

Effects of nanoparticles on neuroinflammation in a mouse model of asthma

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ABSTRACT

The interaction between chronic inflammation and neural dysfunction points to a link between the nervous and immune systems in the airways. In particular, environmental exposure to nanoparticles (NPs), defined as particulate matter having one dimension < 100 nm, is associated with an enhanced risk of childhood and adult asthma. However, the impact of NPs on the neural response in asthma remains to be determined.

This study determined the impact of NPs on neuroinflammation in a mouse model of allergic asthma.

Ovalbumin (OVA) sensitized mice were treated with saline (Sham), OVA challenged and exposed to 200 µg/m³ NPs 1 h a day for 3 days on days 21–23 in a closed-system chamber attached to a ultrasonic nebulizer. The effect of NPs on the levels of neuropeptides, transient receptor potential vanilloid 1 (TRPV1), TRPV4, P2 × 4, and P2 × 7 was assessed by enzyme-linked immunosorbent assays, immunoblotting, and immunohistochemistry.

NP exposure increased airway inflammation and responsiveness in OVA mice, and these increases were augmented in OVA plus NP-exposed mice. The lung tissue levels of TRPV1, TRPV4, P2 × 4, and P2 × 7 were increased in OVA mice, and these increases were augmented in OVA plus NP-exposed mice. The substance P, adenosine triphosphate (ATP), and calcitonin gene-related peptide (CGRP) levels in bronchoalveolar lavage fluid were increased in OVA mice, and these increases were augmented in OVA plus NP-exposed mice. Bradykinin, ATP, and CGRP were dose dependently increased in NP-exposed normal human bronchial epithelial (NHBE) cells. The calcium concentration was increased in NHBE cells exposed to NPs for 8 h.

These results indicate that neuroinflammation can be involved in the pathogenesis of bronchial asthma and that NPs can exacerbate asthma via neuromediator release.

1. Introduction

Asthma is a clinical syndrome comprised of intermittent respiratory symptoms triggered by viral infections, environmental allergens, and air pollutants, and characterized by nonspecific airway hyperresponsiveness and inflammation (Holgate, 2008).

Air pollutants such as particulate matter (PM)_{2.5}, nitric oxide (NO)₂, carbon monoxide (CO), and ozone associated with asthma exacerbations. In children, the association was significant for NO₂, sulfur oxide (SO)₂ and PM_{2.5} (Orellano et al., 2017). Meta-analyses showed statistically significant associations for black carbon, NO₂, PM_{2.5}, PM₁₀ exposures and risk of asthma development (Khreis et al., 2017; Chen

et al., 2006).

Neurogenic inflammation in the lung involves airway obstruction, an increase in vascular permeability, extravasation of plasma and leukocytes, mucus hypersecretion, and the release of additional inflammatory mediators (Butler and Heaney, 2007).

The neurogenic inflammatory pathway is associated with the release and activity of neuropeptides such as tachykinins and calcitonin gene-related peptide (CGRP) as a response of sensory neurons to inflammatory mediators and noxious stimuli (Butler and Heaney, 2007; Banner et al., 2011).

Transient receptor potential vanilloid 1 (TRPV1) is expressed in bronchial and tracheal epithelial cells, airway smooth muscle cells,

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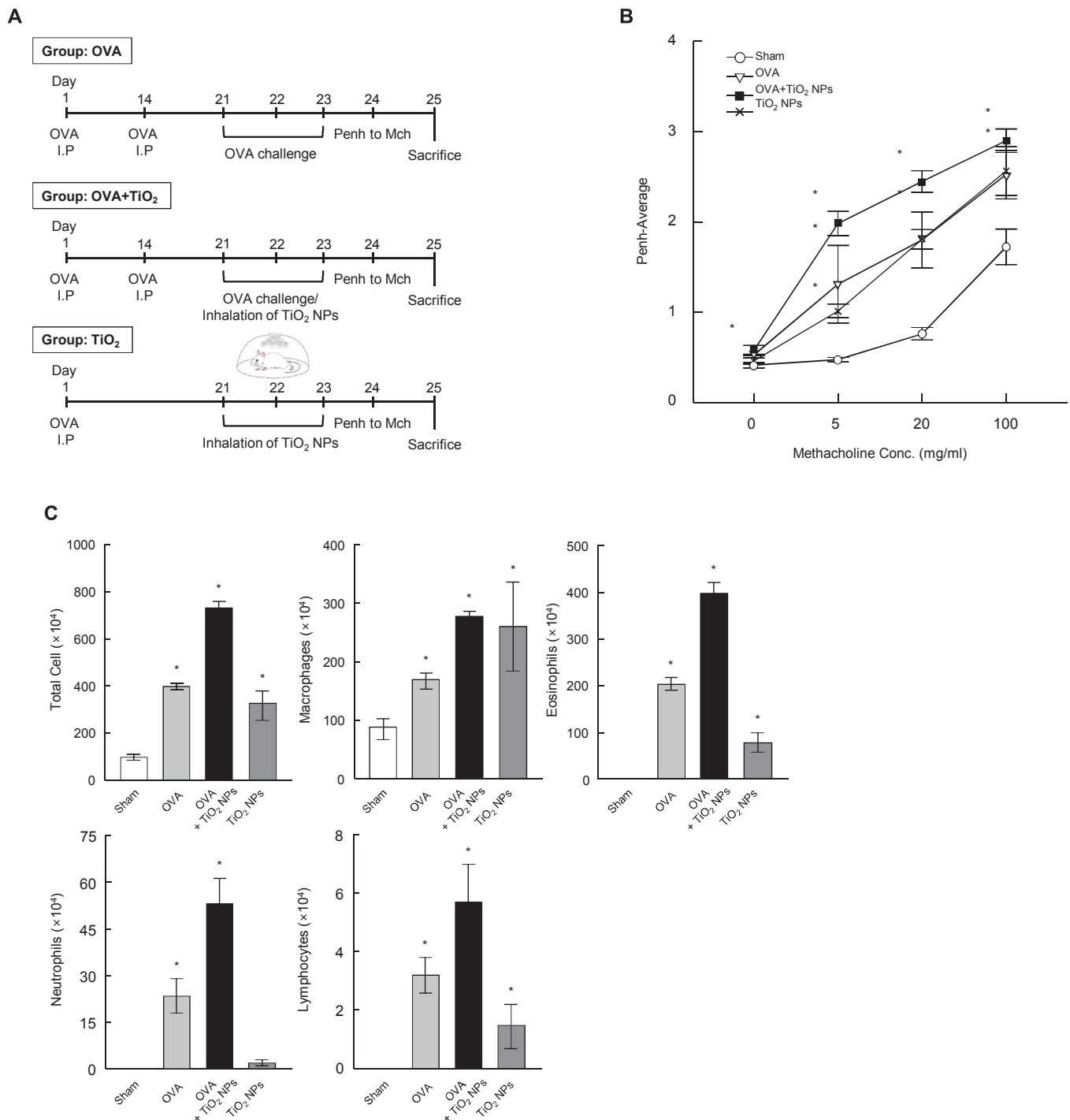


Fig. 1. TiO₂ NP on inflammation in a mouse model. (A) Experimental model of TiO₂ exposure in BALB/c mice. (B) AHR in BALB/c mice exposed to TiO₂ NPs. AHR was measured following the administration of increasing doses of methacholine. (C) Differential inflammatory cell counts in the BALF of TiO₂ NP-exposed mice. TiO₂ NP exposure increased the number of total cells, macrophages, eosinophils, neutrophils, and lymphocytes. The data are the means $\pm$ SEM of 8 mice per group. * $p < 0.05$ compared with the sham group.

primary sensory neurons, and tracheal epithelial cells, and dendritic cells in the lung (Bessac and Jordt, 2008). TRPV1 plays a particularly important role in increasing C-fiber excitability and neuronal inflammatory pathways during airway inflammation (Colsoul et al., 2009). Their activation by noxious temperatures, changes in membrane potential, mechanical or osmotic stress, and a low pH leads to the release of neuropeptides causing to change of airway smooth muscle tone, airway secretion, edema, and the chemoattraction of inflammatory and immune cells (Banner et al., 2011).

P2X receptors are ATP-gated ion channels localized on primary afferent nerves arising from both cranial and dorsal root ganglia (Wang et al., 2014; Burnstock et al., 2012; Kwong et al., 2008; Weigand et al., 2008; Tan et al., 2009). ATP and histamine to tussive stimuli are activated via P2X receptor-mediated mechanisms (Guo et al., 2007; Abdulqawi et al., 2015; Khakh, 2001; Monção-Ribeiro et al., 2014; Wareham et al., 2009; Jacob et al., 2013). P2 $\times$ 7 receptors, which play a role in neuroinflammation, are frequently co-expressed with another P2X receptor, P2 $\times$ 4 (Guo et al., 2007; Abdulqawi et al., 2015; Sakaki

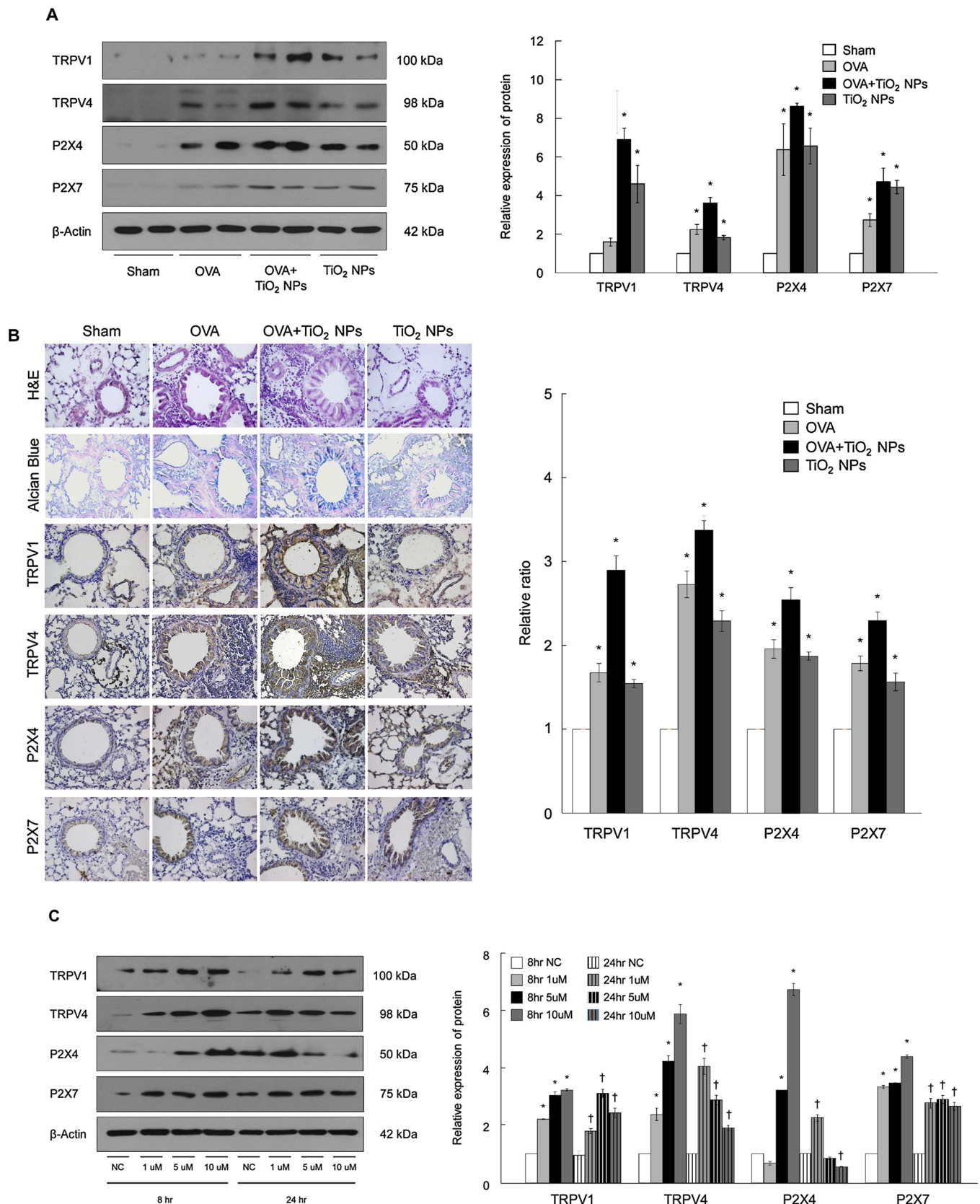


Fig. 2. The expression levels of TRP channels and purinergic receptors in TiO₂ NP-exposed mice and NHBE cells. (A) The TRPV1, TRPV4, P2 × 4, and P2 × 7 levels determined by Western blotting in OVA, TiO₂ NP, and OVA-plus-TiO₂ NP-exposed mice. The band intensities of TRPV1, TRPV4, P2 × 4, and P2 × 7 were compared to that of β-actin. The data are the means ± SEM of 8 mice per group. * *p* < 0.05 compared with the sham group. (B) Immunohistochemical staining of mouse lung tissue using antibodies against TRPV1, TRPV4, P2 × 4, and P2 × 7. Quantitation of the TRPV1, TRPV4, P2 × 4, and P2 × 7 staining intensities. The data are the means ± SEM of 8 mice per group. * *p* < 0.05 compared with the sham group. (C) The TRPV1, TRPV4, P2 × 4, and P2 × 7 levels in NHBE cells exposed to 1, 5, or 10 μM TiO₂ NPs for 8 or 24 h. * *p* < 0.05 compared with the 8-h NC. † *p* < 0.05 compared with the 24-h NC.

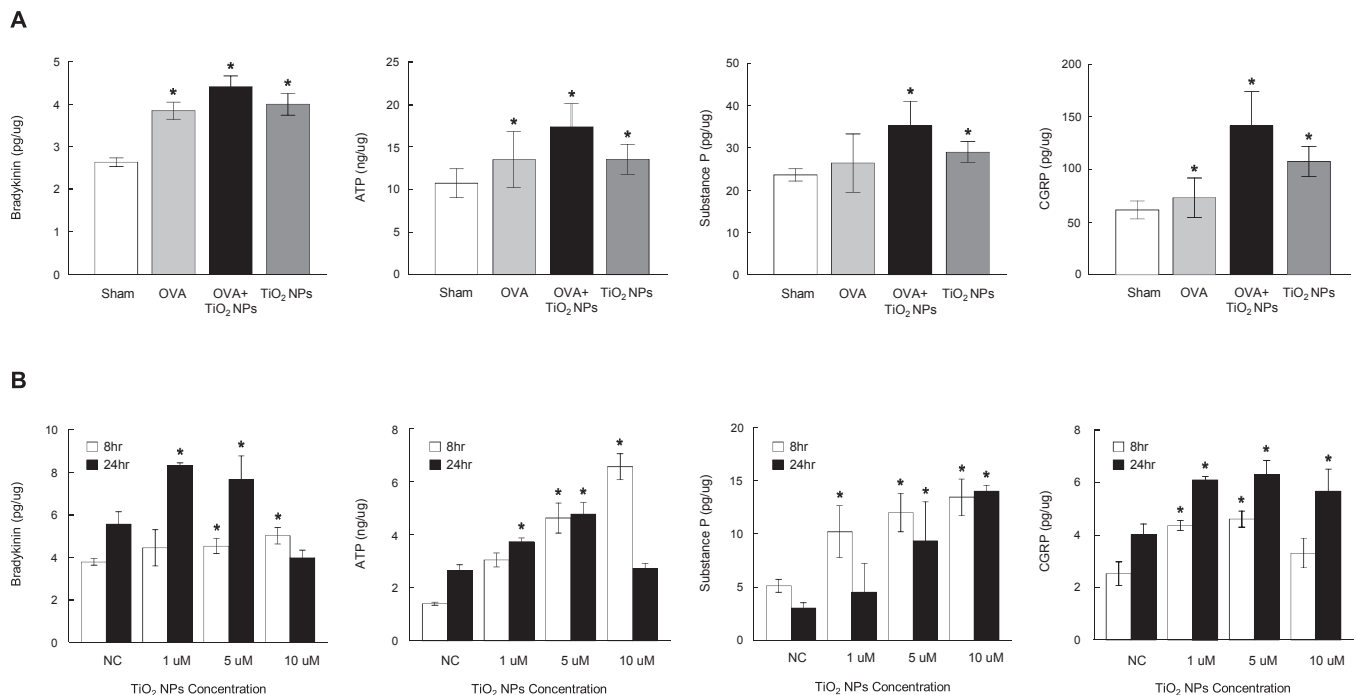


Fig. 3. Neuropeptide levels in the BALF of mice and NHBE cells exposed to TiO₂ NPs. (A) The levels of BK, ATP, SP, and CGRP in OVA, TiO₂ NP, and OVA plus TiO₂ NP-exposed mice. The data are the means $\pm$ SEM of 8 mice per group. (B) The levels of BK, ATP, SP, and CGRP in NHBE cells exposed to 1, 5, or 10 μ M TiO₂ NPs for 8 or 24 h. * $p < 0.05$ compared with the NC. * $p < 0.05$ compared with the sham group.

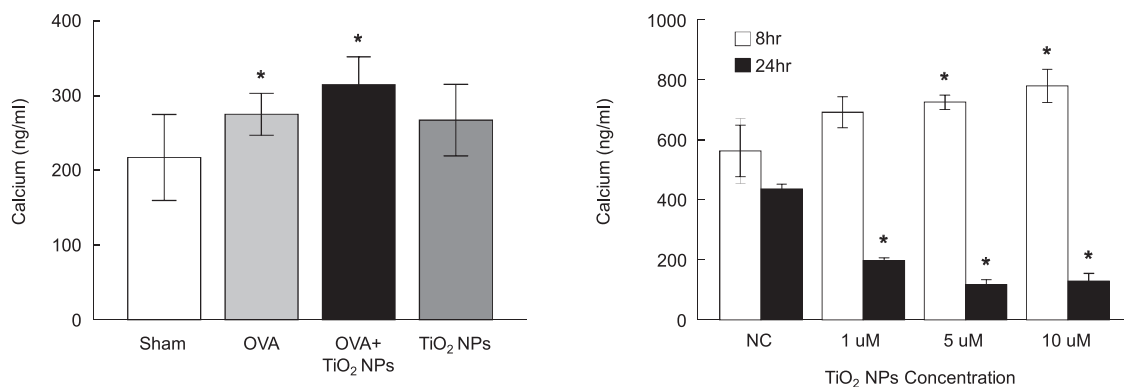


Fig. 4. Calcium deposition in lung tissue and NHBE cells. (A) The calcium concentration in OVA, TiO₂ NP, and OVA plus TiO₂ NP-exposed mice. The data are the means $\pm$ SEM of 8 mice per group. * $p < 0.05$ compared with the sham group. (B) The calcium concentration in NHBE cells exposed to 1, 5, or 10 μ M TiO₂ NP for 8 or 24 h. * $p < 0.05$ compared with the 8-h NC. † $p < 0.05$ compared with the 24-h NC.

et al., 2013). Silica nanoparticles inhibit TRPV4 activation, and impair the positive modulatory action of the stimulation of TRPV4 channel on the ciliary beating frequency in airway epithelial cells (Sanchez et al., 2017). P2 $\times$ 7 receptor is involved in the inflammation triggered by SiO₂ and TiO₂ NPs, by increasing IL-1 β secretion, and likely through the inflammasome pathway (Dekali et al., 2013).

The roles of TRPV1 and TRPV4, and of the purinergic receptors P2 $\times$ 4 and P2 $\times$ 7, and neuromediators in the pathogenesis of asthma are unclear. Using the lungs of mice with ovalbumin (OVA)-induced inflammation exposed to nanoparticles (NPs), we investigated the impact of NPs on neuromediators and neurogenic receptors in a mouse model of asthma.

2. Materials and methods

2.1. Characterization of titanium dioxide (TiO₂) NPs

TiO₂ NPs (Standard Reference Material [SRM] 1898; National

Institute of Standards and Technology [NIST], Gaithersburg, MD) containing 15 g of a mixed phase (anatase and rutile) were used in the form of a dry agglomerated powder according to the manufacturer's instructions. Suspensions of TiO₂ NPs were prepared in D-PBS. The endotoxin concentration of the NPs was less than 0.064 ng/mL (0.32 EU/mL), as determined by the *Limulus* amoebocyte lysate assay (QCL-1000; BioWhittaker/Cambrex Corp., Walkersville, MD) (Grünig et al., 1998).

2.2. Cell culture and stimulation with TiO₂ NPs

Primary normal human bronchial epithelial (NHBE) cells (3500 /cm²) were grown in BEGM™ BulletKit medium (Lonza, Basel, Switzerland) at 37 °C in 5% CO₂. The medium was replaced every 48 h until the cells reached 90% confluence. The cells were seeded in six-well plates, and at 24 h prior to the experiment, stimulated with 1, 5, or 10 μ M/mL bovine serum albumin (BSA)-coated TiO₂ NPs for 8 or 24 h.

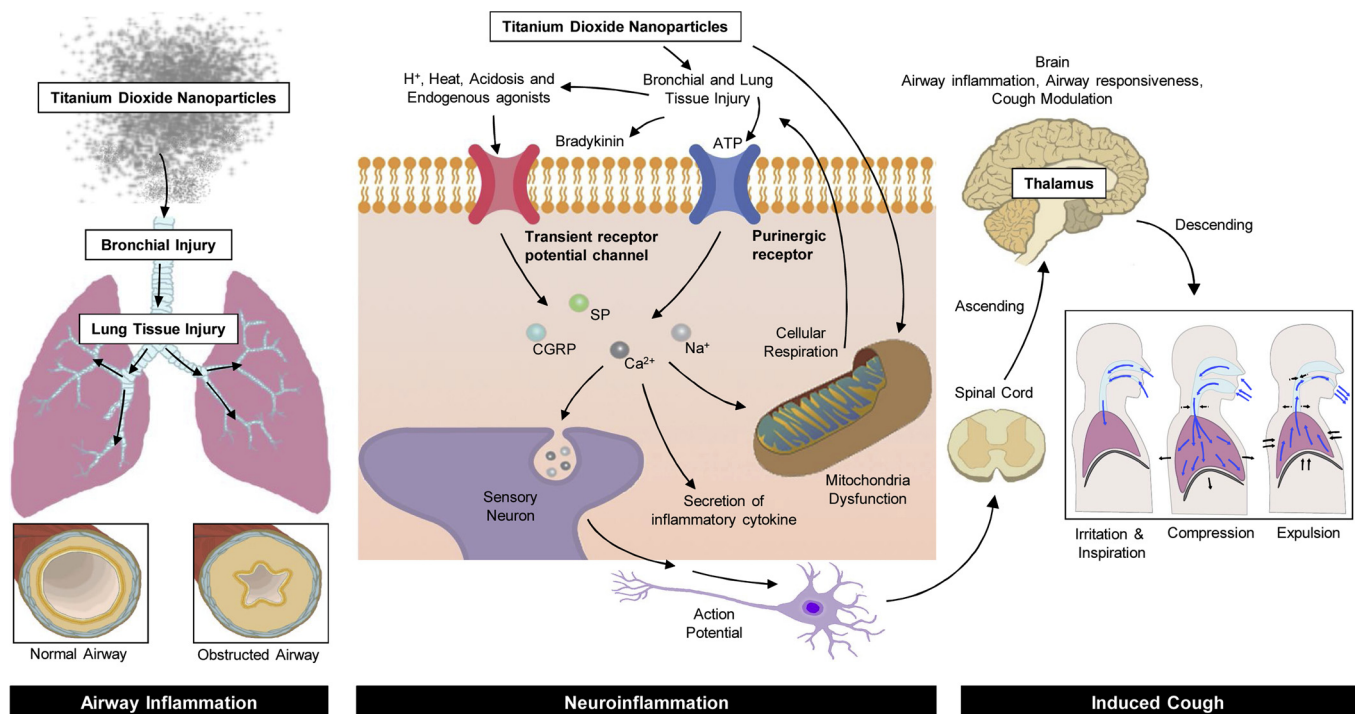


Fig. 5. PM exposure trigger neuroinflammation causing to cough and airway inflammation.

2.3. Animal exposure protocols

Female 6-week-old BALB/c mice ($n = 8$ per group) were sensitized on days 1 and 14 by an intraperitoneal (IP) injection of 50 μg of grade V chicken egg OVA (Sigma-Aldrich, St. Louis, MO) emulsified in 10 mg of hydroxyl aluminum plus 100 μL of D-PBS. At days 21–23, all mice received an intranasal (IN) challenge with 150 μg of grade III OVA (Sigma-Aldrich) in 50 μL of D-PBS. Control mice were sensitized and challenged with saline. Mice in the TiO_2 NP groups were administered 200 $\mu\text{g}/\text{m}^3$ of NPs by inhalation for 2 h before the OVA challenge daily for 3 days. On day 23, airway hyperreactivity (AHR) was measured, bronchoalveolar lavage fluid (BALF) was collected, and lung tissue was processed for protein and reactive oxygen species measurement and Diff-Quick staining (Siemens, Newark, DE). This study was approved by Soonchunhyang University's Institutional Animal Care and Use Committee.

2.4. Analysis of AHR, BALF and morphology

Mice were challenged at baseline with 5, 20, or 100 mg/mL of methacholine. After the methacholine challenge, enhanced pause was measured (Kim et al., 2016). Airway responsiveness was measured using a whole body plethysmograph OCP 3000 (Allmedicus, Anyang) on day 24. Readings were taken and averaged for 3 min after each nebulization, at which time the enhanced pause (Penh) was determined. Breathing pattern was recorded and enhanced pause (Penh) was calculated using a software based on measured parameters such as expiratory time, relaxation time, peak expiratory flow, and peak inspiratory flow. Penh was calculated by the following equation: $\text{Penh} = (\text{Te}/\text{RT}-1) \times \text{PEF}/\text{PIF}$, where Te is expiration time (sec), RT is relaxation time (sec), REF is the peak expiratory flow rate (mL/s), and IF is the peak inspiratory flow rate (mL/s). Penh is a dimensionless value that represents the proportion of maximal expiratory to maximal inspiratory box pressure signals and the timing of expiration. Penh is used as a measure of airway responsiveness to methacholine. The results are expressed as the percentage increase of Penh following challenge with each concentration of methacholine, where the baseline Penh (after

saline challenge) is expressed as 100%.

Mice were anesthetized with an intra-peritoneal injection of a mixture of Zoletil 50 (tiletamine/zolazepam) and Rompun (2% xylazine hydrochloride) at a 4:1 ratio v/v. BALF on 25th day was obtained and stored at -20°C until use. BALF differential cell counts were performed using Diff-Quick cytospin slides; 500 cells were counted per animal. Differential cell counts were calculated as (total cell number of BAL cells) $\times$ (percentage of macrophages, eosinophils, neutrophils and lymphocytes (magnification: X200). A portion of the lung was fixed in 4% buffered paraformaldehyde, embedded in paraffin, cut into 4- μm -thick sections, and stained with hematoxylin and eosin.

2.5. Western blotting

Extracted lung tissue was homogenized in protein lysis solution containing 50 mM Tris-HCl (pH 7.4), 50 mM NaCl, 0.1% sodium dodecyl sulfate (SDS), 1% Triton X-100, 0.5 mM ethylenediaminetetraacetic acid, and 100 mM phenylmethylsulfonyl fluoride in distilled water. The samples were centrifuged at 14,000 rpm for 30 min at 4°C , and the soluble material was collected. Mouse lung proteins were separated by SDS-polyacrylamide gel electrophoresis and transferred to polyvinylidene difluoride membranes. The membranes were blocked in 5% BSA containing 0.1% Tween 20 in Tris-buffered saline for 2 h at room temperature. The membranes were next incubated with rabbit anti-TRPV1 (1:1000; Abcam, Cambridge, MA), rabbit anti-TRPV4 (1:2000; Novus Biologicals, Littleton, CO), rabbit anti-P2 $\times$ 4 (1:1000; GeneTex, Irvine, CA), and rabbit anti-P2 $\times$ 7 (1:1000; Thermo Fisher Scientific, Waltham, MA) antibodies overnight at 4°C . The membranes were then incubated with horseradish peroxidase (HRP)-conjugated goat anti-rabbit and mouse anti-goat (Cell Signaling Technology, Danvers, MA) antibodies for 1 h. Detection was performed using an EzWestLumi Plus Kit (Atto Corp., Tokyo, Japan). The relative protein abundance was determined by quantitative densitometry; data were normalized to β -actin (Sigma-Aldrich); the plasma membrane protein levels were normalized to that of the Na^+/K^+ ATPase.

2.6. Immunohistochemistry

Mouse lung sections were deparaffinized, rehydrated in an ethanol series, and treated with 1.4% hydrogen peroxide in methanol for 30 min to block endogenous peroxidase. Non-specific binding was blocked using 1.5% horse serum before incubating with rabbit anti-TRPV1 (1:1000; Abcam), rabbit anti-TRPV4 (1:1000; Novus Biologicals), rabbit anti-P2 × 4 (1:500; GeneTex), and rabbit anti-P2 × 7 (1:100; Thermo Fisher Scientific) antibodies. The following day, the sections were incubated with an ABC Kit (Vector Laboratories, Burlingame, CA). The color reaction was developed using a liquid DAB + substrate kit (Golden Bridge International Inc., Mukilteo, WA). Finally, the slides were counterstained with Harris's hematoxylin for 1 min. Protein levels were quantified using ImageJ software (National Institutes of Health, Bethesda, MD).

2.7. Determination of neuropeptide levels

The neuropeptide, bradykinin (BK), adenosine triphosphate (ATP), substance P (SP), and calcitonin gene-related peptide (CGRP) levels in mouse BALF and an epithelial cell culture supernatant were measured by an enzyme-linked immunosorbent assay (USCN Science, Inc., Wuhan, China). To compare the results from different plates, the optical densities (ODs) of test samples were adjusted relative to the positive control (PC) and negative control (NC) samples supplied with the kit. The mean OD of duplicate wells was calculated. The index value of each sample was defined by the following formula: index = (OD of test serum – OD of negative control) ÷ (OD of positive control – OD of negative control) × 100. The following were the lower limits of detection: mouse BK kit 0.48 pg/mL, ATP kit 4.93 ng/mL, SP kit 4.63 pg/mL, and CGRP kit 4.41 pg/mL; human BK kit 2.74 pg/mL, ATP kit 4.93 ng/mL, SP kit 5.24 pg/mL, and CGRP kit 4.31 pg/mL, respectively.

2.8. Determination of the calcium concentration in lung tissue

The tissue calcium concentration was indirectly measured using a calcium detection assay kit (Abcam). Lung tissues were homogenized and centrifuged, the supernatant was collected and transferred to a clean tube, and the calcium concentration was assayed.

2.9. Statistical analysis

The data were entered into SPSS statistical software (ver. 20.0; SPSS Inc., Chicago, IL). All data are expressed as means ± standard deviation (SD) or the standard error of the mean (SEM). Group differences were compared by two-sample *t*-tests, Mann-Whitney tests, or Pearson's χ^2 tests for normally distributed, skewed, and categorical data, respectively. One-way ANOVA or two-way ANOVA were using for all data. A *p* value < .05 was considered to indicate statistical significance. A *p*-value < 0.05 was considered to indicate statistical significance.

3. Results

3.1. AHR and inflammation

NP exposure increased airway responsiveness in OVA mice, and these increases were augmented in OVA plus NP-exposed mice (*p* < 0.05; Fig. 1B). The fractions of inflammatory cells in BALF were increased in OVA mice compared with sham mice (*p* < 0.05; Fig. 1C), and the numbers of total cells, macrophages, eosinophils, neutrophils, and lymphocytes in BALF were higher in OVA plus NP-exposed mice (*p* < 0.05; Fig. 1C). Upon histologic examination, the OVA plus NP-exposed mice exhibited marked inflammatory cell infiltration and exudative changes in the peribronchial layers and intraluminal areas of the bronchi (Fig. 2B).

3.2. TRPV and P2X expression

The TRPV1, TRPV4, P2 × 4, and P2 × 7 levels in lung tissue were increased in OVA mice, and these increases were augmented in OVA plus NP-exposed mice (Fig. 2A). Also, neuro receptor levels in NP-exposed NHBE cells were increased (Fig. 2C). Immunohistochemistry revealed that the TRPV1, TRPV4, P2 × 4, and P2 × 7 levels in lung tissue were increased in OVA mice, and these increases were augmented in OVA plus NP-exposed mice (Fig. 2B).

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3.3. Neuromediators levels

The BK, ATP, SP, and CGRP levels in BALF were increased in OVA mice, and these increases were augmented in OVA plus NP-exposed mice (Fig. 4). The BK, ATP, SP, and CGRP levels in NHBE cells increased with increasing NP doses (Fig. 3).

3.4. Calcium concentrations

The calcium concentrations in lung tissue were increased in OVA mice, and these increases were augmented in OVA plus NP-exposed mice (Fig. 3A). The calcium concentration was increased in NHBE cells exposed to NPs for 8 h, but not in cells exposed to NPs for 24 h (Fig. 3B).

4. Discussion

Asthma is a chronic inflammatory disease characterized by AHR involving many inflammatory cells and mediators (Holgate, 2008). The respiratory system represents the main gateway for NP entry into the human body. The levels of particles < 0.1 μm in mass median aerodynamic diameter are increasing in ambient air (Cyrys et al., 2003; Timonen et al., 2004; Zhu et al., 2002). NPs reportedly increase allergic airway inflammation *in vivo* (Shvedova et al., 2005; Last et al., 2004; Al-Humadi et al., 2002). Inhaled NPs are especially important because: (i) this is the main route of unwanted exposure, and (ii) adverse health effects are associated with increased concentrations of ambient fine and ultrafine particles (Laden et al., 2006; Pope, 2004). In this study, we found that NP exposure increased airway inflammation and AHR in a mouse model of asthma.

The dense network of sensory nerve fibers in the epithelial layer from the upper to the lower airways comprises the first and fundamental line of defense that, following the sensing of irritant and potentially harmful agents and chemicals in the respiratory system, initiates a complex and coordinated responses designed to protect the lung from injury. These reflex responses encompass, for example, bronchoconstriction, mucus secretion from nasal and tracheobronchial glands, sneezing, and coughing (Geppetti et al., 2010; Couto et al., 2013). The discovery that a number of TRP channels are expressed in sensory neurons innervating the upper and lower airways as well as in non-neuronal cells in the airways and lungs, has initiated a quest to do their role in the pathophysiology of the respiratory tract (Nassini et al., 2012). Various members of the TRP vanilloid subfamily (TRPV1 and TRPV4) and TRP ankyrin 1 (TRPA1) promote airway neurogenic inflammation (Nassini et al., 2012). The identification of TRP channels as major targets of oxidative/nitrative stress and a variety of irritant environmental agents, suggesting that neurogenic inflammation plays an important role in workrelated inflammatory diseases, and that antagonists for such channels may be novel therapeutic options for the treatment of these diseases (Geppetti et al., 2014). TRP ion channels are

implicated in the afferent sensory loop of the cough reflex and in the heightened cough sensitivity seen in disease (Grace and Belvisi, 2011). Agonists of the TRPV1 capsaicin receptor as the most potent chemical stimuli can initiate cough (Grace and Belvisi, 2011). The respiratory tract has primary sensory C-fibers that are activated by mechanical and chemical stimuli. Activation of vagal sensory afferents leads to central reflexes, including coughing, dyspnea, and changes in breathing pattern. A high parasympathetic tone causing to bronchoconstriction and neurogenic inflammation is thought to play an important role in the pathogenesis of asthma. A major determinant of neurogenic inflammation, TRPV1 functions as a sensor for irritants in the lung by transmitting noxious stimuli to the central nervous system and inducing the release of a variety of proinflammatory neuropeptides at the peripheral terminals. A challenge with inhaled capsaicin, an exogenous agonist of TRPV1, has been used to measure the sensitivity of the cough reflex (Couto et al., 2013). Our results demonstrate that TRPV1 is expressed in the airway and that NPs augment TRPV1 expression, suggesting that TRPV1 in the airways plays a role in the neuromodulation of airway inflammation after NP exposure and contributes to asthma. P2X receptor activate and enhance responsiveness to a large range of stimuli, either through the priming of sensory afferent nerve terminals in airways or modulating the activity at their central synapses (Monção-Ribeiro et al., 2014; Khakh and North, 2006; Burnstock, 2016). In this study, the TRPV1, TRPV4, $P2 \times 4$, and $P2 \times 7$ levels were increased in mice and epithelial cells exposed to TiO_2 NPs, suggesting that TiO_2 NPs trigger neurogenic inflammation in the lungs.

In this study, BK, ATP, SP, and CGRP levels in BALF (Fig. 5) were increased in OVA mice, and these increases were augmented in OVA plus NP-exposed mice and in NHBE cells with increasing NP doses, suggesting that NP can activate TRPVs and $P2 \times 7$, and secrete neuro-mediators leading to airway inflammation, and causing to aggravation of asthma.

In conclusion, our data suggest that TRPV1, TRPV4, $P2 \times 4$, and $P2 \times 7$ are involved in the pathogenesis of bronchial asthma and that NPs can exacerbate asthma via a neurogenic mechanism.

Declaration of Competing Interest

The authors declare that they have no conflict of interest.

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