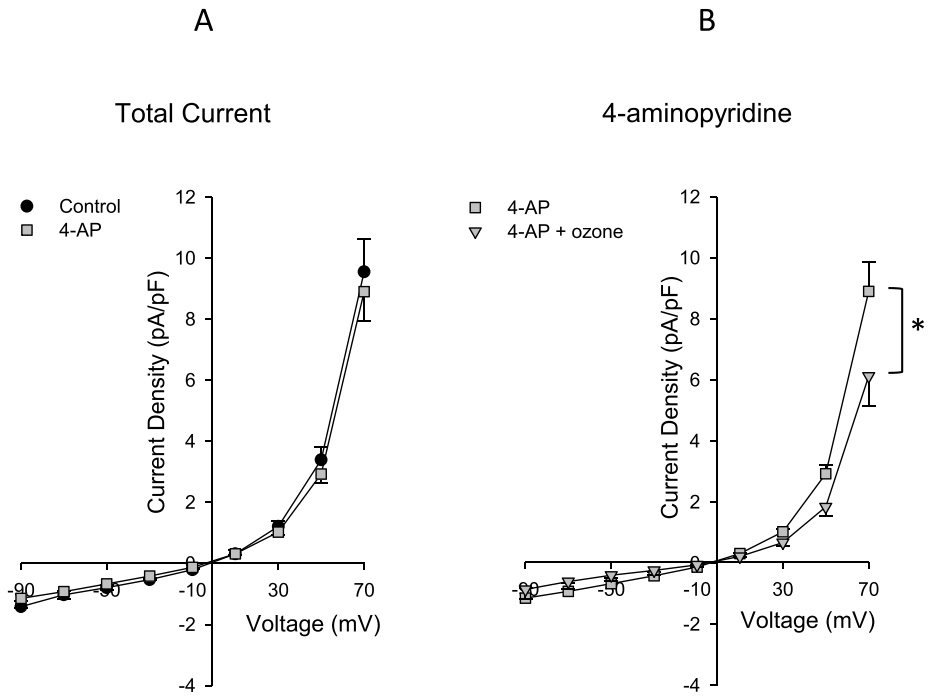


Fig. 6. Panel A: Current-voltage relationship of control (n = 23) and treated with 4-AP (n = 7) cells (Tukey's test p = ns). Panel B: Current-voltage relationship of 4-AP treated cells (n = 7) and 4-AP treated cells exposed to ozone (n = 10). The results were expressed as mean ± SEM; comparisons between curves were performed by Tukey's test (* = $p < 0.05$).

lining fluid (ELF), avoiding its penetration inside the cells [64]. Therefore, cellular effects of O_3 exposure may be mediated in large part by reaction products of ozone with cellular membrane. For example, various lipid ozonolysis products initiate signal transduction [65] and can induce inflammatory responses. Pryor and Church [12] indicated also that O_3 can induce a peroxidation process in the cells, leading to the formation of several derivatives due to its interaction with lipids among which aldehydes are among the most reactive compounds. Our group has previously shown that O_3 is able to increase the levels of lipid peroxidation products such as 4HNE which can covalently bind to proteins and affect their function [13,66]. Thus, ion channel protein chains may be important targets of ozone adducts. As many physiological processes regulated in the cells, the activation or inhibition of ionic channels can alter cellular functions and modify cellular homeostasis [67].

We focused on potassium current ($I_{potassium}$) because of its fundamental importance for a variety of type II pneumocytes functions, including the control of membrane potential and consequently of ion flow. Therefore, the ionic composition of the ELF, the first barrier against air pollutants [68], is strictly linked to the maintenance of membrane potential in a physiological range.

Our experiments indicated a significantly inhibitory effect of O_3 exposure on the total membrane current (Fig. 2A; $p < 0.01$; Tukey's test). Although with a physiological level of K^+ in the intracellular solution the total current decreased, experiments in which the intracellular KCl was substituted with CsCl indicated that, in absence of K^+ ions, the total membrane current significantly increased after treatment ($p < 0.01$; Tukey's test). We previously demonstrated [29] that the total membrane current increase is due to the activation of the Outward Rectifier Chloride Channels (ORCC), a channel turned on by oxidative stress and depolarized voltage. This indicated that the interplay between I_{Cl} and $I_{potassium}$ during ozone exposure tries to minimize its effect on the driving force acting on ions, but never less the I_{Cl} increase does not completely cancel the decrease of the total current. By a mathematical approach we calculated the contribution of K^+ to the total current, in control and after O_3 exposure demonstrating that the treatment almost completely cancels $I_{potassium}$ (it is significantly different from 0 only at +70 mV) and we tried to identify the K^+ channel type mainly involved in the cellular response.

A variety of K^+ channels has been described in A549 cells: Kir3.x, Kv9.3, KCa3.1, TREK1 and BK channels [30,32]. As BK channel is responsible of a big K^+ conductance and has been described in the literature as sensible to ROS [40,52], we investigated its role in cellular reaction to ozone.

Immunocytochemistry indicated the expression of the KCNMA1 gene, the gene coding for BK alfa-subunit, in our cells and the treatment of the cells with IbTx, a specific inhibitor of BK channel activity [59,69,70], demonstrated the functionality of the channel. Indeed, the total current significantly decreased ($p < 0.0001$; Tukey's test) when IbTx was added to the bath and the blockage of almost all the type of K^+ channels by TEA-Cl demonstrated that BK are the main active K^+ channels of these cells.

Western blot analyses confirmed the presence of the protein in A549 cell and indicated that the protein level was not affected by ozone exposure. Nevertheless, we demonstrated that O_3 exposure can induce the formation of 4HNE protein adducts with KCNMA1. Previous studies have suggested that 4HNE affects the functionality of different ion channels, inducing the decrease of ions current amplitudes [71,72]. Based on these considerations, we may assume that the altered functionality of BK channel and the resulted decrease in total current can be due to 4HNE-KCNMA1 protein adducts formation upon O_3 exposure.

To exclude that some other K^+ channel type little active in control condition, could be activated by ozone exposure we treated the cells with 4-aminopyridine (4-AP), an inhibitor of voltage dependent K^+ channels but not of BK [61,73] showing that this inhibitor minimally affect the current. When the cells treated with 4-AP were exposed to O_3, the total membrane current decreased and the K^+ current cancelled by ozone exposure were not significantly different from the K^+ current eliminated by the pollutant in control cells. These results indicate that most of the K^+ current in control cells is due to the BK channels activity and that the other K^+ channels are leakage channels whose action is important to maintain the physiological membrane potential (Em ≈ −30 mV) for e variety of events, but that cannot counteract important

shifts of ion flow because of their little conductance. Moreover, exposure to O_3 in presence of 4-AP induced a significantly decrease of the current ($p < 0.05$ Tukey's; Fig. 6B) demonstrating the strong involvement of BK channels in the cell response to ozone exposure.

In conclusion, the present work brings new insights on the effect that pollutants such as O_3, can have on cellular response, by the modulation of cellular ions currents, compromising therefore the physiological cellular homeostasis. These findings can help us to reconstruct what happens when a disturbing event alters the membrane potential of the cell. It will be interesting to continue the research on the ozone adducts responsible for the inhibition of the BK channel functionality and the molecular pathway involved to identify a pharmacological intervention acting on the interplay of $I_{potassium}$ and I_{Cl} that could rebalance the driving force on ion flow limiting the cellular damage.

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CRediT authorship contribution statement

Rita Canella: Conceptualization, Methodology, Validation, Investigation, Writing – review & editing. **Mascia Benedusi:** Conceptualization, Methodology, Validation, Investigation, Writing – original draft. **Andrea Vallese:** Formal analysis, Investigation. **Alessandra Pecorelli:** Investigation, Validation. **Anna Guiotto:** Investigation, Data curation. **Francesca Ferrara:** Investigation, Data curation. **Giorgio Rispoli:** Software, Methodology. **Franco Cervellati:** Resources, Software. **Giuseppe Valacchi:** Conceptualization, Writing – review & editing, Supervision, Project administration, Funding acquisition.

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