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Role of cathepsin S in ozone-induced airway hyperresponsiveness and inflammation

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Running title: Cathepsin S and ozone-induced airway effects

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Abstract

Ambient ozone has been linked to the worsening of symptoms of patients with obstructive diseases such as chronic obstructive pulmonary disease (COPD) and asthma. We investigated the role of cathepsin S on ozone-induced airway hyperresponsiveness (AHR) and inflammation, using the selective cathepsin S inhibitor, Compound A. Balb/c mice were exposed to ozone at a concentration of 3ppm or air for 3h, following administration by gavage of Compound A or vehicle. Bronchoalveolar lavage (BAL) was performed 3h and 20-24h following exposure, AHR was measured at 20-24h only. Ozone exposure, compared to air exposure increased BAL cathepsin S levels, AHR and BAL inflammatory cells. Compound A (30 mg kg^{-1} p.o) dosing compared to vehicle dosing inhibited ozone-induced AHR (-$logPC_{100}$ vehicle: -0.70±0.12, n=8 vs. cathepsin S inhibitor: -1.30±0.06, $P<0.001$, n=8) at 20-24h and BAL neutrophilia at 3h and 20-24h ($P<0.05$, n=6). Ozone exposure increased levels of BAL cytokines IL-6, TNF-α and IFN-γ. Compound A reduced IL-6 at 3h and 20-24h ($P<0.05$, n=5) and TNF-α, at 20-24h ($P<0.05$, n=6). These data indicate an important role for cathepsin S in the regulation of ozone-induced AHR and neutrophil cell recruitment and suggest that cathepsin S may be a target in the treatment of oxidative stress-induced AHR and inflammation.

Keywords

Airways hyperresponsiveness, airway inflammation, cathepsin S, neutrophilia, oxidative stress, ozone.

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Introduction

Ozone is a potent oxidising pollutant. Experimental ozone exposure induces oxidative stress, airways hyperresponsiveness (AHR) and lung neutrophilia(1-6). The mechanisms underlying ozone-induced AHR and inflammation are unclear, although the influx of neutrophils may be due to their recruitment by ozone-induced release of pro-inflammatory cytokines and chemokines. These include cytokine induced neutrophil chemoattractant (CINC), MIP-2, TNF-α and IL-1β (3;7-11).

Cathepsin S is a papain-like cysteine protease that is highly homologous to other cysteine protease family members, with 57% homology to cathepsin L, 49% to cathepsin K and 16% to cathepsin B. In contrast to cathepsins B and L, the tissue distribution of cathepsin S is restricted to spleen, heart and lung. Therefore, its localisation in the lung may pinpoint to an important function for cathepsin S. Thus, cathepsin S is also expressed in antigen-presenting cells (APC), such as macrophages and dendritic cells, and in MHC Class II cells, and could mediate $CD4^+$ T-cell mediated immune responses. In addition, it is expressed in non-professional APCs, such as epithelial cells(12-14). Such distribution strongly suggests a role for cathepsin S in immune and inflammatory processes such that provoked by ozone exposure. While other cathepsin family members are limited to operating within the alkaline conditions of the lysosome, cathepsin S is less restricted and is bioactive in the acidic conditions of the extracellular matrix. This property of cathepsin S enables it to be active in the acidic sites of inflamed tissues, potentially exacerbating inflammatory processes and causing tissue destruction.

Previous studies indicate that cathepsin S may have an important role in lung remodelling(15-17). It has been proposed that the protease-antiprotease imbalance

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may contribute to the development of emphysema(18). Increases in cathepsin S may be IFN-γ dependent, and IFN-γ stimulates cathepsin S release from smooth muscle cells and inhibits the antiprotease secretory leukocyte protease inhibitor (SLPI)(19). Cathepsin S-knockout mice bred with IFN-γ transgenic mice are protected against IFN-γ mediated epithelial cell apoptosis and emphysema (16). Interestingly, macrophages from cathepsin $E^{-/-}$ mice exhibit decreased surface receptors for TLR2 and TLR4 indicating a role for cathepsins in innate immune recognition (20). We have recently highlighted the importance of TLRs and Myeloid Differentiation Factor (MyD)-88 in ozone-induced AHR and neutrophilia(21). Overall, these data indicate that cathepsin S may be important in the process of lung tissue destruction in disease and may have a role in the response to oxidative stress. We therefore investigated the role of cathepsin S in ozone-induced AHR, neutrophilia and inflammation, using a selective cathepsin S inhibitor, Compound A.

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Methods

Ozone exposure

Pathogen-free, 6-8 week old male BALB/c mice (Harlan, UK) were housed within 'maximiser' filter-topped cages (Maximiser, Theseus caging system Inc., Hazelton, PA, USA) and were used throughout all experiments. Mice were exposed to ozone produced by an Ozoniser (Model 500 Sander Ozoniser, Germany), mixed with medical air (BOC, UK) for 3 hours at a concentration 3 parts per million (ppm) in a sealed Perspex container. Ozone concentration was continually monitored with an ozone probe (ATi Technologies, Oldham, UK) placed within the box. Control animals were exposed to air.

Protocol

We first studied the effect of ozone on release of Cathepsin S. Mice were exposed to ozone (3 ppm) or air (control) for 3 hours and were studied at 3 h, 20-24 h and 48 h after exposure. Bronchoalveolar lavage (BAL) was performed at all time-points. Lung resistance was measured at 20-24h only. Secondly, we studied the role of cathepsin S in ozone-induced inflammation.

Compound A is 4-Morpholinecarboxamide, *N*-[(1*R*)-2-[[(1*S*)-1-(2-benzoxazolylcarbonyl) propyl] amino]-1-[[(cyclopropylmethyl)sulfonyl]methyl]-2-oxoethyl] and is an inhibitor of cathepsin S previously reported as 05141(17). It was kindly provided by Sanofi-Aventis, New Jersey, USA. Compound A displays excellent selectivity and activity for cathepsin S over the other cathepsin family members L, K and B(17). The dissociation constant for Compound A for inhibitory

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binding of cathepsin S, K_i , was 0.64 nM, while with regard to cathepsins L, K and B, the K_i values were 17,000, 37,000 and 200 nM, respectively (17). In mice administered with a dose of 30 mg/kg orally, a maximum plasma concentration of 8,850 ng/ml with a half-life of 1.47 hours and a bioavailability of 47% were found from assays of plasma and lung levels of Compound A taken at regular time-points after gavage.

We used four groups of mice: two groups were exposed to air, dosed with vehicle or Compound A, and two groups were exposed to ozone, dosed with vehicle or Compound A. For each group, mice were studied at 3 hours or 20-24 hours, following a 3 hour exposure to ozone at a concentration of 3 ppm or air. Compound A was administered by gavage (30mg kg^{-1} in 0.2ml vehicle) or vehicle alone (0.5% methylcellulose, 0.2% Tween 80 in PBS), was dosed in the same volume as the active inhibitor. Compound A or vehicle was administered daily for the 2 days prior to air or ozone exposure, and 1 hour before and 6 hours and 16-18 hours after exposure to ozone or air.

Measurement of AHR

Twenty-four hours following exposure, mice were anesthetized with an intraperitoneal injection of anesthetic solution containing midazolam (Roche Products Ltd., Welwyn Garden City, UK) and Hypnorm (0.315mg/ml fentanyl citrate and 10mg/ml fluanisone; Janssen Animal Health, Wantage, UK). Mice were tracheostomised and ventilated (Mini Vent type 845, Hugo Sach Electronic, Germany; rate: 250 breaths/min and tidal volume: 250µl). Mice were monitored in a whole body plethysmograph with a pneumotachograph connected to a transducer (EMMS, Hants, UK). Transpulmonary pressure was assessed via an esophageal catheter (EMMS, Hants, UK). Instantaneous calculation of pulmonary resistance (R_L) was obtained.

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Increasing concentrations of acetylcholine chloride (ACh) (Sigma, Dorset, UK) (4-256 mg ml^{-1}) were administered with an ultrasonic nebulizer, and R_L was recorded for a 5-min period following each concentration. R_L after each concentration was expressed as percentage change from baseline R_L measured following nebulized phosphate buffered saline (PBS) (Sigma, Dorset, UK). The concentration of acetylcholine required to increase baseline R_L by 100% from baseline was calculated (PC_{100}), and the results are reported and analysed as –log PC_{100}.

Bronchoalveolar Lavage

We used a previously-published method (22). Briefly, following an overdose of anaesthetic, mice were lavaged with one 0.8ml aliquot of PBS via the endotracheal tube, and retrieved as the bronchoalveolar lavage fluid (BALF). Total cell counts and differential cell counts from cytospin preparations stained by May-Grünwald-Giemsa stain were determined under an optical microscope (Olympus BH2, Olympus Optical Company Ltd., Tokyo, Japan). At least 400 cells were counted per mouse and identified as macrophages, eosinophils, lymphocytes and neutrophils according to standard morphology under x400 magnification.

Cathepsin S Activity Assay

Cathepsin activities were measured in BALF collected in the previous step, using a previously published method (16). Briefly, solutions of cathepsins in varying concentrations were prepared in 10µl of DMSO and then diluted into 40 µl of the appropriate assay buffer. The following assay buffers were used: cathepsin S assay: MES, 50 mM (pH6.5); EDTA, 2.5 mM; NaCl, 100 mM; cathepsin K or L assay: MES, 50mM (pH 5.5); EDTA, 2.5 mM; DTT, 2.5 mM; cathepsin B assay: *N*,*N*bis(2-

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hydroxyethyl)-2-aminoethanesulfonic acid, 50 mM (pH 6.0); polyoxyethylenesorbitan monolaurate, 0.05%; and DTT, 2.5 mM. The assay solutions were incubated for 30 min at ambient temperature. Substrates for cathepsin S (Z-Val-Val-Arg-AMC, 9 nmol), cathepsin K (Z-Phe-Arg-AMC, 4 nmol), cathepsin L (Z-Phe-Arg-AMC, 1 nmol), or cathepsin B (Z-FR-AMC, 20 nmol) in 25 µl of assay buffer were added to the assay solutions. Hydrolysis was followed spectrophotometrically (l460 nm) for 5 min.

Luminex Analysis of Cytokines

We used a previously-published method (23). A standard multiplex assay kit, 8-plex (with eight bead sets) (RCYTO-60K-PMX8, Linco Research Inc., St. Charles, MO, USA) was used with the Luminex Flowmetrix system (Luminex, Austin, TX, USA) to determine the levels of cytokines in BAL supernatant. Individual bead sets in the 8-plex assay were coupled with monoclonal antibodies to IL-6, IFN-γ or TNF-α. Firstly, the beads were incubated with diluted standards or BALF overnight and then with a detector antibody cocktail for 60 min each at room temperature. After two washes in PBS supplemented with 0.02% Tween 20, 0.1% BSA, and 0.02% $NaNO_3$, the beads were incubated for 30 min with fluorescent dye-conjugated streptavidin. Cytokine levels were measured using a flow cytometer and analyzed with Flowmetrix software (Luminex, Austin, TX, USA). Standard curves for each cytokine were generated on a log-log plot for each assay, and the cytokine concentrations in each sample were calculated from the corresponding curve-fitting equations. Cytokine levels were measured from standard curve constructed from serial dilutions of the reference standard provided with the assay kit. The threshold of detection for IFN- γ was 0.7 pg ml^{-1}, for IL-6 0.7 pg ml^{-1} and for TNF-α 0.9 pg ml^{-1}.

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Data Analysis

Data are presented as mean±S.E.M. For multiple comparisons of different groups, we used the Kruskall-Wallis test for analysis of variance. If the Kruskall-Wallis test for analysis of variance was significant, we performed the Dunns test for comparison between two individual groups or Mann-Whitney test. A p value of less than 0.05 was accepted as significant.

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Results

Effect of ozone on AHR, inflammatory cell recruitment and cathepsin S Activity

Compared to air exposure, mice exposed to ozone required a lesser concentration of ACh to cause a 100% increase in baseline R_L (-LogPC_{100} Ozone: -1.47±0.06 vs. Air: -2.12±0.11; $P<0.001$; Fig. 1B). Ozone increased total cells recovered from the bronchoalveolar lavage (BAL) fluid, greatest at 20-24h following exposure ($P<0.05$), as compared to air exposure. These numbers were reflected by increases in macrophages, maximal at 48h ($P<0.05$) (Fig1. D.), while neutrophil numbers were increased at 3h, 20-24h and 48h, maximal at 20-24h ($P<0.05$) compared to air exposure (Fig. 1E). Compared to air, ozone exposure increased cathepsin S activity in the BAL fluid at 3h ($P<0.05$), 24h ($P<0.05$) and 48h ($P<0.05$) (Fig. 1F). Increases in activity of cathepsin K, L, or B were not detected.

Effect of Cathepsin S Inhibitor on ozone-induced AHR and inflammation

Compound A-dosed mice were protected against ozone-induced AHR, as shown by the R_L and PC_{100} data (Fig. 2A). Compound A-dosed mice required a higher concentration of ACh to reach a 100% increase in R_L (-logPC_{100} Cathepsin S inhibitor: -1.47±0.13 vs. vehicle: -0.71±0.11, $P<0.001$) (Fig. 2B).

Compared to vehicle dosing, Compound A administration reduced total BAL cells ($P<0.01$) 20-24h after ozone exposure (Fig. 3A). This was reflected by a reduction in macrophage ($P<0.05$) (Fig. 3B) and neutrophil ($P<0.05$) (Fig. 3C) numbers.

Effect of Cathepsin S Inhibition on BALF cytokines

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In vehicle-dosed mice, ozone increased BAL IL-6 and IFN-γ at 3h and 20-24h and TNF-α at 20-24h, compared to air exposure (Fig. 4A-C). TNF-α was not detected at 3h in any treatment or exposure group. Compared to vehicle dosing, Compound A reduced ozone-induced BAL IL-6 levels at 3h ($P<0.05$) and 20-24h ($P<0.05$) and TNF-α levels were inhibited at 20-24h ($P<0.05$). Compound A did not affect ozone-induced IFN-γ.

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Discussion

We have demonstrated that inhibition of cathepsin S using Compound A reduces ozone-induced airway hyprerresponsiveness, BAL fluid neutrophil and macrophage numbers and reduces the inflammatory mediators, IL-6 and TNF-α, in BAL fluid. These results indicate the important contribution of cathepsin S in mediating ozone-induced effects in the airways including AHR and inflammation.

The demonstration of cathepsin S involvement was achieved by using a relatively selective inhibitor of cathepsin S that also had a favourable pharmacokinetic profile in mice when administered orally with maximum plasma levels of 8.9 mg ml^{-1} and a half-life of nearly 90 minutes with 47% bioavailability at the dose of 30 mg kg^{-1} that we used. Zheng et al also used the same cathepsin S inhibitor at the lower dose of 10 mg kg^{-1} per day to inhibit IFNγ-induced DNA injury, apoptosis and emphysema in mice (17). We used the higher dose of 30 mg kg^{-1} because of the improved pharmacokinetics at that increased dose. We demonstrated that cathepsin S activity was selectively increased in BAL fluid of mice exposed to ozone, accompanied by a 2.1-fold increase in cathepsin S expression in lung tissue homogenates as measured by Affymetrix gene array (unpublished observation). These observations gave further support to an important role for cathepsin S in the lungs of ozone-exposed mice.

Previous studies have reported that cathepsin S release from pulmonary macrophages and epithelial cells is IFN-γ-dependent and that using cathepsin S knockout mice, cathepsin S may mediate DNA injury, apoptosis and emphysema induced by IFNγ (17;19). The significant reduction in ozone-induced lung inflammation caused by compound A was not accompanied by any changes in levels of IFN-γ in BALF. This difference may be accounted for by only a partial inhibition

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achieved by Compound A even at the high dose, in comparison to the situation in cathepsin S knockout mice which cannot produce cathepsin S. Additionally, cathepsin S causes the breakdown of extracellular matrix.

The effects of Compound A on AHR, neutrophil recruitment and inflammatory cytokine production induced by ozone illustrates the complex interrelationships between these parameters. The inhibition of BALF levels of IL-6 and TNF-α by compound A may account for the reduction of neutrophils observed in the present study. This is supported by the observation that the reduction in BAL neutrophils at 3h and at 20-24h after ozone exposure was paralleled by the reduction in IL-6 and TNFα levels. The increased levels of IL-6 in BALF following ozone exposure were accompanied by simultaneous increases in IL-6 mRNA in lung tissue (24;25), indicating that changes in BALF concentration of IL-6 were also reflected in the lung expression of IL-6. However, although BALF neutrophils were reduced following 3ppm exposure to ozone for 2 hours in IL-6 knockout mice compared to IL-6 transgenic mice, AHR was not affected (26), indicating a dissociation of IL-6 expression and AHR. Similar dissociation between AHR and TNFα expression has been reported. Although TNF-α is chemotactic for neutrophils and enhance the movement of inflammatory cells (27), ozone-induced neutrophil migration was not affected in p55/p75 TNF-receptor knockout mice following a 2ppm exposure for 3 hours, but ozone-induced AHR was reduced (28), implicating a non-neutrophilic TNF-α dependent AHR. Furthermore, ozone-induced changes in tracheal permeability which may contribute to airway wall oedeama and may also facilitate the recruitment of inflammatory cells into BALF and tissues (9) are inhibited by an anti-TNF-α blocking antibody (29). Taken together, our data confirm previously-published data and support in part the premise that IL-6 is primarily involved in

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ozone-induced neutrophil recruitment but not AHR; conversely, TNF-α, may be involved in ozone-induced AHR, but not neutrophil recruitment. Our current data using a cathepsin S inhibitor would indicate that cathepsin S is involved in both AHR, neutrophil recruitment and induction of IL-6 and TNFα, likely to be because cathepsin S is involved at a more proximal point of the inflammatory cascade induced by ozone. There may be other factors involved in the neutrophilic response observed after ozone exposure and the exact mechanisms as to how cathepsin S is involved in the mediation of these effects remain unclear and require further investigation.

Apart from ozone exposure, the Th2 cytokine IL-13 has been shown to induce the expression of cathepsin S in the lungs. Thus, inducible expression of IL-13 in the respiratory epithelium of the lungs of mice led to the development of emphysema with enhanced lung volumes and compliance, mucus metaplasia, and inflammation, associated with the induction of cathepsins including cathepsin S (16). Administration of cysteine protease inhibitors to these mice inhibited emphysema and inflammation. More direct evidence for cathepsin S involvement in the IL-13 induction of emphysema, inflammation and airway remodelling comes from the observation that cathepsin $S^{-/-}$ mice are protected from the increases in lung volumes associated with the IL-13 overexpressing phenotype (16). Interestingly, recent data from our laboratory indicated that IL-13 is involved in ozone-induced neutrophilic inflammation and AHR(30), and therefore in our ozone model, IL-13 could actually contribute to cathepsin S expression. The interactions between ozone exposure and IL-13 in the expression and activation of cathepsin S deserve further studies.

Cathepsin S can also be upregulated by TNF-α in endothelial cells and cathepsin S induces endothelial cell motility, invasion and migration. Lack of

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cathepsin S as in cathepsin S-knockout mice leads to impaired invasion of collagens by endothelial cells, that in turn lead to defective angiogenic development (31), indicating a role for cathepsin S in angiogenesis. This effect has not been studied in pulmonary epithelial cells, but similar results may pertain regarding the motility of cells other than endothelial cells when cathepsin S is blocked. This could be a plausible explanation for the reduction of inflammatory cells recovered in BAL fluid induced by Compound A.

This study highlights an important role for cathepsin S in mediating ozone-induced AHR, inflammatory cell recruitment and cytokine release. Inhibition of cathepsin S may be beneficial in the treatment of airways diseases such as asthma and COPD, particularly where lung oxidative stress may be an important component.

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Legend to figures

Figure 1. Airways hyperresponsiveness (AHR). (Panel A): Percent increase in lung resistance (R_L) to increasing concentrations of aerosolised acetylcholine (ACh) for air-exposed (☐) or ozone-exposed (▲) Balb/c mice (Panel B): Provocative dose of ACh causing a 100% increase in baseline R_L (PC_{100}) was converted to -LogPC_{100} to represent bronchial responsiveness. Horizontal bar indicate mean values. Ozone exposed mice (n=10) exhibited significant increase in R_L (P<0.001) compared to air-exposed mice (n=10). Ozone induced time-dependent increases in total BAL cells (Panel C), macrophages (Panel D) and neutrophils (Panel E) compared to air-exposed mice (n=10). Ozone induced a time-dependent increase in total cells, macrophages and neutrophils. Cathepsin S-levels peaked 3 h after ozone-exposure (Panel F) (P<0.05). Data are expressed as mean ± S.E.M. * P<0.05; ** P<0.01; *** P<0.001 compared to air-exposure.

Figure 2. Panel A shows mean percentage increase in lung resistance (R_L) to increasing concentrations of acetylcholine for compound-dosed, air- (☐) or ozone-exposed (■) mice and vehicle-dosed, air- (Δ) or ozone-exposed (▲) mice. R_L was increased in vehicle-dosed and ozone-exposed mice compared to air-exposed mice at each concentration of ACh. Dosing with Compound A significantly inhibited ozone-induced R_L at each concentration of ACh. Panel B shows **-log PC_{100}** data. Compound A-dosed mice showed a significant attenuation of bronchial hyperresponsiveness induced by ozone . Data are expressed as mean ± SEM. Horizontal bars indicate mean. ** P<0.01; *** P<0.001, compared to vehicle-dosed air-exposed mice. # P<0.05; ## P<0.01; ### P<0.001, compared to ozone-exposed vehicle-dosed mice.

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Figure 3. Time-dependent effect on mean numbers of total cells (Panel A), neutrophils (Panel B) and macrophages (Panel C) recovered in bronchoalveolar lavage fluid (BALF). Compound A decreased ozone-induced total cells ($P<0.01$, n=6) and macrophages ($P<0.05$, n=6) 20-24h after exposure, and neutrophil numbers at 3h ($P<0.05$, n=6) and at 20-24h ($P<0.05$, n=6). Data presented as mean ± S.E.M. * $P<0.05$; ** $P<0.01$; *** $P<0.001$ compared to corresponding air-exposed mice; # $P<0.05$; ## $P<0.01$; ### $P<0.001$ compared to ozone-exposed, vehicle-dosed mice.

Figure 4. Expression of bronchoalveolar lavage fluid (BALF) IL-6 (Panel A), TNF-α (Panel B) or IFN-γ (Panel C), by after ozone and after administration of Compound A. Compound A inhibited ozone-induced BAL fluid IL-6 levels at 3h ($P<0.05$, n=5) and 20-24h ($P<0.05$, n=5), and TNF-α levels at 20-24h ($P<0.05$, n=6). It had no affect on IFN-γ levels. Data presented as mean ± S.E.M. * $P<0.05$; ** $P<0.01$ compared to corresponding air-exposed mice; # $P<0.05$; ## $P<0.01$ compared to ozone-exposed, vehicle-dosed mice. ND = not detected.

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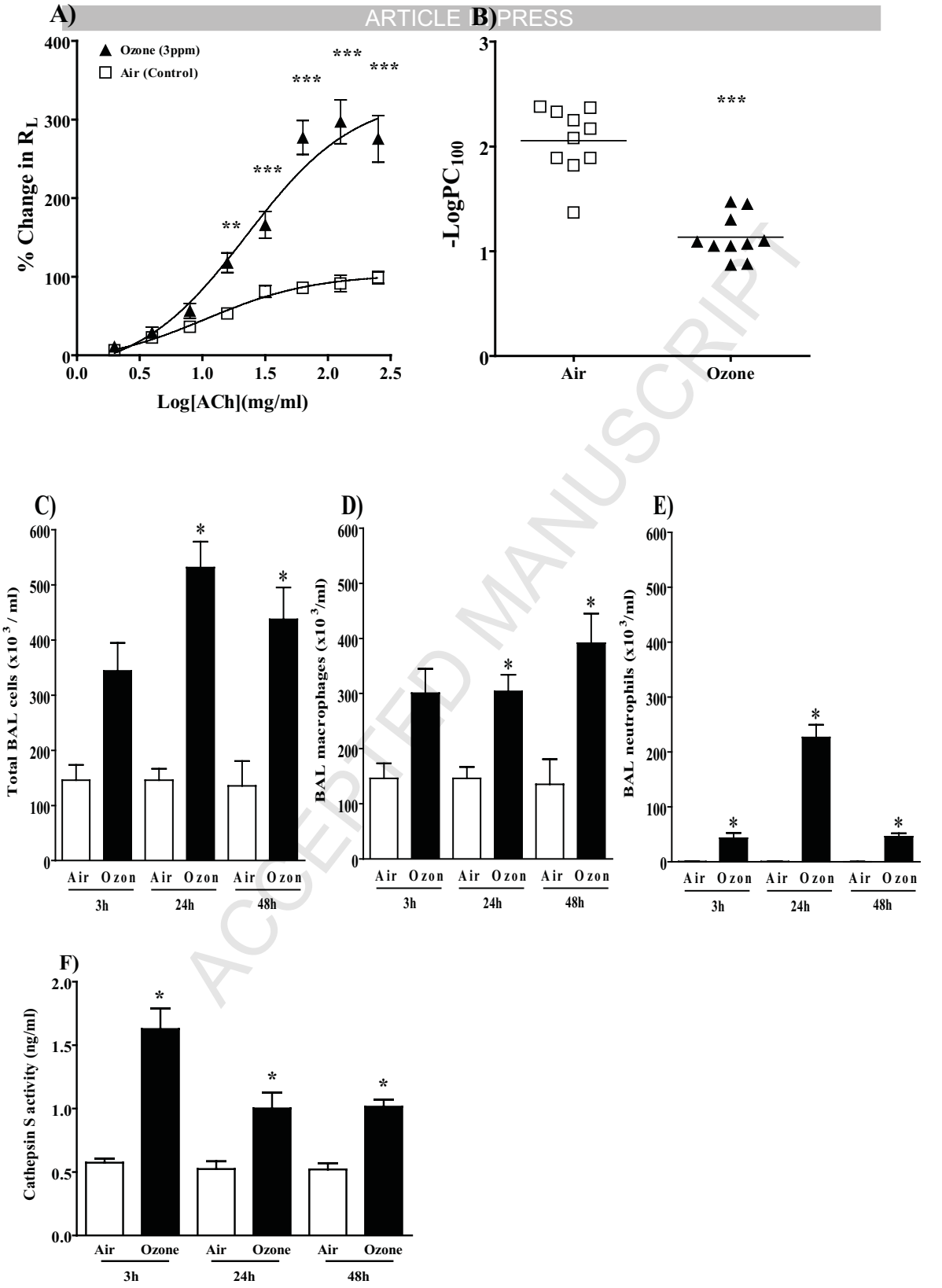

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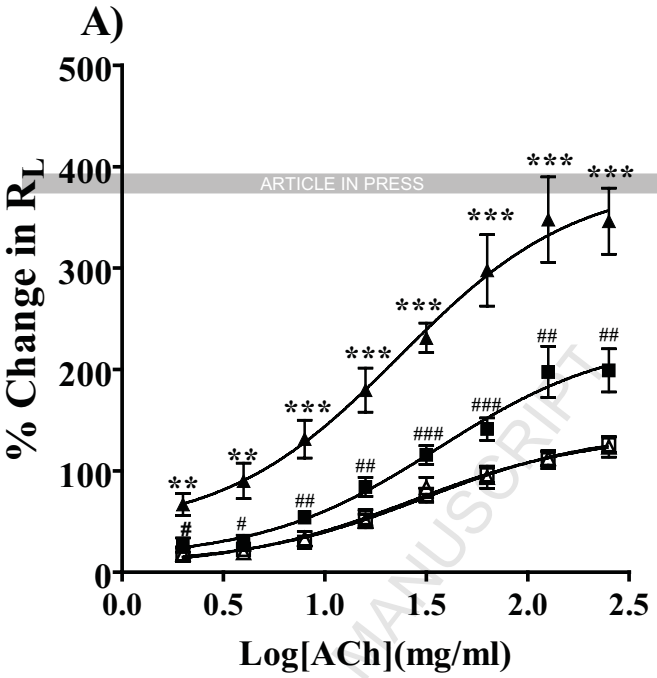

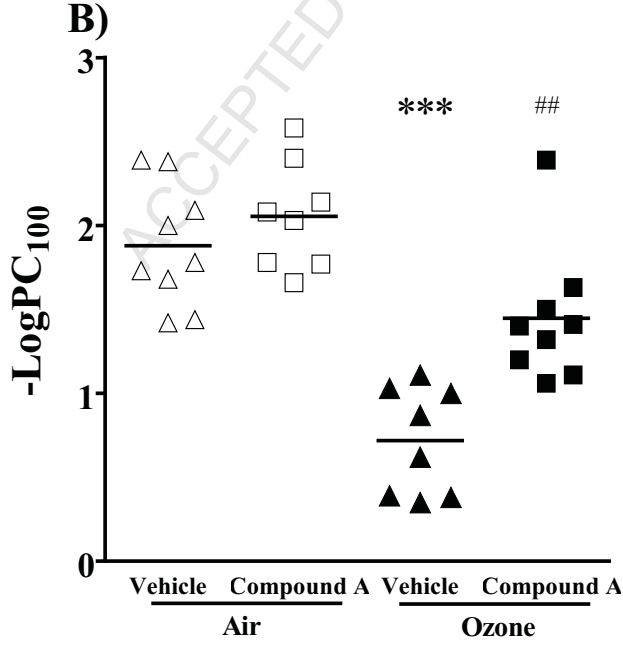

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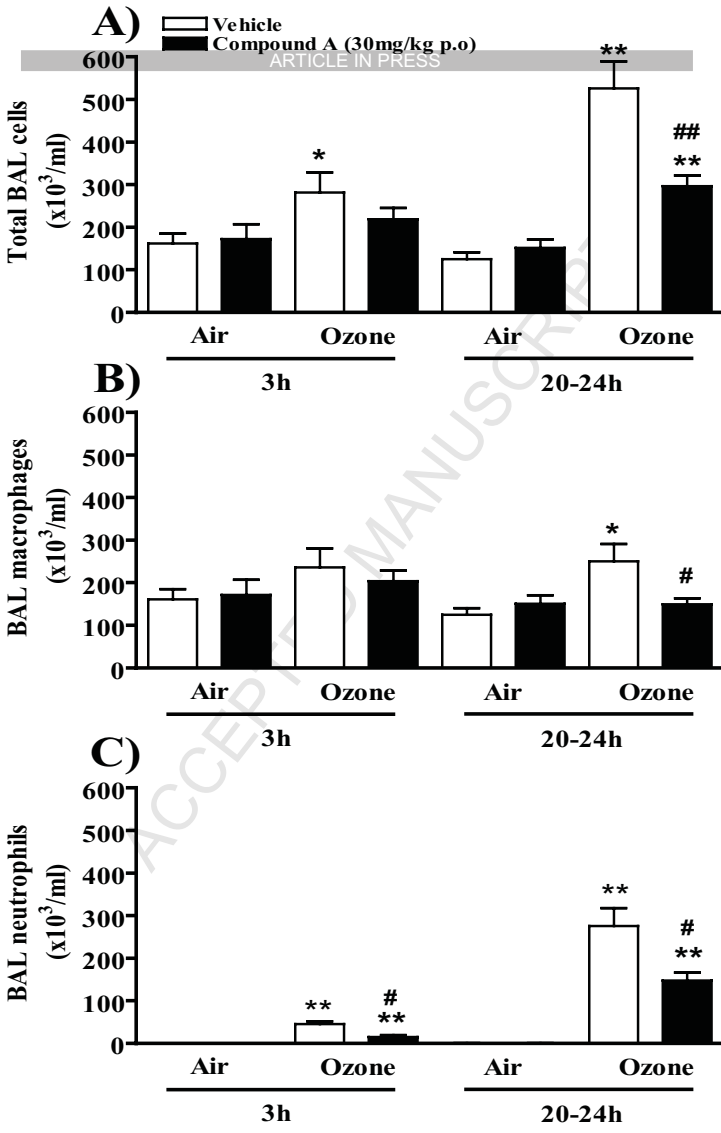

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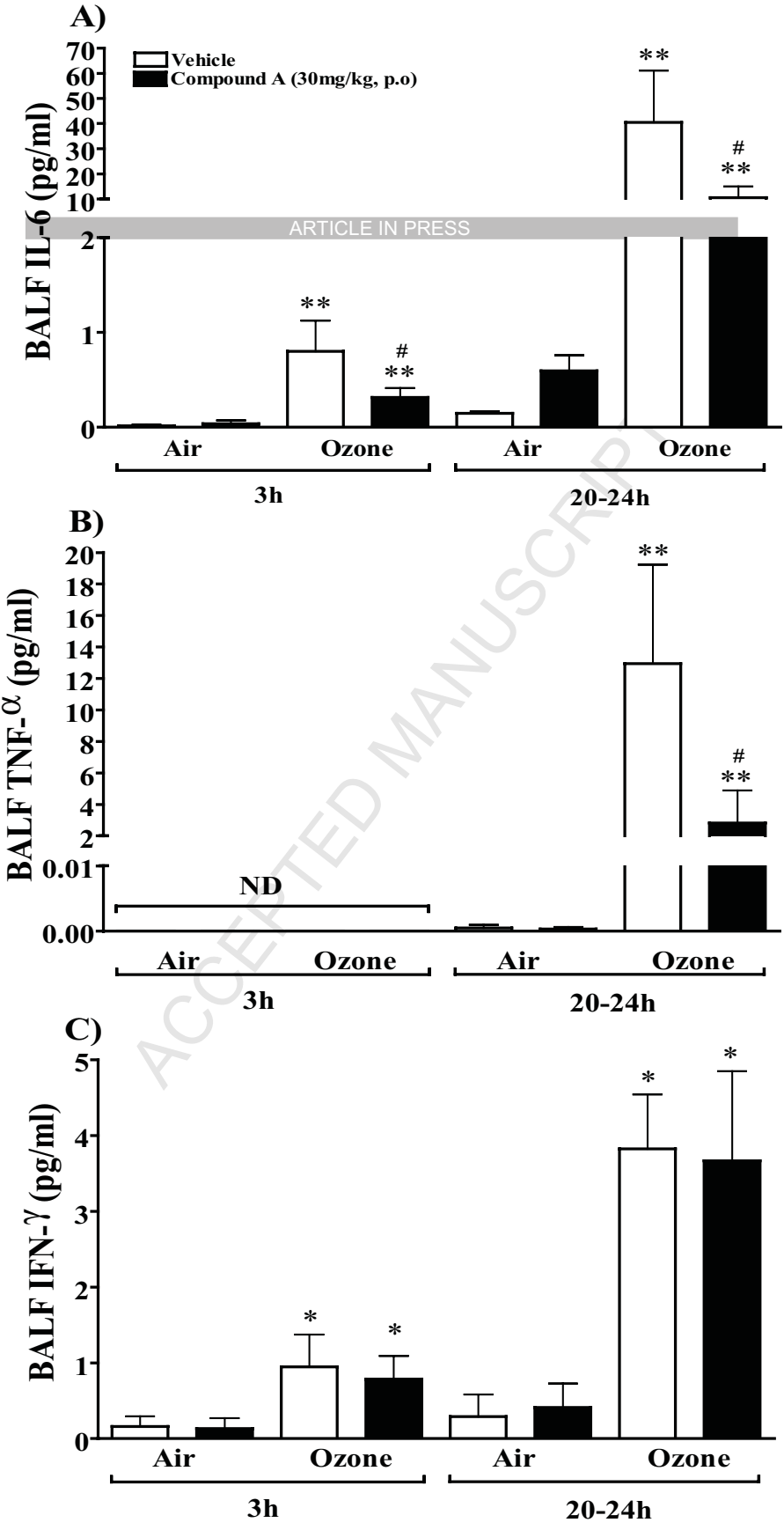

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