Original Research Article

Modulation of chloride currents in human lung epithelial cells exposed to exogenous oxidative stress†

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ABSTRACT

Air pollution continues to be a major public health concern affecting 9 out of 10 individuals living in urban areas worldwide. Respiratory tract is the organ most exposed to gas pollution and ozone has been shown to be one of the most noxious pollutants to which living organisms are exposed. In the present work, we have investigated the effects of 0.1 ppm of ozone on chloride currents in human lung epithelial cells (A549 line) and whether this effect could be modulated by vitamin E pre-treatment.

Whole-cell patch clamp technique was applied to not excitable cells in order to obtain information about chloride currents behavior, important for epithelial lung cells homeostasis. Significant alteration of the I-V curve after ozone treatment was observed, with the appearance of a large outward rectifier component decreasing over time and returning to the basal state levels after 24 hours. Statistical analysis indicated a modification of the amount of ions passing the membrane in the unit of time as a possible cause of this difference. RT-qPCR analysis showed an increase in ClC-2 and ORCC mRNA after ozone exposure.

In addition, pre-treatment with vitamin E was able to suppress the outward rectifier component induced by ozone, bringing back the current values to the control level and preventing ozone induced chloride channels up regulation.

Our data suggest that ozone exposure is able to modify chloride current density and the use of vitamin E can prevent the above mentioned damage.

INTRODUCTION

Air pollution is a major environmental risk to health and its exposure has been associated directly or indirectly to several pathologies such as stroke, heart disease, lung cancer, asthma and both chronic and acute respiratory diseases (Sun et al., 2010; Cui et al., 2016; Bowatte et al., 2016; Malhotra et al., 2016; Ogawa and Kishi, 2016; Tomczak et al., 2016; Seaton et al., 1995; Guarnieri and Balmes, 2014).

When we breathe in dirty air, we bring air pollutants deep into our lungs, so it's no surprise that air pollution causes serious damage to the respiratory tract which is the first target of gasseous stressors. It has been well documented that pollution exposure can trigger new cases of asthma, exacerbate (worsen) a previously-existing respiratory illness, and provoke development or progression of chronic illnesses including lung cancer, chronic obstructive pulmonary disease, and emphysema (Lopez-Campos et al., 2016; Ling and van Eeden, 2009.). Air pollutants also negatively and significantly harm lung development, creating an additional risk factor for developing lung diseases later in life (Kurt et al., 2016).

Among the several environmental stressors to which we are daily exposed Ozone (O_3) is one of the most reactive and toxic. O_3, one of the most widespread air pollutants, is formed when volatile organic compounds react with nitrogen oxides in the presence of sunlight. It irritates the lungs at concentrations that are fairly common in urban settings, particularly in summer months (Lockwood, 2009).

O_3 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. It oxidizes lipids directly to ozonides, which decompose to aldehydes, many of them are very reactive and noxious to cells (Pryor and Church, 1991). Epithelial lung lining fluids (ELF) may react with O_3 producing various byproducts, such as organic and hydrogen peroxides, aldehydes, and organic radicals, which may promote oxidative damage. The respiratory tract, is one of the most vulnerable targets to oxidative stress in the body.

In general ELF represents the first line of defense against microbial and environmental aggressions. Its ion composition and osmolarity control the ciliary beat frequency and may also regulate mucin exocytosis (Widdicombe and Widdicombe, 1995) and the bactericidal capacity of neutrophils (Mizgerd et al., 1995). It has been shown that lung epithelial lining fluid (ELF) contains lipophilic and hydrophilic antioxidants, among which vitamin E has been shown to be the most effective in preventing the formation of oxidized lipids and for this reason tocopherol has been defined as the most important lipophilic antioxidant in the lung (Sabat et al., 2001). The exceptional role of vitamin E in the defense system of the lung is underlined by the findings that extreme oxidative burdening of the lung such as smoking, O_3 or paraquat, does not lead to an expected decrease but rather to an increase of the concentration of α-tocopherol in the lung. This result has been interpreted as mobilization of vitamin E from other tissues to maintain adequate its levels in the lung (Elsayed, 2001). Investigations on relation of serum levels of vitamin E and other antioxidants with the pulmonary function in the general population show that α-tocopherol correlates better with lung function than vitamin C or retinol (Schunemann et al., 2001). Kolleck et al. (2002) demonstrated that vitamin E reaches the alveolar space as an integral constituent of ELF secreted by type II pneumocytes.

Type II cells are the only cells involved in surfactant secretion in the respiratory tract and their damage can

affect the defensive system presents in the lung against environmental stressors (Akella and Deshpande, 2013). Surfactant secretion is a critical regulated process in the metabolism of pulmonary surfactant. Presumably, because this process is vital to the survival of the organism, there are several independent pathways for stimulating secretion that works through different cell surface receptors and signaling mechanisms. As many physiological processes regulated in the cells the activation of ionic channels can alter cellular functions and modify cellular homeostasis (Rao et al., 2015).

In the lungs, epithelial chloride channels mediate *trans*-cellular Cl^- secretion, a major driving force for fluid secretion (Pitkanen, 2001). At birth, chloride secretion becomes significantly decreased, facilitating perinatal lung fluid clearance and preparing the lung for air breathing (Kitterman et al., 1979; Dickson et al., 1986). Although Cl^- secretion is reduced in adult lungs, it is essential for the maintenance of airway innate immunity (Widdicombe and Widdicombe, 1995). The mRNA for ClC-2 is present in fetal and adult human lung (Cid et al., 1995; Sherry et al., 1997) and it appears to provide one of the pathway for chloride transport across the lung epithelia (Cuppoletti et al., 2000). On the other hand, the ORCC channels (Outward Rectifyer Chloride Channel), often identified as VSOR (Volume Sensitive Outward Rectifyer) are ubiquitous (Martins et al., 2011) and activated by oxidative stress (Shimizu et al. 2004).

Therefore, in the present study we want to evaluate the effect of O_3, at doses found in polluted cities (0.1 ppm), on chloride currents via an elettrophysiological approach and whether pre-treatment with vitamin E (the most potent antioxidant able to prevent lipid peroxidation) could modulate O_3 effect on chloride currents.

MATERIALS AND METHODS

Cell culture

A549 cells were purchased from ATCC (Rockville, MD). Ham's F-12, fetal bovine serum, RPMI-1640, penicillin/streptomycin and l-glutamine were obtained from Lonza (Milan, Italy). Cell suspension containing 5×10^6 viable cells/ml were used. Cells were incubated at 37 °C for 24 h in 95% air/5% CO2 until 80% confluency.

For Vitamin E experiments cells were pretreated with 100 µM of α-tocopherol 48 hrs before exposure as previously described (Nardini et al., 2002).

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. After pretreatment with different antioxidant mixtures, cells ($1x10^6$ cells/well in 1.2 ml of media in 6 cm Petri dishes) were exposed to filtered air or to O_3 0.1ppm, for 30 minutes. 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 (Valacchi et al., 2015). Temperature and humidity were monitored during exposures (37°C and 45–55%, respectively).

RT-QPCR (Reverse Transcription Quantitative Real-Time PCR)

Quantitative real-time PCR was carried out as previously described (Pecorelli et al. 2016). Briefly, total RNA was extracted, using an AURUM total RNA Mini Kit with DNase digestion (BioRad), according to the manufacturer's recommended procedure. First-strand cDNA was generated from 1 pg 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 nMeach primer, 1 p l of cDNA, and 7 p l of iQ SYBR Green Supermix (Bio-Rad), with RNase-free water being used to bring the reaction mixture volume to 15 Pl. All reactions were run in triplicate. Real-time PCR was initiated with a 3-min hotstart denaturation step at 95°C and then performed for 40 cycles at 95°C for 3 s and 60°C for 5 s. During the reaction, fluorescence, and therefore the quantity of PCR products, was continuously monitored by Bio Rad CFX Manager software (Bio-Rad). Primers were initially used to generate a standard curve over a large dynamic range of starting cDNA quantities, permitting calculation of the amplification efficiency (a critical

value for the correct quantification of expression data) for each of the primer pairs. Ribosomal proteins L13a (RPL13a) and L11a (RPL11a) and GAPDH were employed as housekeeping genes. Samples were compared using the relative cycle threshold (CT). After normalization to more stable mRNA RPL13a, RPL11a, and GAPDH, the fold increase or decrease was determined with respect to control, using the formula $2^{-\Delta\Delta}$ CT, where Δ CT is (gene of interest CT) (reference gene CT), and ΔΔ CT is (Δ CT experimental) (Δ CT control).

Patch Clamp Technique

Patch pipettes were pulled from glass capillaries with 1.0 mm outer diameter using a micropipette puller (NARISHIGE Instruments, Japan, mod PP-830), fire-polished (tip resistance between 2 and 5 M) and filled with an intracellular solution. In order to characterize the overall membrane current response, the following solutions were employed:

6 Intracellular solution containing (in mM): 145 KCl, 1 MgCl2, 10 HEPES and 5 EGTA; the pH was adjusted with KOH up to the value of 7.4;

6 Extracellular solution containing (in mM) 145 NaCl, 1.8 CaCl2, 1 MgCl2, 5.4 KCl, 10 glucose and 10 HEPES; pH was adjusted with NaOH until the value of 7.35.

To isolate chloride current intra and extracellular solutions were changed as follows:

6 Intracellular solution containing (in mM) 1 MgCl2, 10 HEPES, 5 EGTA and 130 CsCl; pH was adjusted with TEAOH up to the value of 7.4;

6 Extracellular solution containing (in mM) 135 NaCl, 1.8 CaCl2, 1 MgCl2, 5.4 KCl, 10 glucose and 10 HEPES; the pH was adjusted with NaOH until the value of 7.35.

Osmolality was adjusted with sucrose to obtain values between 300 and 310 mOsm/Kg.

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.

After a gigaseal had been formed, intracellular access was established by suction.

Whole cell currents were elicited by voltage-clamp pulses (1,400 ms duration) between +70 and -110 mV in 20mV increments from a beginning holding potential of -30 mV.

The voltage protocol and data acquisition were performed with Digidata card 1322A and pClamp package (version 9.0). 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 5 (total current) and 10 kHz (chloride current) and stored on disk.

The cell samples exposed to O_3 were electrophysiologically examined at time 30 minutes, 6 hours and 24 hours after treatment.

4-(2-butyl-6,7-dichloro-2-cyclo pentyl-indan-1-on-5-yl) oxobutyric acid (DCPIB; Tocris) was dissolved in ethanol and added to the perfusion solution to achieve a final concentration of 10□M; tunicamicyn (Sigma-Aldrich) and arachidonic acid (AA; Sigma-Aldrich) were dissolved in dimethylsulfoxide (DMSO) and added to the perfusion solution to achieve a final concentration of 3□M and 2□M respectively; $CdCl_2$ (Sigma-

Aldrich) was dissolved in water and added to the perfusion solution to achieve a final concentration of 50 M.

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.6, and the significance of P values were reported in the text and figures (Student's t test for means, and two-way ANOVA; significant differences for $P < 0.05$).

RESULTS

Electrophysiological characterization

Passive parameters of A549 cells under each experimental conditions were measured. The resting membrane potential was -43.17 ± 5.20 mV in 6 cells studied, according to published data (Jovanović et al., 2003); average membrane resistance was 2,70 ± 0,64 G and the capacity was 34,65 ± 1,83 pF.

Compound current were evoked in control, applying the voltage-clamp protocol described in Methods without any selective blocker. The obtained current recordings are shown in Fig. 1, panel A (mean traces, n = 7).

As it is shown in Fig.1 the compound current rapidly built up in to a steady state level maintained over time. This stationary level varied proportionally with voltage command, up to +30 mV, but its magnitude increased for more positive voltages, giving rise to a strong outward component in the I-V relation.

Accordingly, we isolated the chloride current by using intracellular CsCl as a blocker of potassium channels. Figure 1, panel B (mean trace, n = 5) shows the recorded traces. The tracing time courses became a monotonic relation of current to voltage with the disappearance of the outward component of the current of control cells. Apparently, it is cancelled by CsCl at very positive command potentials, showing to be mainly K^+ current.

Ozone exposure effect on the chloride current

After exposing the cells to 0.1ppm of O_3 for 30 minutes we evaluated the electrophysiological effects on A549 cells, 30 minutes (time 0, panel B), 6 hours (panel C) and 24 hours (panel D) after the exposure. As it is shown in Fig. 2, it appeared that the exposure to O_3 produced marked changes in the steady-state current amplitude for each group of treated cells, that tend to disappear over time after O_3 exposure.

Analysis of ozone exposure on current

Figure 3 shows that the inward component of the current is substantially the same in all experimental conditions, while it is evident a strong outward rectifier effect of the treatment at t0 (30 min after O_3 exposure), decreasing gradually over time, and going back to the steady level after 24 hours.

The statistical analysis (2-way ANOVA) confirmed a significant diversity between controls and time 0 ($P < 0.01$) and time 6 hours ($P < 0.05$) of the O_3 exposed cells. On the other hand, the comparison between controls and time 24 hours was not significant. Carrying out the analysis up to +50 mV (range of more physiologic voltage), the comparison between controls and time 0 was still significant ($P < 0.05$).

To understand whether the action of O_3 involves other ion species, we compared the equilibrium potential of the mean currents studied at each experimental condition with the control. Student's *t* values were not significant for any comparison, indicating that the treatment does not alter the ionic composition of the activated current, but only its conductance.

Subsequently, we evaluated the effect of O_3 on the cell conductance. As it is shown in Fig.3B the average conductance of each group of cells, with SEM (n = 5), indicates a significant difference between controls and time 0 ($P < 0.01$) and between controls and time 6 hours ($P < 0.05$).

We attempted to clarify whether oxidative stress affects the functionality of ClC-2 and/or ORCC channels. RT-QPCR for ClC-2 mRNA indicates that O_3 exposure induces a significant increase in the mRNA levels, at all the time points considered after the exposure (Fig. 3C; $P < 0.01$ for 0 and 24 hours; $P < 0.001$ for 6 hours).

Despite ORCC channel have been discovered about thirty years ago, its gene has not yet been identified (Verkman and Galietta, 2009); some authors have proposed that it matches the ClC-3 channel, but the evidence is contradictory (Duan et al., 1997; Hara-Chikuma et al. 2005). Other authors have demonstrated the presence of anoctamin 6 as an essential component of this channel (Martins et al., 2011). Therefore, we have analyzed the mRNA levels of anoctamin 6. The results are shown in Fig. 3D and indicate that the O_3 treatment was able to affect also the mRNA levels of anoctamine 6. Of note it that at time 0 the amount of mRNA had dramatically decreased ($P < 0.001$) and it rises again at t6 and t24 significantly respect to the control ($P < 0.01$ at t6 and $P < 0.05$ at t24).

To verify whether the electrophysiological results were a consequence of the activation of ClC-2 and/or ORCC channels, we performed experiments with specific inhibitors for the two channels. As it is shown in Fig.4A, treating the control cells with tunicamycin, a selective activator of ORCC channels (Zhang et al., 2011; Shen et al., 2014), I-V curve was not statistically different from t0 curve. On the other hand, administrating DCPIB (selective inhibitor of ORCC, Shen et al., 2014) to the cells after O_3 exposure, prevents the appearance of the outward component of the current. Figure 4B shows that the action of the activator (arachidonic acid, Cuppoletti et al., 2001) and the inhibitor of ClC-2 channels (Duan et al., 1997; Verkman and Galietta, 2009) alters the control and t0 curves to a lesser extent than the tunicamycin and DCPIB.

These tests let us to suggest a prevalent involvement of ORCC channel in the observed phenomenon.

Protective effect of vitamin E

In order to verify whether the observed effect of O_3 treatment on chloride current was oxidative stress mediated, A549 cells were pre-treated with 100 µM of vitamin E 48h before O_3 exposure. The dose of 100 µM was chosen based on dose-viability preliminary studies (not shown) and on our previous work (Nardini M et al. 2002). Electrophysiological experiments were performed 30 minutes after O_3 exposure. Figure 5 displays example traces of control chloride and t0+vitE chloride currents. No changes of amplitude, rising and falling phase are evident.

Analysis of the vitamin E effect on chloride current

The I-V curves for control, and O_3 + vit.E (t0+vitE), are shown in Figure 6A.The graph clearly shows that treatment with vitamin E eliminates the outward rectifier current component activated by oxidative stress.

The statistical analysis (2-way ANOVA) between controls and t0+vitE was not significant, indicating the protective effect of tocopherol.

The protective effect of vitamin E with respect the O_3 exposure, on chloride conductance is shown in Fig. 6B. The average conductance of control cells is not significantly different from that of t0+vit.E group.

In addition, as it is shown in Fig.6C and D, pre-treatment with vitamin E was able to counteract the mRNA

induction by O_3 treatment for both ClC-2 and anoctamine 6, although the latter seems to be more susceptible to vitamin E treatment.

The comparison by Student's t, between mRNA levels at all the time points between O_3 treated cells and Vit.E + O_3 exposed cells was consistently significant. In regards to both ClC-2 and anoctamine 6, in the presence of vitamin E the mRNA levels at each time points were significantly lower respect to O_3 alone.

DISCUSSION

The focus of the present work was the study of the possible redox modulation of chloride current in in lung epithelial cell line. To do so we have exposed cells to a well known environmental stressor (O_3) and evaluated the protective effect of vitamin E pretreatment.

O_3, is a strong oxidant it has been shown that in the lung reacts rapidly and completely within the epithelial lining fluid (ELF), avoiding its penetration inside the cells (Pryor, 1992). Therefore, cellular effects of O_3 exposure may be mediated in large part by reaction products of O_3 with cellular membrane. For example, various lipid ozonolysis products initiate signal transduction (Kafoury et al., 1998) and are able to induce inflammatory environments. O_3 is able to induce a peroxidation process in the cells and the products derived from the interaction between O_3 and lipids process include aldehydes (Pryor and Church, 1991) which are sufficiently stable. Indeed, it has been suggested that molecules able to prevent O_3 induced lipid peroxidation would be beneficial. One potential target of O_3 oxidation could be α-tocopherol because it is quantitatively the major lipid-soluble antioxidant in plasma (Ingold et al., 1987), although under some circumstances α-tocopherol showed a pro- rather than an antioxidant activity (Ingold et al., 1993). Several controversial studies have shown different effects of O_3 on tocopherol lung concentration. For instance, Kadiiska et al. (2011) did not notice any difference in lung tocopherol concentration in animals exposed to 2 and 5 ppm of O_3 and also an animal study from Vasu et al (2010) was not able to demonstrate a variation in tocopherol levels after 0.5 ppm of O_3 exposure for a week in mouse lung tissue homogenates. On the other hand, other murine studies reported evidence of α-tocopherol depletion in the lung and BALF (Bronchoalveolar Lavage Fluid) after O_3 exposure (Valacchi et al., 2004; Kodavanti et al., 1996). In addition, there are studies that have even found increase of vitamin E levels after O_3 exposure (Elsayed et al., 1990) justifying these phenomena as a recruitment of the lipophilic antioxidant from the plasma compartment to the lining fluid levels. The controversy of the data present in the literature can be a consequence of the different doses of O_3 studied and also of the different mouse strain and time points chosen. Indeed it has been clearly shown that the different mouse strains have the own peculiar susceptibility to environmental pollutans (Gardi and Valacchi 2012).

Our data have shown that pre-treatment with tocopherol can rescue the chloride current alteration induced by O_3 confirming indirectly, the ability of O_3 to induce oxidative stress damage. Indeed, as our group has shown before, O_3 is able to increase the levels of lipid peroxidation products such as 4HNE which is able to covalently bind to proteins and affect their function (Valacchi et al., 2004). Of note is the fact that O_3 is able to compromise the levels of genes involved in tocopherol tissue transport, confirming its ability to compromise lung antioxidant levels not only by its direct interaction with the antioxidants but also indirectly, by affecting its plasma-to-cells transport (Valacchi et al., 2007). In our study we have performed experiment in A549 cells that share many characteristics with both type I and II pneumocytes which are one of the main tropospheric O_3 target. In these cells the chloride current is fundamental for a variety of cellular functions, among which stands out the formation of the ELF that is the first barrier against air pollutants (Pitkanen, 2001). CFTR and ClC-2 channels were indicated, in A549, as main responsible for the chloride current. In patch-clamp experiment,

CFTR activation in intact cells, requires stimulation of the endogenous PKA by increasing the cytoplasmic concentration of cAMP (Moran and Zegarra-Moran, 2008). Since our experiments were performed in absence of cAMP or PKA activators, we assumed that the chloride current observed in our control experimental conditions is attributable mainly to the ClC-2 channel. The bath and pipette solutions were similar to the solutions used for most studies of chloride currents (Fahlke et al., 1997; Fahlke et al., 1995; Park et al., 1998). Control chloride currents amplitude and trend were in agreement with published data (Cuppoletti et al., 2001).

Treating the cells with O_3, causes a remarkable alteration of the I-V curve specially in the cells analyzed immediately after treatment (time 0, ANOVA, $P < 0.01$), with the appearance of an outward rectifier component. This modification decreases after 6 hours from the treatment while remaining significant ($P < 0.05$) until it disappears after 24 hours. For t0 cells the difference was significant also in the more physiological range - 110/+50 mV. The comparison of the reversal potential of the I-V curves obtained at the different experimental conditions, indicated that the difference between the curves is not due to a modification of the type of ion crossing the membrane but to their amount.

ClC-2 channel has been described in the literature, as inward rectifier in cardiomyocytes (Bi et al., 2014), even if this feature varies according to experimental conditions and cell type. In the work by Cuppoletti et al. (2001), the authors did not see inward rectification in HEK-293, Calu-3, BEAS-2B and A549 cells. In addition, further studies (Schwiebert et al., 1998) observed that in human cells overexpressing ClC-2 at physiological pH, the I-V curves showed only a slight inward rectification. This phenomenon was not observed in the present study. However, all the cell types mentioned above showed a linear I-V without outwardly rectifying current also after activation of ClC-2 channels.

Instead, A549 cells treated with O_3 showed current traces similar to those observed for the ORCC channels in other tissues (Shimizu et al., 2004; Martins et al., 2011; Shen et al., 2014). In fact, this chloride channel sensitive to the volume, outward rectifier, ubiquitous (sometimes referred to as ORCC, other as VSOR, even if there is no certainty that it is the same channel) has been described in several papers. In HeLa cells, this channel is involved in apoptosis (Shimizu et al., 2004) and it is activated by oxidative stress in rat cardiomyocytes (Shen et al., 2014). Tests performed with inhibitors and activators of the two types of channels, indicate that ORCC is definitely the most involved in the modification of the I-V curve due to O_3 exposure, although the involvement of ClC-2 channels cannot be completely excluded.

RT-QPCR indicates that O_3 exposure was able to induce an increased level of the mRNA for both ClC-2 and anoctamine 6 genes. This effect it most likely linked to the oxidative effect of O_3 since pre-treatment with vitamin E was able to partially abolished the mRNA induction. Although there are no data on the effect of oxidative stress on ClC-2 channel, our results are in line with a recent work showing that the increased oxidative stress present in a diabetic model was able to induce the expression of chloride channel ClC-3 and that the pre-treatment with trolox (the water soluble analog of vitamin E) was able to prevent chloride channel induction (Ramana KV et al., 2004).

In addition, pre-treatment with vitamin E was not only able to prevent the mRNA induction of chloride channel but also to abolish the modification of the I-V curve due to O_3 exposure (Fig.6), suggesting a possible

direct link between oxidative stress and chloride current.

It should be mentioned that O_3 is not the only pollutant to which we are exposed, for instance, there are environmental stressors such as cigarette smoke that have the ability to induce similar "bioactive molecules" to the one derived by the interaction between O_3 and biological systems. Therefore, it is possible that the effect on chloride channel by O_3 could be detectable also after the exposure to other pollutants present in the outdoor environment (Gardi and Valacchi, 2012) .

In conclusion, the present work brings new insight on the effect and damage that pollutants, and in this specific case O_3, can have on cellular response, such as the modulation of cellular ions currents, compromising the physiological cellular lung homeostasis and this effect can be rescue by tocopherol supplementation. Moreover, since ORCC channels are ubiquitous and are activated during apoptosis, further studies may be aimed to clarify the protective role of vitamin E during cell death in other cell types. In fact, many evidence has demonstrated that oxidative stress-induced cell death is an essential step in the pathogenesis of a variety of diseases such as atherosclerosis, myocardial infarction, hypertension and tumorigenesis.

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Figure legend

Figure 1. Voltage-clamp recordings: voltage-clamp pulses (1400 ms duration) between +70 and -110 mV in 20mV increments from a beginning holding potential of -30 mV A) total current recordings (mean traces of 7 cells) without K^+ current blockers; B) registrations of chloride current (mean traces of 5 cells), in the presence of intracellular CsCl as a blocker of the potassium channels.

Figure 2. Representative families of chloride recording currents in control conditions (panel A) and at various times after ozone exposure (panels B, C and D).

Figure 3. Panel A: Current-voltage relationship for the chloride current in 5 cell samples in control and at the appointed times. The difference between the curves was evaluated by two-way ANOVA (** = P <0.01; * = P <0.05). Panel B: Mean conductance of the four groups of cells analyzed. Student's t between means is significant for CTL vs t0 and for CTL vs t6 (P < 0.01 and P <0.05). Panel C: RT-QPCR results for ClC-2 mRNA. Student's t comparison between CTL and treated cells is significant at any time after treatment.-Panel C and D: RT-QPCR results for ClC-2 mRNA and RT-QPCR results for anoctamin 6 mRNA.Student's t comparison between CTL and treated cells is significant at any time after treatment (*** = P<0.001; ** = P <0.01; * = P <0.05).

Figure 4. Panel A: Current-voltage relationship for the chloride current in 5 cell samples before and after treatment with O_3, tunicamycin and O_3+DCPIB. Panel B: Current-voltage relationship for the chloride current in 5 cell samples before and after treatment with O3, Arachidonioc acid and O_3+CdCl2.
The comparison between curves have been evaluated by two-way ANOVA .

Figure 5. Voltage-clamp recordings: A) example traces of control chloride currents; B) example traces of ozone t0+vitE chloride current.

Figure 6. Panel A: Current-voltage relationship for the chloride current in control (n=5), O_3 t0 (n=5) and ozone t0+vitE (n=6) treatments. The difference between the curves evaluated by two-way ANOVA was not significant. Panel B: Mean conductance of CTL, t0 and t0+vit.E groups of cells. Student's t between means of controls and cells treated in vitamin E is not significant; Panel C and D: RT-QPCR results for ClC-2 mRNA and RT-QPCR results for anoctamin 6 mRNA.. Student's t comparison between cells treated with O_3 and cells treated vit.E + O_3 are significant (*** = P<0.001; ** = P <0.01; * = P <0.05).

Table 1

Gene	Primer sequence	T_a °C	Product length NP)	QPCR Amplification Efficiency	n° of cycles	Ref. Primer Bank
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 - R : 5'- ggctggtggtccaggggtct -3	60	134	94.6	39	GenBank Accession NM 002046.3
CCl-2	F : 5'- tcctcaccctggtcatcttc -3' R : 5'- gcaggtagggcagtttcttg -3	60.2	402	96.5	39	GenBank Accession NM_001243372.1
RPL13A	F : 5'-cccgtccggaacgtctataa- 3' R : 5'-ctagcgaaggctttgaaattcttc-3'	60.2	203	97.3	39	GenBank Accession NM 000977.2
RPL11A	F : 5'- tgcgggaacttcgcatccgc-3' R : 5'- gggtctgccctgtgagctgc-3'	60.1	108	96.5	39	GenBank Accession NM 000975.2
Anoct-6	F : 5'- ctgagcccaagcgacaca -3 R : 5'- ccattaaattcttccgatatcccc -3'	59.9	204	94.6	39	GenBank Accession XM 005268707.3

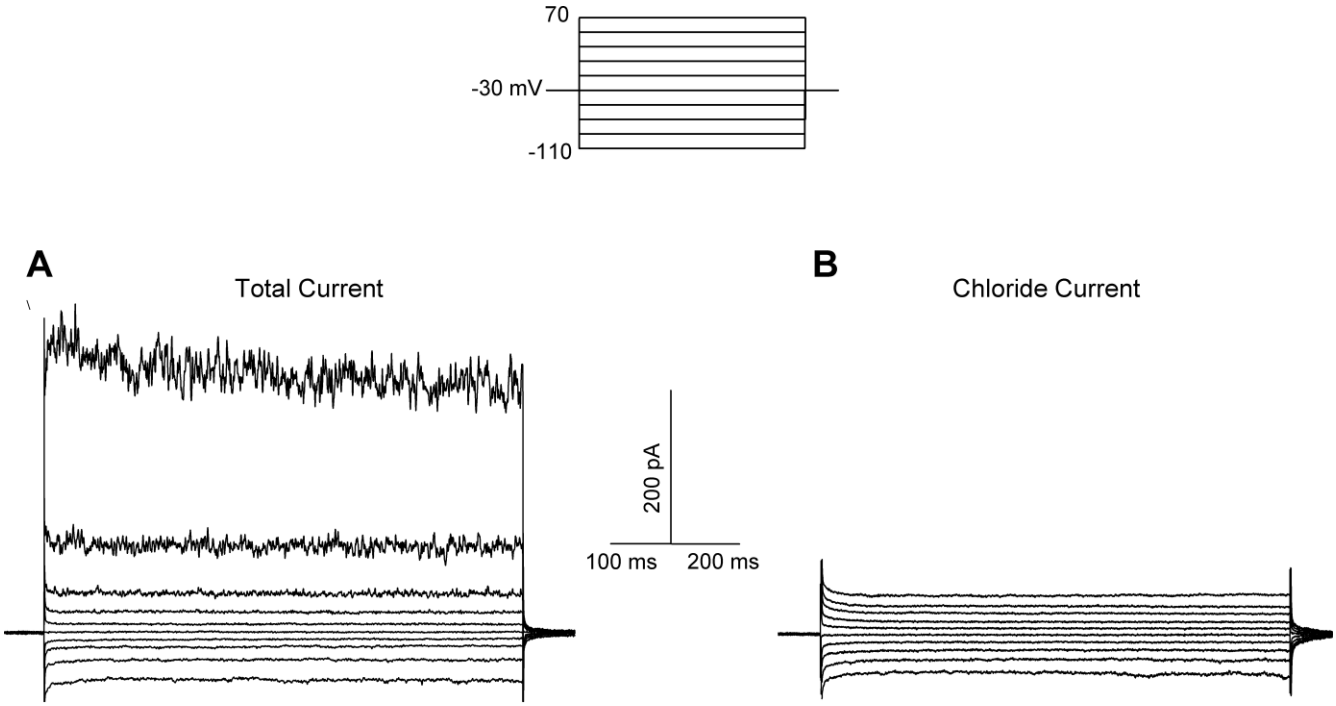
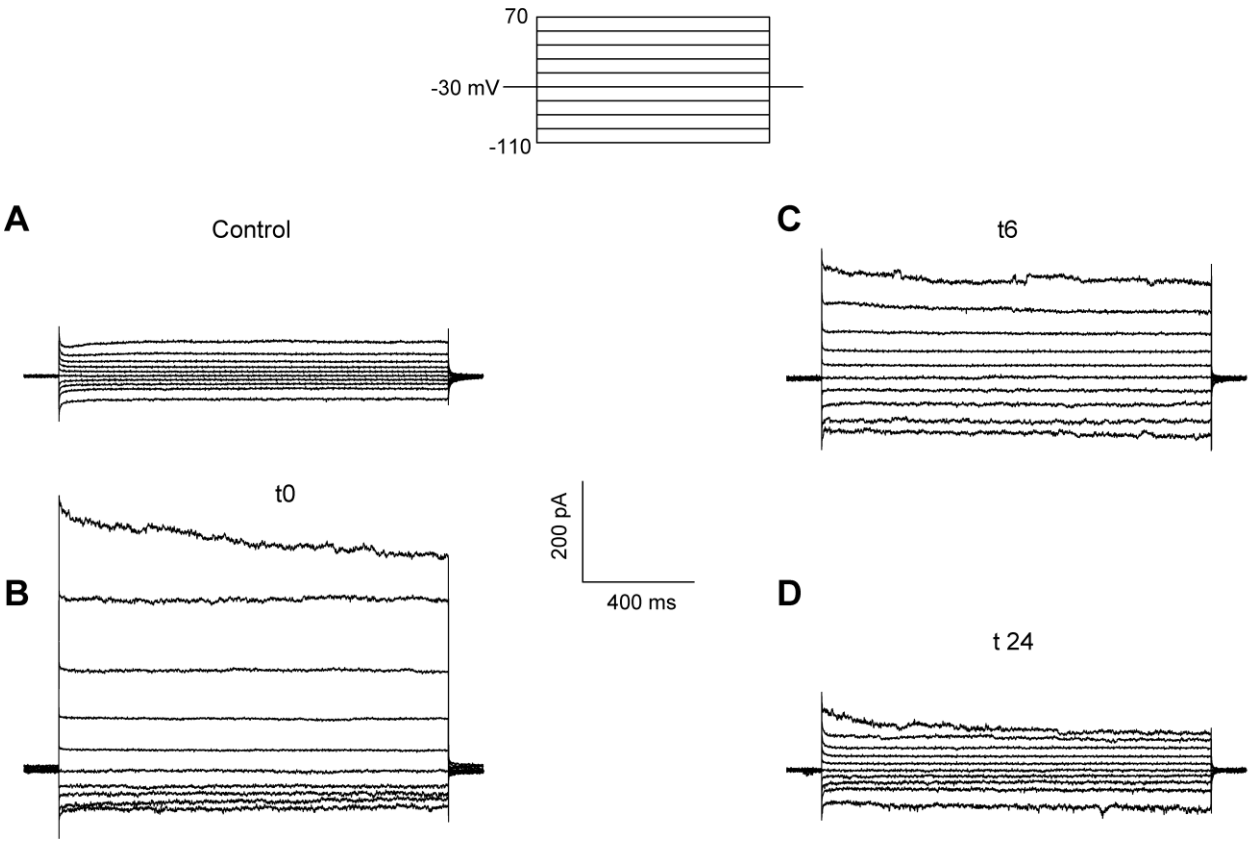


Figure 1

Figure 2



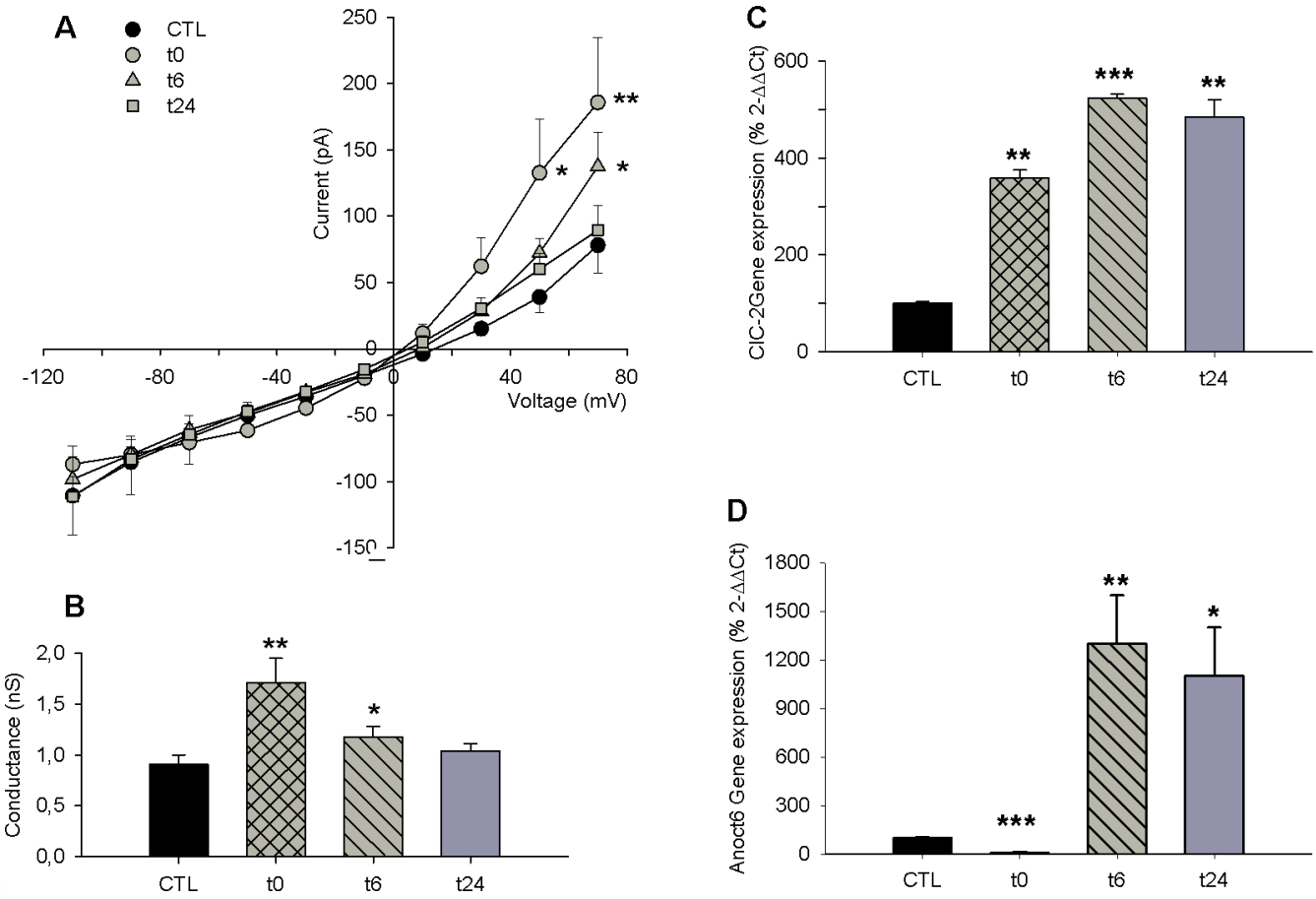


Figure 3

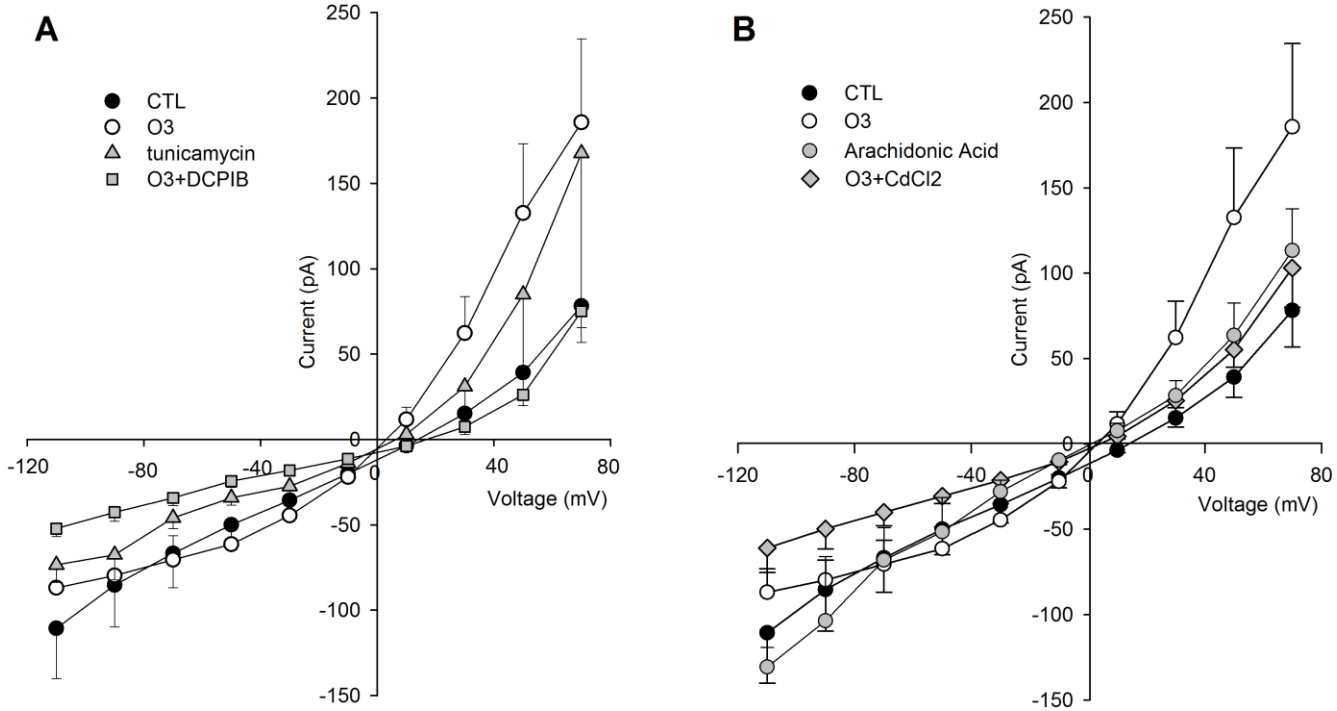


Figure 4

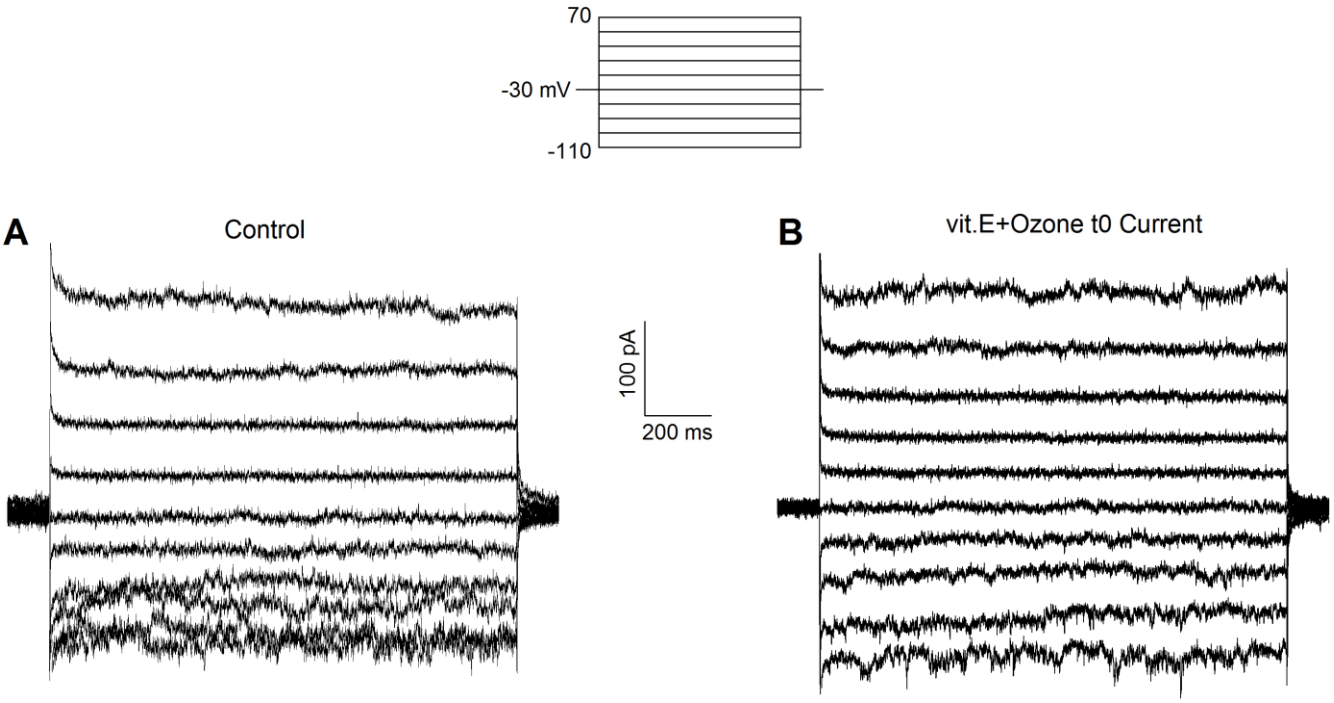


Figure 5

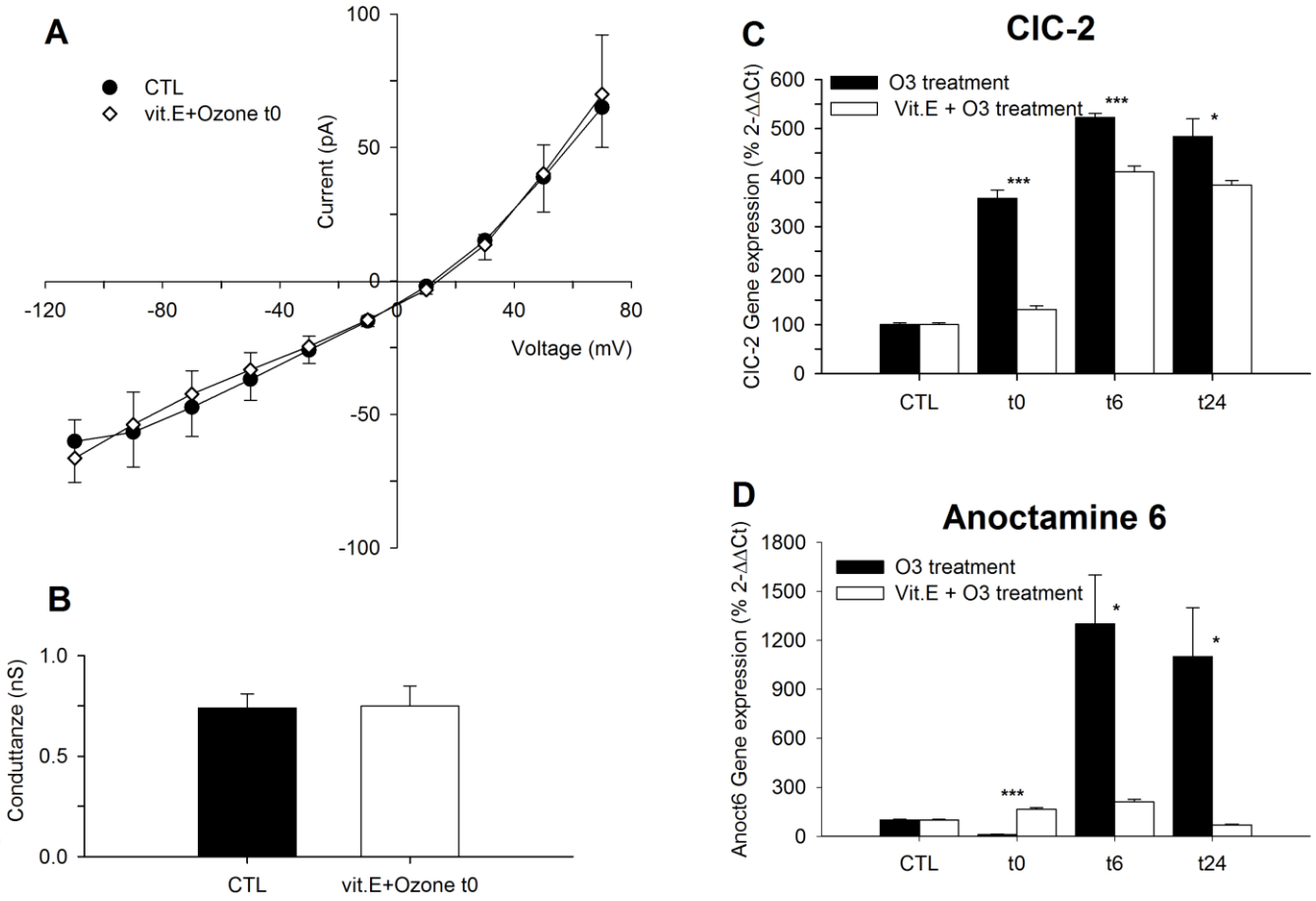


Figure 6