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The role of the non-ciliated bronchiolar cell in tolerance to inhaled vanadium of the bronchiolar epithelium

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Summary. The Non-Ciliated Bronchiolar Cell (NCBC) is responsible for the defense and maintenance of the bronchiolar epithelium. Several cellular defense mechanisms have been associated with an increase in the secretion of CC16 and changes in the phenotype of the cell; these mechanisms could be linked to tolerance to the damage due to exposure to inhaled Particulate Matter (PM) of the epithelium. These defense mechanisms have not been sufficiently explored. In this article, we studied the response of the NCBC to inhaled vanadium, an element which adheres to PM. This response was measured by the changes in the phenotype of the NCBC and the secretion of CC16 in a mouse model. Mice were exposed in two phases to different vanadium concentrations; 1.56 mg/m^3 in the first phase and 2.57 mg/m^3 in the second phase. Mice were sacrificed on the 2nd, 4th, 5th, 6th and 8th weeks. In the second phase, we observed the following: sloughing of the NCBC, hyperplasia and small inflammatory foci remained without changes and that the expression of CC16 was higher in this phase than in phase I. We also observed a change in the phenotype with a slow decrease in both phases. The increase in the secretion of CC16 and the phenotype reversion could be due to the anti-inflammatory activity of CC16. The changes observed in the second phase could be attributed to the tolerance to inhaled vanadium.

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Introduction

The non-ciliated bronchiolar cell (NCBC) performs numerous essential functions in the lung. These cells are located mainly in the bronchioles, and in rodents they constitute about 80% of the population of the bronchiolar epithelium (Wong et al., 2009). They function as stem cells (Crosby and Waters, 2010; Reynolds and Malkinson, 2010; Zuo et al., 2018), perform immunomodulatory activities through their secretion products (Snyder et al., 2010; Hiemstra and Bourdin, 2014) and metabolize xenobiotics using P450 mono-oxygenates (Hukkanen et al., 2002; Chang et al., 2006). They also have anti-inflammatory, immuno-suppressive (Chen-Chen et al., 2001; Snyder, 2010; Liu et al., 2013) and antioxidant activities (Mango et al., 1998; Pilon et al., 2016). The NCBC can change its phenotype to mucosecreting (PAS+ and / or AB / PAS+) when exposed to toxic agents (Alessandrini et al., 2010; Curran and Cohn, 2010).

These cells produce the CC16 protein (club cell protein, CC10 or CCSP) that participates in the maintenance of the integrity of the bronchiolar epithelium and in its repair, mainly when the lung is exposed to toxic substances (Broeckaert et al., 2000; Stripp et al., 2002; Wong et al., 2009); under these stimuli, a hyper-production and hyper-secretion of CC16 have been reported (Xiao et al., 2007, 2013).

There is evidence of the participation of the NCBC

in the development of tolerance to the damage caused by exposure to different types of toxic substances and environmental pollutants such as naphthalene, coumarin, methylene chloride, trichloro-ethylene, ozone and zinc oxide. Tolerance is a recognized phenomenon in toxicology, and has been defined as the mechanisms an organ develops, the lung in this case, against a substance as a consequence of pre-exposure to lower doses of the same substance; the importance of this response is that it allows the cells to "resist" toxic exposure and survive (Fairchild, 1967; Sutherland et al., 2012). The mechanisms by which this type of response occurs have not yet been established with precision; however, the participation of the NCBC is suggested (Lakritz et al., 1996; Born et al., 1999; West et al., 2002; Vassallo et al., 2010).

Vanadium (V) is a pro-oxidant element to which we are exposed through inhalation due to occupational or environmental exposure and because of its presence in the particulate matter (PM) (Rodríguez-Mercado and Altamirano-Lozano, 2006; Assem and Levy, 2012). *In vivo* it has been reported that compounds of this element, such as vanadium pentoxide (V_2O_5), produce hematotoxicity, hepatotoxicity, neurotoxicity and pulmonary toxicity (Fortoul et al., 2011, 2014). Previously, Knetch (1992), in a model of subchronic exposure to V_2O_5 found that repeated exposure to this compound did not trigger a cellular immune response, nor did it increase bronchial hyperreactivity or cause exacerbation of an acute response that had been previously reported by the same group. This suggests a possible tolerance response to this metal without further details about the possible mechanisms that favored this response (Knetch et al., 1992).

Due to the important protective role of the NCBC in the lung and its possible participation in the tolerance mechanism, the objective of this study was to evaluate the response of this cell in relation to the change in its phenotype to mucosecreting and the production of CC16 as a possible mechanism related to the tolerance to inhaled vanadium (Rodríguez-Mercado and Altamirano-Lozano, 2006; Fortoul et al., 2011).

Materials and methods

Mice

Male mice of strain CD-1 weighing 33±2 g obtained from the vivarium of the Faculty of Medicine of the National Autonomous University of Mexico were used. The mice were randomly distributed in special plastic boxes and maintained in light-dark cycles (12:12 h), with water and food (Purina rodent chow) *ad libitum*. The animals were handled in accordance to the Laboratory Animal Care and Use Guide (Institute of Laboratory Animal Resources, Commission on Life Sciences, National Research Council) and the Official Mexican Standard (NOM-062-ZOO-1999), for the production, care and use of laboratory animals. This study was approved by the Research and Ethics Commissions of the Faculty of Medicine of the National Autonomous University of Mexico (UNAM) with the number FM/DI/071/2017.

Experimental protocol

The inhalation protocol was performed according to Fortoul et al., (2014), with some modifications. A total of 90 mice were used and distributed in two groups (A and B) of 45 animals each. Group A was the control group; these mice were exposed to 0.9% saline inhalation for one hour, twice a week, for eight weeks. Group B was exposed in two phases to the inhalation of V_2O_5 (99.99% purity, Sigma-Aldrich, St. Louis, MO); in the first phase, the exposure was performed using a V_2O_5 [0.01 M] solution, the inhalations were applied for one hour, twice a week, for four weeks. The second phase began when a [0.02 M] solution of V_2O_5 was used with the same exposure scheme as before. A total of 18 animals (9 controls and 9 exposed) were sacrificed on the 2nd, 4th, 5th, 6th and 8th weeks.

Exposure in all the groups was performed in a transparent acrylic chamber (45x21x35 cm) connected to an ultra Yuehua WH 2000® nebulizer, with a maximum nebulization rate of 4mL/minute with 80% of the nebulized particles with aerodynamic diameters from 1 to 5.0 μm.

Determination of vanadium concentration

The determination of vanadium concentrations in the inhalation chamber was made by analyzing the filters placed at the nebulizer outlet, with a nebulization rate of 4 mL/ min. After each exposure, the filters were removed and then placed in an oven until a constant weight was obtained (Riossa HS-33); afterwards, they were fractionated into small pieces to be digested. One hundred mg of the weight of the filters were used, then, suprapur nitric acid was added, and the digestion process was performed in a microwave oven (CEM model MDS 2000). Once the digestion was completed, the resulting solutions were transferred to 10 mL volumetric flasks for analysis.

A calibration curve of 5 points, from 20 to 100 ppb (ng/mL) of vanadium, was prepared. These solutions were analyzed and then used to build the calibration curve. Afterwards, the samples were evaluated. For the preparation of the curve, the certified standard QCS-27 ICP 27 (High Purity Standards, Charleston, South Carolina) was used. The samples were analyzed by mass spectrometry of induction-coupled plasma (ICP-MS) using a Bruker equipment, model Aurora M90 with coupled autosampler.

Determination of the concentration of vanadium in the blood

The determination of the concentrations of vanadium

in the blood was performed following the same analysis protocol that was used to determine the concentrations during the exposure. The concentration of the metal was reported as parts per billion (ppb) or nanograms of vanadium per gram of dry weight of tissue (ng/g).

Euthanasia and organ harvesting

For each analysis (the determination of vanadium in the blood, the histopathological evaluation and the immunohistochemistry, the western blot WB) three mice were sacrificed per week of exposure for each group (A and B).

The euthanasia of the animals for the determination of the concentrations of vanadium in the blood and the WB was performed by cervical dislocation. In the case of the histopathological and the immunohistochemical evaluation, euthanasia was performed by a lethal dose of sodium pentobarbital administered intraperitoneally (Pisabental, Pisa Agropecuaria) in a concentration of 35 mg/kg of weight, and subsequently perfused with 0.9% saline and 4% of buffered paraformaldehyde solution intracardially. The lungs were extracted and insufflated intratracheally with buffered paraformaldehyde to be processed by the histological technique in paraffin.

Histopathological and morphometric analysis of the lung tissue

Slices 3 μm thick were obtained from the lungs and were stained afterwards. The histopathological evaluation of the lung tissue was performed using two staining techniques: hematoxylin-eosin (HE) to analyze the general structure of the lung tissue and histochemistry with Peryodic Acid Schiff (PAS) for the identification of PAS+ mucosecreting cells in the terminal bronchioles.

The morphometric analysis was performed using photomicrographs obtained from the lungs that were stained with PAS. An Olympus microscope (model BH2-RFCA) with a camera of the same brand was used for the analysis. Once obtained, the images were analyzed using ImageJ (V1.50i, National Health Institutes Bethesda, Maryland USA) to count the cells and record the number of mucosecreting PAS+ cells with a length of 100 μm in the terminal bronchioles. The cell count was performed in 10 terminal bronchioles per lung, per mouse, and was transformed into a percentage of PAS+ cells to do the statistical analysis.

Determination of CC16 in the lung tissue

The tissues were dewaxed with xylol and dehydrated in a train of alcohols in increasing order (70%, 96%, 100%). Subsequently, an antigenic recovery was performed by incubation in a 2% Diva Decloacker recovery solution (Diva Decloaker 20x, BioCare Medical, Pacheco, California) at 15 psi. The endogenous peroxidase was inhibited with 3% hydrogen peroxide for 15 minutes (JT Baker, Phillipsburg, New Jersey). Nonspecific antigens were blocked with a 5% bovine albumin solution (MP Biomedicals, Santa Ana, California). The tissues were incubated with primary anti-CC16 antibody (anti-CC16 antibody, 1:1000 dilution, Santa Cruz Biotechnology, Inc.) at 4°C overnight. Subsequently, the tissues were incubated with a secondary anti-goat antibody for 30 minutes at room temperature and then incubated with a drop of the streptavidin-horseradish peroxidase (HRP) complex (Goat on rodent HRP Polymer, BioCare Medical, Pacheco, California). The immunoreactivity was identified by incubation with 0.05% diaminobenzidine tetrachloride (Invitrogen, Camarillo, California). Finally, the sections were counterstained with hematoxylin. Some samples were only incubated with the secondary antibody and served as negative controls.

Determination of CC16 in lung homogenates

For this technique, the lungs of the mice were homogenized with a Heidolph DIAX 900 homogenizer (Sigma-Aldrich, St. Louis, MO). The homogenate was centrifuged at 30,000 RPMI (9000 G) for 15 minutes; the supernatant was recovered and subsequently centrifuged at 34,800 RPMI (105,000 G), recovering the supernatant (cytosolic fraction). Subsequently, the protein concentration obtained in the cytosolic fraction was determined with a Bio-Rad Protein Assay Dye Reagent Concentrate (Bio-Rad, Hercules, California); albumin V was used as positive control. Samples were prepared under denaturing conditions (in charge buffer with 2-mercaptoethanol, 10% SDS, glycerol, bromophenol blue, 90°C for five minutes); 20 mg of protein per sample were loaded on 17 and 10% sodium polyacrylamide-dodecylsulfate electrophoresis (SDS-PAGE) gels. Then, they were transferred to nitrocellulose-PVDF membranes (Merck Millipore, Tullagreen, Cork, Ireland) in a semi-wet transfer system for one hour at 120 mA. The membranes were blocked with a solution of 5% albumin in Tris buffer (TBS), washed twice in TBS and incubated with the primary anti-CC16 antibody (1:1000, Santa Cruz Biotechnology Inc., Dallas, Texas) and an anti-actin primary antibody (1:1000, kindly donated by Dr. José Manuel Hernández, CINVESTAV-México) in Tween20/TBS solution (TTBS) overnight at 4°C. Subsequently the membranes were washed again with TTBS and incubated with the secondary antibodies anti-goat (1:2500, secondary rabbit anti-goat, Invitrogen, Camarillo, California) and anti-mouse (1:1000 secondary rabbit anti-mouse, Abcam, Cambridge, Massachusetts) for the recognition of the primary antibodies against CC16 and actin respectively. Both antibodies were coupled to HRP and diluted in TTBS for one hour at room temperature. The membranes were washed three times with TTBS and once with TBS before detection of antibody binding by chemiluminescence detected with the Amersham ECL Prime Western Blotting Detection Reagent Kit (General

Electric Company, Uppsala, Sweden).

Processing of the images

The lung slides were observed under an Olympus microscope (model BH2-RFCA, Tokyo, Japan) and photomicrographs of lung parenchyma and terminal bronchioles were taken at 20x and 40x respectively, with a digital camera (Evolution MP Color, Media Cybernetics). The images were later processed with Adobe Photoshop CC 2019 (Ps. V. 20.04), and the processing included adjusting the brightness and contrast, in addition to changing the background to white.

Densitometric analysis

The photomicrographs were processed and separated in three-color channels for the examination of the density of the mark obtained in the immunohistochemistry, but only the yellow channel was used to perform the evaluation with the MathLab DensiFe software 1.0.0.0. We analyzed 5 mice per group; for each mouse, 10 fields of 0.088 mm^2 were chosen randomly with the 40x objective.

The quantitative analysis of the bands obtained in the WB was performed using ImageJ 1.50i (National Health Institutes Bethesda, Maryland USA). This software allowed us to calculate the intensity of the bands by measuring the average density in pixels.

Statistical analysis

The data were analyzed with one-way analysis of variance (ANOVA), and the differences between the groups were identified with a Tukey *post hoc* test, considering $p \leq 0.05$ as statistically significant. All the data were reported as mean ± standard error. All the analyses were performed with Prisma v. 6.0c (GraphPad, San Diego, CA).

Results

Determination of the V concentration in the box

To achieve vanadium exposure in two different concentrations, low and high, the average concentration of V in the inhalation chamber during exposure was determined using a solution of [0.01 M] V_2O_5, the concentrations recorded were of 1.56 mg/m^3 of vanadium, while the concentration using a V_2O_5 solution with a [0.02 M] was of 2.57 mg/m^3.

Determination of the concentration of V in the blood

Once the mice were exposed, the average concentration of V in the blood was determined to ensure the presence of the metal in the organism. The concentration recorded in the controls was of 59.19±16.23 ppb (ng/g dry weight); during week two of V exposure, higher levels of V were detected, but these were not different from controls (180.5±24.87 ppb), this concentration increased significantly from week 4 of exposure (245.4±31.13 ppb). At the beginning of phase II of the exposure, on week 5, a significant increase in the concentration of the metal (436.0±40.97 ppb) was observed, but no changes were registered in week 6 (348.1±72.62 ppb) of the treatment. At the end of the exposure, on week 8, the concentration of the metal decreased significantly (146.3±40.84 ppb). Fig. 1 shows the behavior of the concentration of the metal throughout the treatment.

Histopathological findings and morphometric analysis

In the histological analysis with hematoxylin-eosin of the lungs of the control mice, the typical structure of the tissue was identified. Fig. 2A shows the cut of a bronchiole in which we observed the simple cubic epithelium formed mainly by NCBC and scarce ciliated cells. The NCBC presented its typical cubic shape with a characteristic apical protrusion. The intact alveolar sacs constituted by type I pneumocytes (flat cells) and type II pneumocytes (cubic) were observed adjacent to the bronchioles. No relevant changes in tissue structure were identified.

Regarding the histopathological analysis of the lung tissue of the exposed mice, several alterations were observed. We identified on week 2 of inhalation, small foci of perivascular and peribronchiolar inflammatory infiltrate, areas of bronchiolar epithelial hyperplasia and sloughing of the NCBC. In some cells the movement of the nuclei towards the apical zone was observed. All the changes remained the same during the rest of the exposure weeks and qualitatively they were similar throughout. At the beginning of phase II bronchial

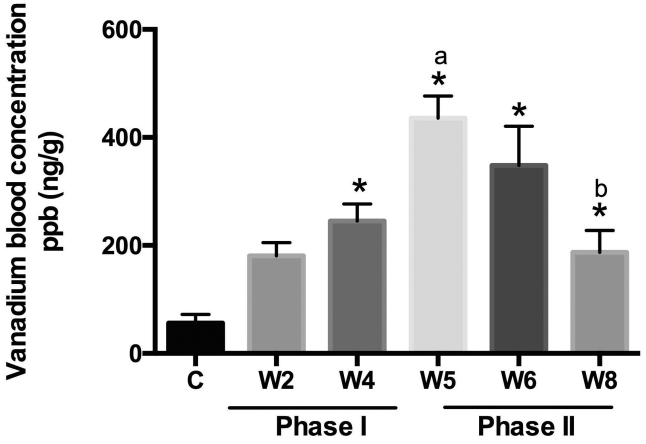

Fig. 1. Vanadium levels (ng/g dry weight) detected in the blood samples of the exposed and unexposed mice. The values are expressed as the mean concentration of V in ng/g of dry weight±standard error, ANOVA $p \leq 0.05$ post hoc (Tukey). *: control versus exposed; a: difference versus W2; b: difference versus W5.

epithelial cell sloughing was identified, as well as enlargement of NCBCs (Fig. 2B-F).

Regarding the morphometric analysis with the PAS stain, in the lungs of the controls, a few mucosecreting cells were found in the bronchi, whereas in the bronchioles none was observed (Fig. 3A). Mucous metaplasia developed during the treatment with V, it started on the second week of exposure of phase I and increased with time (Fig. 3B,C). During phase II of exposure, mucous metaplasia continued but was observed to a lesser extent (Fig. 3D). Graph 3 shows the results of the percentages of positive PAS cells in the

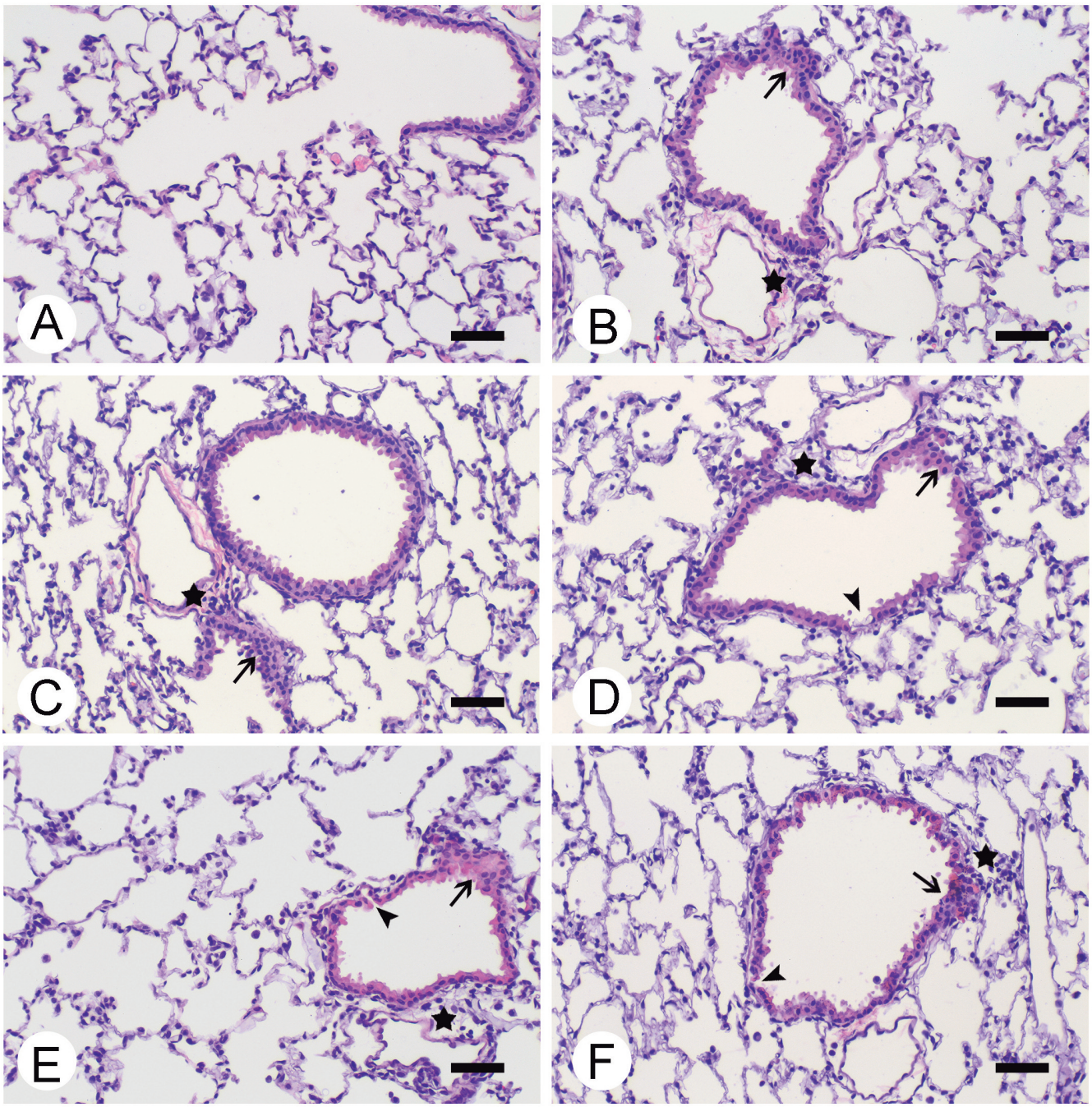

Fig. 2. Pulmonary histopathological changes produced by V inhalation. The arrows indicate the areas where bronchiolar hyperplasia was identified; the arrowheads indicate the areas where bronchiolar epithelial sloughing was observed and the stars (*) small foci of inflammatory infiltrate. **A.** Control lung. **B.** 2 weeks of exposure. **C.** 4 weeks. **D.** 5 weeks. **E.** 6 weeks. **F.** 8 weeks. Hematoxylin-eosin stain. Scale bars: 50 µm.

terminal bronchioles; in the controls, the percentage of cells was zero, while exposure to V led to the appearance and increase of mucosecreting cells in the bronchioles. On week 2 of the exposure, the percentage was 1.27±0.24%, and increased significantly on week 4 (33.36±2.66%). At the beginning of phase II, on week 5 of exposure, the percentage of cells decreased significantly to 15.47±1.68% and this decrease remained until week 6 (4.36±0.47%) and 8 (5.71±0.91%) without significant changes.

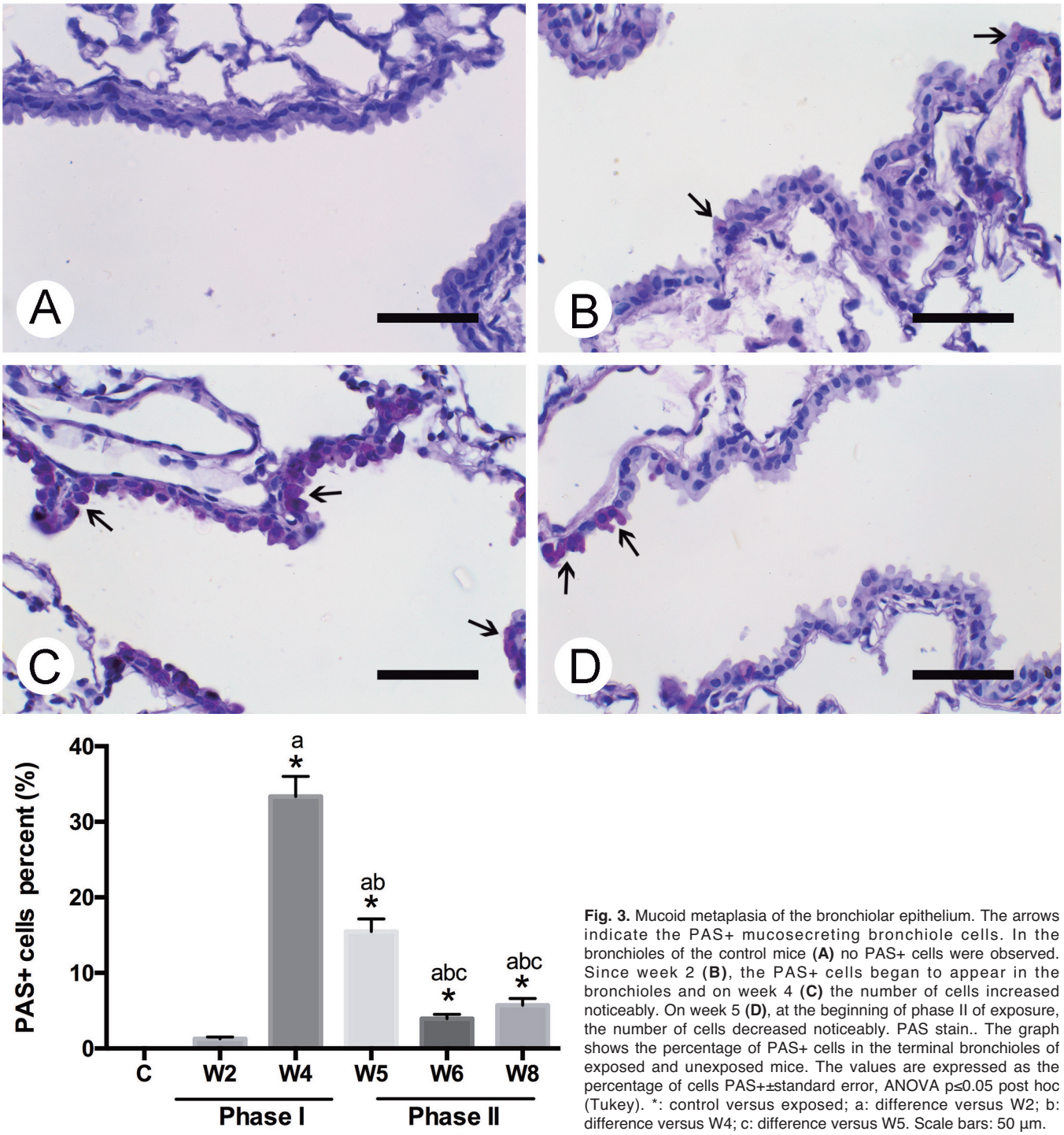

Fig. 3. Mucoid metaplasia of the bronchiolar epithelium. The arrows indicate the PAS+ mucosecreting bronchiole cells. In the bronchioles of the control mice **(A)** no PAS+ cells were observed. Since week 2 **(B)**, the PAS+ cells began to appear in the bronchioles and on week 4 **(C)** the number of cells increased noticeably. On week 5 **(D)**, at the beginning of phase II of exposure, the number of cells decreased noticeably. PAS stain.. The graph shows the percentage of PAS+ cells in the terminal bronchioles of exposed and unexposed mice. The values are expressed as the percentage of cells PAS+±standard error, ANOVA p≤0.05 post hoc (Tukey). *: control versus exposed; a: difference versus W2; b: difference versus W4; c: difference versus W5. Scale bars: 50 µm.

Determination of CC16 in the lung tissue

Immunohistochemistry showed the presence of the CC16 marker in the non-ciliated bronchiolar cells in the control group in all weeks of exposure. The marker in this group was relatively homogeneous (Fig. 4A), while the inhalation of V resulted in an increase in the presence of CC16. At the beginning of phase I of exposure, CC16 in the bronchiolar epithelium remained as in control group; this pattern was observed on week 2 of inhalation without significant changes. On week 4 CC16 increased its presence (Fig. 4B). On week 5, at the

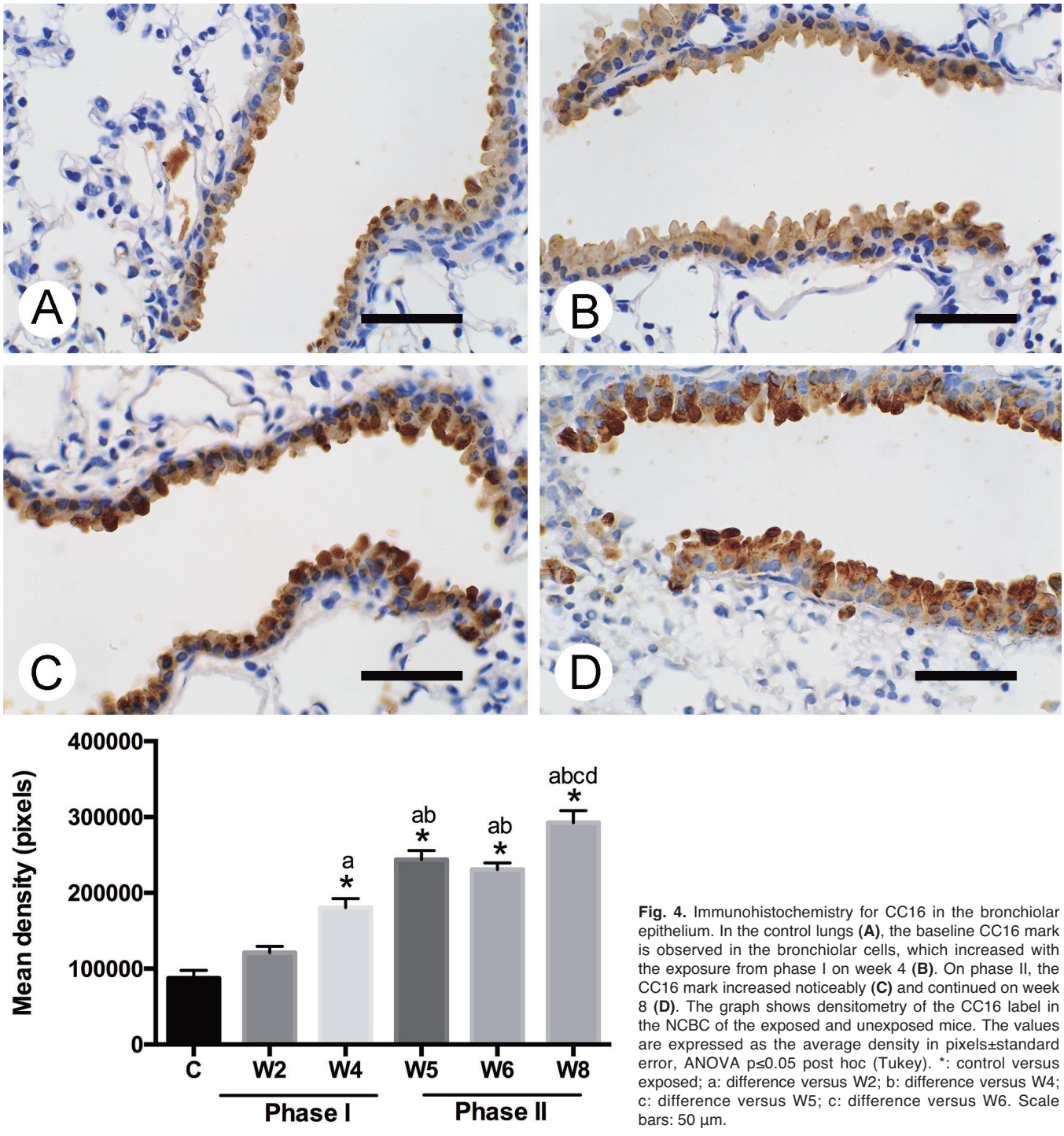

Fig. 4. Immunohistochemistry for CC16 in the bronchiolar epithelium. In the control lungs **(A)**, the baseline CC16 mark is observed in the bronchiolar cells, which increased with the exposure from phase I on week 4 **(B)**. On phase II, the CC16 mark increased noticeably **(C)** and continued on week 8 **(D)**. The graph shows densitometry of the CC16 label in the NCBC of the exposed and unexposed mice. The values are expressed as the average density in pixels±standard error, ANOVA p≤0.05 post hoc (Tukey). *: control versus exposed; a: difference versus W2; b: difference versus W4; c: difference versus W5; c: difference versus W6. Scale bars: 50 µm.

beginning of phase II, the presence of CC16 increased again (Fig. 4C) and remained so until the end of the exposure (Fig. 4D).

The densitometric analysis showed baseline levels of CC16 in the controls and an increase in the density of the protein from the second week of the exposure to V, without it being significant. At week 4, the density of CC16 was significantly higher and at the beginning of phase II, CC16 increased significantly again and remained constant on week 6. At the end of the exposure on week 8, the density of the marker increased again (Graph 4).

Determination of CC16 in the lung homogenates

WB detected the expression of CC16 in the lungs of the control mice but this was modified by the inhalation of V (Fig. 5). On weeks 2 and 4 of phase I of exposure, no significant difference was detected in the expression of the marker (it was the same as in the controls) while on weeks 5, 6 and 8 of the exposure, during phase II, the expression of CC16 increased significantly while remaining stable (Graph 5).

Discussion

The results showed that vanadium inhaled at the two concentrations (1.56 and 2.57 mg/m^3) produced alterations in the lung such as peribronchiolar and perivascular infiltrate, and various modifications of the bronchiolar epithelium such as hyperplasia and sloughing of the NCBC. These alterations occurred during the entire exposure time, without relevant changes when modifying the exposure concentration.

On the other hand, the change in the phenotype of the NCBC to mucosecreting was identified and the cell quantification evidenced the gradual increase in the number of PAS+ cells during phase I of exposure. However, increasing the concentration of the element during phase II showed a significant decrease in the number of mucosecreting cells.

As for CC16, it increased significantly in the bronchiolar epithelium in phase I of exposure and afterwards, during the second phase, the increase in the marker expression was significantly higher than in phase I.

Vanadium concentration

The exposure concentration of V was higher when using the (0.02 M) solution compared to the (0.01 M). With this data, the animals were exposed to two different concentrations, one lower than the other, establishing an adequate model to study tolerance. Regarding the blood levels, a gradual increase in the concentration of the metal was observed from weeks 4, 5 and 6. However, we observed that during week 8, the levels of V in the blood unexpectedly decreased. This could be explained by the deposition and accumulation of V in soft organs such as the lung and the liver, and in hard organs such as the bone (Rhoads and Sanders, 1985; Rodríguez-Mercado and Altamirano-Lozano, 2006).

Another possible explanation could be that

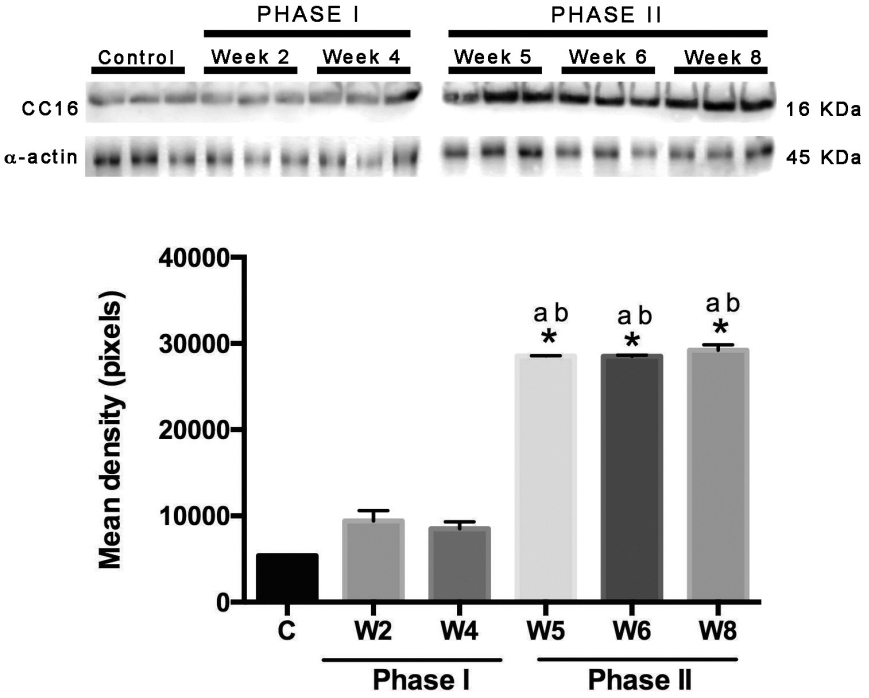

Fig. 5. The effect of V on the expression of CC16 in the lung homogenates. By observing the bands from the controls, the expression of CC16 was identified and in phase I the expression of the marker did not change. In phase II an increase in the marker was observed. The graph shows the analysis of the expression of CC16 in lung homogenates as an effect of V. The α-actin protein was used as a loading marker. The values are expressed as the mean density in pixels and represent the mean±standard error, ANOVA $p \leq 0.05$ post hoc (Tukey). *: control versus exposed; a: difference versus W2; b: difference versus W4.

metallothioneins, particularly in the liver, could be acting as metal chelators according to what has been demonstrated by Kobayashi et al. (2006), using mass spectrometry (HPLC / ICP-MS), by testing the binding of V to an unidentified low-molecular-weight protein with characteristics similar to metallothionein II that could have the ability to bind with this element and retaining it in the liver (Kobayashi et al., 2006). The latter has not yet been explored.

The interaction with metallothioneins could also be happening in the lung, since it is an organ in which these proteins are expressed in an important way. It has been proven that during exposure to oxidizing agents such as ozone, nitrogen dioxide and oxygen, and to metals such as cadmium, their expression can be induced in response to these (Mango et al., 1998; Johnston et al., 2001; Lau et al., 2006).

It is also possible that vanadium interacts with other antioxidant proteins, such as CC16, which increased significantly in our model, and whose antioxidant function will be explained later on. CC16 could contribute in an important way in the detoxification of this metal in the lung.

Lung and non-ciliated bronchiolar cells changes

The histopathological changes reported in this study have been identified in other murine models of exposure to V. Regarding the inflammatory response, it has been reported that the inhalation of the two different concentrations of V (NTP, 2002; Ress et al., 2003) and the intratracheal instillation (Pierce et al., 1996, Toya et al., 2001; Wang et al., 2003) produce the activation of the pulmonary inflammatory response. In our inhalation model, perivascular and peribronchiolar inflammatory foci were observed and it apparently remained unchanged during the whole exposure, independently of the exposure concentration (1.56 and 2.57 mg/m^3 of V); meaning that the increase in the concentration of the metal in phase II of exposure did not exacerbate the response observed during phase I.

The CC16 protein may be behind the possible explanation of why in phase II the inflammatory response did not increase. In our model, we observed that CC16 increased in the lung tissue when the organisms were exposed to the inhalation of V at a concentration of 1.56 mg/m^3 as detected by immunohistochemistry in the bronchiolar cells, and subsequently, when exposed to a higher concentration (2.57 mg/m^3), the density of CC16 increased again significantly. In addition, the redistribution of the protein was observed concentrated mainly in the apical region of the cells. This increase in CC16 was confirmed by WB in the lung homogenates and could explain why the mice did not present an exacerbated inflammatory infiltrate in the second phase of the exposure, as observed in the histopathological results.

The importance of this protein as an immunomodulator in the lung was previously mentioned. *In vitro* it has been shown that CC16 attenuates inflammation by decreasing the production of IL-8 (a pro-inflammatory cytokine) in bronchial cells, which attenuates the infiltration of cells such as neutrophils (Tokita et al., 2014). *In vivo*, it has been shown that mice deficient in the production of CC16 have a greater inflammatory response when they are sensitized with ovalbumin, in addition to expressing higher levels of pro-inflammatory cytokines such as IL-4, IL-5, IL-9 and IL1-13 (Chen-Chen et al., 2001). When chronic exposure to different classes of pollutants decreases the expression of CC16 in the bronchiolar epithelium, organisms become more susceptible to develop an inflammatory response in the lung. Gowdy (2008) reported that the inhalation of particles derived from diesel (DEP) that decrease of CC16 and surfactant proteins (SP-A and SP-D) in the lung homogenates and bronchoalveolar lavage coincides with over-expression of pro-inflammatory cytokines such as IL-6, IL-13 and TNF-α (Gowdy et al., 2008), demonstrating the relation between the decrease in CC16 and inflammation.

Dodge (1994) indicated that in the development of tolerance to ozone, which is a pro-oxidant agent, the density of CC16 in the bronchiolar cells increases when animals are exposed to this gas in comparison to the controls; also, the cytoplasmic distribution of the granules that contain the protein is modified and accumulates in the apical region of the cells (Dodge et al., 1994); these results coincide with our findings.

Regarding bronchial cell hyperplasia, it has been reported that V-inhalation produces this alteration in the bronchioles and alveoli (NTP, 2002; Ress et al., 2003), as well as the instillation of compounds of the same element (Toya et al., 2001). This change has been linked with the induction of pro-inflammatory mediators such as IL-6, IL-8, and TNFα that can also trigger cell proliferation (Bonner et al., 2000). In our model, bronchiolar epithelial hyperplasia was identified from the first week of exposure in phase I, and it remained constant throughout the exposure without any relevant changes during the rest of the treatment. The change in the concentration of V did not cause the increase in hyperplasia. It would be interesting to further explore the effect of the CC16 on the expression of pro-inflammatory cytokines in our model.

Many of the alterations produced by the exposure to V are attributed to the generation of oxidative stress (Rodríguez-Mercado and Altamirano-Lozano, 2006). Previous reports show that CC16 has the ability to function as a molecule with antioxidant properties through the interaction of methionines that are part of its structure (Pilon et al., 2016) which can attenuate the toxic effects of vanadium in the lung in our model. It could also be involved in the detoxification of vanadium; however further research is needed.

In addition to the antioxidant activity of CC16, it is important to take into consideration the activity of metallothioneins in the lung as regulators of oxidative stress. As mentioned above, metals induce the

expression of these proteins that act as chelators and trappers of reactive oxygen species (ROS); also, it has been reported that these play an important role in the development of tolerance to metals such as zinc (Wesselkamper et al., 2001) and cadmium in the lung (Lau et al., 2006).

Furthermore, in addition to metallothioneins, the lung has a large variety of enzymatic and non-enzymatic antioxidant mechanisms that contribute to the defense against oxidizing agents (Rahman et al., 2006). The main non-enzymatic antioxidants in the lung are glutathione, vitamins C and E, carotenoids and uric acid (Rock et al., 1993; Comhair and Erzurum, 2002; McFadden et al., 2005). Superoxide dismutases (MnSOD and CuSOD), catalase and peroxidases are the major enzymatic antioxidants in this organ (Rahman et al., 2006).

There is evidence that suggests that an increase in the levels of glutathione favors tolerance to oxidizing compounds exposure such as coumarin in the lung, decreasing its susceptibility (Vassallo et al., 2010). The relationship between tolerance and the activity of other antioxidants in the lung has not been sufficiently studied; hence, it would be relevant and interesting to study this more as well as its relationship with tolerance to vanadium exposure and other metals.

It is important to note that the qualitative evaluation of the lung tissue showed that during phase I the aforementioned changes occurred in a subtle way. In phase II of the experiment, in which the concentration of V increased, the changes remained stable. As previously mentioned, a similar response was reported by Knetch (1992) noting that in his model of subchronic exposure to 0.5 mg/m^3 (V_2O_5) (6 h/ for 5 days/ for 26 weeks), the repeated exposure to V did not trigger a cellular immune response, nor did it increase bronchial hyperreactivity or cause exacerbation of the acute response that had been previously reported by the same group (Knetch et al., 1992). Although our exposure model differs from Knetch's model, both suggest that animals develop some type of tolerance as a result of repeated exposure to V_2O_5.

The increase in the concentration of CC16 coincides in the second phase of exposure with the decrease in the number of mucosecreting PAS+ cells. It has been reported that exposure to V produces a change in the phenotype of these cells that manifests itself with the synthesis of AB/PAS+ mucins; this mucoid metaplasia has been directly linked to the inflammation produced by the treatment through the production of TNFα and IL-6 (Toya et al., 2001; Bonner et al., 2000). Other agents that produce bronchiolar mucoid metaplasia by inhalation are PM and ozone. *In vivo* it has been reported that the exposure to ultrafine particles induces mucoid metaplasia in the lower airways, in addition to mucosal hypersecretion (Alessandrini et al., 2010), while Kumagai et al. reported bronchiolar mucoid metaplasia in mice exposed to ozone (Kumagai et al., 2017).

Since it is well known that the inflammatory response is directly related to the changes in the phenotype of the NCBC and this inflammation is associated to the decrease in the expression of CC16, it can be inferred that the increase in CC16 decreases inflammation and this will directly influence the phenomenon of metaplasia. This decrease in PAS+ mucosecretory cells in phase II could be attributed to a reversal in the phenotype of the cells favored by the increase in the expression of CC16 that begins on week 3 of the exposure on phase I and remains stable during phase II.

As it was previously mentioned, tolerance is a phenomenon that has been observed in lungs exposed to various contaminants and substances. However, the factors that could contribute to the development of this response have not yet been clearly established. Our model shows that the pre-exposure to V at 1.56 mg/m^3 induces the increase of CC16, which could have a direct effect on the inflammatory changes and on the modification in the mucosecreting phenotype of the NCBC observed at 2.57 mg/m^3.

Conclusions

The V-inhalation model proposed in this study was useful for identifying the changes that suggest tolerance in the bronchiolar epithelium and highlights the importance of CC16 in the protection of the lung as our results demonstrated. These phenomena could in part explain the effects that favored the "resistance" of the lung when exposed to pollution and helps to understand the importance of NCBC and its protein CC16 in the development of this tolerance.

One limitation of this study is that CC16 was not identified in the bronchoalveolar lavage, which could support the findings of its increase as part of the epithelium's defense. To confirm its participation, it would be interesting to explore its modification in the bronchoalveolar lavage, in addition to the direct evaluation on the inflammation in this model.

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References

Alessandrini F., Weichenmeier I., van Miert E., Takenaka S., Karg E., Blume C., Mempel M., Schulz H., Bernard A. and Behrendt H. (2010). Effects of ultrafine particles-induced oxidative stress on Clara cells in allergic lung inflammation. Part. Fibre Toxicol. 7, 11-24.

Assem F.L. and Levy L.S. (2012). Inhalation toxicity of Vanadium. In: Vanadium. Biochemical and molecular biological approaches. Michibata H. (ed). Springer Science+Bussiness Media. Netherlands. pp. 209-224.

Bonner J.C., Rice A.B., Moomaw C.R. and Morgan D.L. (2000). Airway fibrosis in rats induced by vanadium pentoxide. Am. J. Physiol. Lung Cell. Mol. Physiol. 278, L209-L216.

Born S.L., Fix A.S., Caudill D. and Lehman-McKeeman L.D. (1999). Development of tolerance to Clara cell necrosis with repeat administration of coumarin. Toxicol. Sci. 309, 300-309.

Broeckaert F., Clippe A., Knoops B., Hermans C. and Bernard A. (2000). Clara cell secretory protein (CC16): features as a peripheral lung biomarker. Ann. NY Acad. Sci. 923, 68-77.

Chang H., Chang L.W., Cheng Y., Tsai W., Tsai M. and Lin P. (2006). Preferential induction of CYP1A1 and CYP1B1 in CCSP-positive cells. Toxicol. Sci. 89, 205-213.

Chen Chen L.C., Zhang Z., Myers A.C. and Huang S.K. (2001). Cutting edge: Altered pulmonary eosinophilic inflammation in mice deficient for Clara cell secretory 10-kDa protein. J. Immunol. 167, 3025-3028.

Comhair S.A. and Erzurum S.C. (2002). Antioxidant responses to oxidantmediated lung diseases. Am. J. Physiol., Lung Cell. Mol. Physiol. 283, L246-L255.

Crosby L.M. and Waters C.M. (2010). Epithelial repair mechanisms in the lung. Am. J. Physiol. Lung Cell Mol. Physiol. 298, 715-731.

Curran D.R. and Cohn L. (2010). Advances in mucous cell metaplasia. Am. J. Respir. Cell Mol. Biol. 42, 268-275.

Dodge D.E., Rucker R.B., Pinkerton K.E., Haselton C.J. and Plopper C.G. (1994). Dose-dependent tolerance to ozone: III. Elevation of intracellular Clara cell 10-kDa protein in central acini of rats exposed for 20 Months. Toxicol. Appl. Pharmacol. 127, 109-123.

Fairchild E.J. (1967). Tolerance mechanisms. Arch. Environ. Occup. Health. 14, 111-126.

Fortoul T. I., Rodriguez-Lara V., Gonzalez-Villalva A., Rojas-Lemus M., Cano-Gutierrez G., Ustarroz-Cano M., Colín-Barenque L., Montaño L.F., García-Pelaez I., Bizarro-Nevares P., López-Valdez N., Falcón-Rodríguez C.I., Jimenez-Martínez R.S., Ruíz-Guerrero M.L., López-Zepeda and Muñiz-Rivera-Cambas A. (2011). Vanadium inhalation in a mouse model for the understanding of air-suspended particle systemic repercussion. J. Biom. Biotechnol. 2011, 951043.

Fortoul T. I., Rodriguez-Lara V., González-Villalva A., Rojas-Lemus M., Cano-Gutiérrez G., Ustarroz-Cano M., Colín-Barenque L., Bizarro-Nevares P., García-Pelaez I., Montaño L.F., Jimenez-Martínez R.S., López-Valdez N., Ruíz-Guerrero M.L., Meléndez-García N.A., García-Ibarra F.A., Martínez-Báez V., Zapata Alfaro D., Muníz-Rivera-Cambas A., López-Zepeda L.S., Quezada-Maldonado E.M. and Cervantes-Yépez S. (2014). Inhalation of vanadium pentoxide and its toxic effects in a mouse model. Inorganica Chim. Acta. 420, 8-15.

Gowdy K., Krantz Q.T., Daniels M., Linak W.P., Jaspers I. and Gilmour M.I. (2008). Modulation of pulmonary inflammatory responses and antimicrobial defenses in mice exposed to diesel exhaust. Toxicol. Appl. Pharmacol. 229, 310-319.

Hiemstra P.S. and Bourdin A. (2014). Club cells, CC10 and self-control at the epithelial surface. Eur. Respir. J. 44, 831-832.

Hukkanen J., Pelkonen O., Hakkola J. and Raunio H. (2002). Expression and regulation of xenobiotic-metabolizing Cytochrome P450 (CYP) enzymes in human lung. Crit. Rev. Toxicol. 32, 391-411.

Johnston C.J., Oberdörster G. and Finkelstein J.N. (2001). Recovery from oxidant-mediated lung injury: response of metallothionein, MIP-2, and MCP-1 to nitrogen dioxide, oxygen, and ozone exposures. Inhal. Toxicol. 13, 689-702.

Knecht E.A., Moorman W.J., Clark J.C., Hull R.D., Biagini R.E., Lynch D.W., Boyle T.J. and Simon S.D. (1992). Pulmonary reactivity to vanadium pentoxide following subchronic inhalation exposure in a non-human primate animal model. J. Appl. Toxicol. 12, 427-434.

Kobayashi K., Himeno S., Satoh M., Kuroda J., Shibata N., Seko Y. and Hasegawa T. (2006). Pentavalent vanadium induces hepatic metallothionein through interleukin-6-dependent and -independent mechanisms. Toxicology 228, 162-170.

Kumagai K., Lewandowski R.P., Jackson-Humbles D.N., Buglak N., Li N., White K., Van Dyken S.J., Wagner J.G. and Harkema J.R. (2017). Innate lymphoid cells mediate pulmonary eosinophilic inflammation, airway mucous cell metaplasia, and type 2 immunity in mice exposed to ozone. Toxicol. Pathol. 45, 692-704.

Lakritz J., Chang A., Weir A., Nishio S., Hyde D., Philpot R., Buckpitt A. and Plopper C. (1996). Cellular and metabolic basis of Clara cell tolerance to multiple doses of cytochrome P450-activated cytotoxicants. I: Bronchiolar epithelial reorganization and expression of cytochrome P450 monooxygenases in mice exposed to multiple doses of naphthalene. Pharmacol. Exp. Ther. 278, 1408-1418.

Lau A., Zhang J., and Chiu J. (2006). Acquired tolerance in cadmium-adapted lung epithelial cells: Roles of thec-Jun N-terminal kinase signaling pathway and basal level of metallothionein. Toxicol. Appl. Pharmacol. 215,1-8.

Liu Y., Yu H.J., Wang N., Zhang Y.N., Huang S.K., Cui Y.H. and Liu Z. (2013). Clara cell 10-kDa protein inhibits TH17 responses through modulating dendritic cells in the setting of allergic rhinitis. J. Allergy Clin. Immunol. 131, 387-394.

Mango G.W., Johnston C.J., Reynolds S.D., Finkelstein J.N., Plopper C.G. and Stripp B.R. (1998). Clara cell secretory protein deficiency increases oxidant stress response in conducting airways. Am. J. Physiol. 275, 348-356.

McFadden S.L., Woo J.M., Michalak N. and Ding D. (2005). Dietary vitamin C supplementation reduces noise-induced hearing loss in guinea pigs. Hear. Res. 202, 200-208.

National Toxicology Program (2002). NTP toxicology and carcinogensis studies of vanadium pentoxide in F344/N rats and B6C3F1 mice (inhalation). Natl. Toxicol. Program Tech. Rep. Ser. 507,1-35.

Pierce L.M., Alessandrini F., Godleski J.J. and Paulauskis J.D. (1996). Vanadium induced chemokine mRNA expression and pulmonary inflammation. Toxicol. Appl. Pharmacol. 138, 1-11.

Pilon A.L., Winn M.E., Clayton R.S. and Hariprakasha H. (2016). Modification of CC10 protein by reactive oxygen species: A novel anti-inflammatory mechanism. Am. J. Respir. Crit Care Med. 193, 5907.

Rahman I., Biswas S.K. and Kode A. (2006). Oxidant and antioxidant balance in the airways and airway diseases. Eur. J. Pharmacol. 533, 222-239.

Ress N.B., Chou B.J., Renne R., Dill J., Miller R., Roycroft J.H., Hailey J.R., Haseman J.K. and Bucher J.R. (2003). Carcinogenicity of

inhaled vanadium pentoxide in F344/N rats and B6C3F1 mice. Toxicol. Sci. 74, 287-296.

Reynolds S.D. and Malkinson A.M. (2010). Clara cell: Progenitor for the bronchiolar epithelium. Int. J. Biochem. Cell. Biol. 42, 1-4.

Rhoads K. and Sanders C.L. (1985). Lung clearance, translocation, and acute toxicity of arsenic, beryllium, cadmium, cobalt, lead, selenium, vanadium, and ytterbium oxides following deposition in rat lung. Environ. Res. 36, 359-378.

Rock C.L., Rodriguez J.L., Khilnani R., Lown D.A. and Parker R.S. (1993). Carotenoids and antioxidant nutrients following burn injury. Ann. NY Acad. Sci. 691, 274-276.

Rodríguez-Mercado J.J. and Altamirano-Lozano, M.A. (2006). Vanadio: contaminación, metabolism y genotoxicidad. (Vanadium: Pollution, metabolism and genotoxicity). Rev. Int. Contam. Ambient. 22, 173-189.

Snyder J.C., Reynolds S.D., Hollingsworth J.W., Li Z., Kaminski N. and Stripp B.R. (2010). Clara cells attenuate the inflammatory response through regulation of macrophage behavior. Am. J. Respir. Cell Mol. Biol. 42, 161-171.

Stripp B.R., Reynolds S.D., Boe I., Lund J., Power J.H.T., Coppens J.T., Wong V., Reynolds P.R. and Plopper C.G. (2002). Clara cell secretory protein deficiency alters Clara cell secretory apparatus and the protein composition of airway lining f luid. Am. J. Respir. Cell Mol. Biol. 27, 170-178.

Sutherland K.M., Edwards P.C., Combs T.J. and Van Winkle L.S. (2012). Sex differences in the development of airway epithelial tolerance to naphthalene. Am. J. Physiol. Lung Cell Mol. Physiol. 302, 68-81.

Tokita E., Tanabe T., Asano K., Suzaki H. and Rubin B.K. (2014). Club cell 10-kDa protein attenuates airway mucus hypersecretion and inflammation. Eur. Respir. J. 44, 1002-1010.

Toya T., Fukuda K., Takaya M. and Arito H. (2001). Lung lesions induced by intratracheal instillation of vanadium pentoxide powder in rats. Industrial Health 39, 8-15.

Vassallo J.D., Kaetzel R.S., Born S.L., Lewis C.L., Lehman-McKeeman L.D. and Reed D.J. (2010). Gamma-glutamyl transpeptidase null mice fail to develop tolerance to coumarin-induced Clara cell toxicity. Food Chem. Toxicol. 48, 1612-1618.

Wang L., Medan D., Mercer R., Overmiller D., Leornard S., Castranova V., Shi X., Ding M., Huang C. and Rojanasakul Y. (2003). Vanadium-induced apoptosis and pulmonary inflammation in mice: Role of reactive oxygen species. J. Cell. Physiol. 195, 99-107.

Wesselkamper S.C., Chen L.C. and Gordon T. (2001). Development of pulmonary tolerance in mice exposed to zinc oxide fumes. Toxicol. Sci. 60,144-151.

West J., Williams K. J., Toskala E., Nishio S.J., Fleschner C., Forman H.J. and Plopper C.G. (2002). Induction of tolerance to naphthalene in Clara cells is dependent on a stable phenotypic adaptation favoring maintenance of the glutathione pool. Am. J. Pathol. 160, 1115-1127.

Wong A.P., Keating A. and Waddell T.K. (2009). Airway regeneration: the role of the Clara cell secretory protein and the cells that express it. Cytotherapy 11, 676-687.

Xiao C., Guo L., Qi R. and Xi S. (2007). Effects of air mixed certain pollutants on the expression of CC16 and certain cytokine in pulmonary tissue of rats. Wei Sheng Yan Jiu 36,679-682.

Xiao C., Li S., Zhou W., Shang D., Zhao S., Zhu X., Chen K. and Wang R. (2013). The effect of air pollutants on the microecology of the respiratory tract of rats. Environ. Toxicol. Pharmacol. 36, 588-594.

Zuo W.L., Shenoy S.A., Li S., O'Beirne S. L., Strulovici-Barel Y., Leopold P.L., Wang G., Staudt M.R., Walters M.S., Mason C., Kaner R.J., Mezey J.G. and Crystal R.G. (2018). Ontogeny and biology of human small airway epithelial club cells. Am. J. Respir. Crit. Care Med. 198, 1375-1388.

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