Online supplementary material

Neuro-immune interactions in chemical-induced airway hyperreactivity

Fien C. Devos[1], Brett Boonen[2], Yeranddy A. Alpizar[2], Tania Maes[3], Valérie Hox[4], Sven Seys[4], Lore Pollaris[1], Adrian Liston[5], Benoit Nemery[1], Karel Talavera[2], Peter H.M. Hoet[1], and Jeroen A.J. Vanoirbeek[1]

[1]Centre for Environment and Health, Department of Public Health and Primary Care, KU Leuven, [2]Laboratory for Ion Channel Research and TRP Research Platform (TRPLe), Department of Cellular and Molecular Medicine, KU Leuven, [3]Laboratory of Pneumology, Department of Respiratory Medicine, UGent, [4]Laboratory of Clinical Immunology, Department of Microbiology and Immunology, KU Leuven, [5]Laboratory of Genetics of Autoimmunity, Department of Microbiology and Immunology, KU Leuven

Corresponding author:

Jeroen Vanoirbeek, PhD
KU Leuven
Department of Public Health and Primary Care
Centre for Environment and Health
Herestraat 49 mailbox 706
3000 Leuven, Belgium
Phone: +32 16 33 01 96, Fax.: +32 16 33 08 06
E-mail: jeroen.vanoirbeek@med.kuleuven.be

Supplemental Materials and Methods

Reagents

Toluene-2,4-diisocyanate (TDI) (98%; Fluka, CAS 584-84-9), Toluene-2,4-diamine (TDA) (98%; CAS 95-80-7), acetyl-β-methylcholine (methacholine), ketotifen fumarate salt (CAS 34580-14-8), acetone, allyl isothiocyanate (AITC) and capsaicin (CAP) were obtained from Sigma-Aldrich (Bornem, Belgium). The TRPA1 antagonist HC030031 (CAS 349085-38-7) and the NK1R antagonist RP67580 (CAS 135911-02-3) were obtained from Tocris Bioscience (Bristol, United Kingdom). Pentobarbital (Nembutal®) was obtained from Sanofi Santé Animale (CEVA, Brussel, Belgium). The vehicle (acetone/olive oil, AOO), used to dissolve TDI consisted of a mixture of 2 volumes of acetone and 3 volumes of olive oil (Extra, Carbonell, Madrid, Spain) for both the dermal sensitization and the intranasal challenge. Concentrations of TDI are given as percent (v/v) in AOO.

Mice

Male WT C57Bl/6 mice (25-30 grams) were obtained from Harlan. *Trpa1* KO and *Trpv1* KO mice with a C57Bl/6 background (25-30 grams) were kindly provided by Prof. Rudi Vennekens (Laboratory of Ion Channel Research, KU Leuven). Mast cell KO mice were obtained from Charles River. All mice were housed in a conventional animal facility with 12-h dark/light cycles. They were housed in filter top cages and received lightly acidified water and pelleted food *ad libitum*.

In vitro Ca^{2+} imaging experiments

TDI-specific activation of TRPA1 was investigated by intracellular Ca^{2+} imaging experiments on Chinese hamster ovarian (CHO) cells selectively expressing murine TRPA1 (mTRPA1) and mTRPV1. To induce the expression of mTRPA1 and mTRPV1 in CHO cells, a tetracycline-regulated system was used [1]. TRPA1- and TRPV1-expressing CHO cells were seeded in 18 mm glass cover slips coated with poly-L-lysine (0.1 mg/ml) and grown in their specific medium at 37 °C in a humidity controlled incubator with 10 % CO_2. Prior to the photometric measurements, cells were incubated with 2 mM Fura-2 acetoxymethyl ester (Biotium, Hayward, CA) for 30 min at 37 °C. The intracellular Ca^{2+} concentration was monitored using the ratio of the fluorescence intensity measured upon alternating illumination at 340 and 380 nm using an MT-10 illumination system and the Cell M software (Olympus, Planegg, Germany). A standard Krebs solution (NaCl 150 mM, KCl 6 mM, $CaCl_2$ 1.5 mM, $MgCl_2$ 1 mM, HEPES 10 mM, glucose 10 mM, pH 7.4) was used as control solution and vehicle for TDI and TDA.

Patch-clamp

We used a tetracycline-regulated system for inducible expression of TRPA1 in CHO cells. To induce expression of TRPA1, 0.5µg/ml tetracycline was added to the culture medium, and cells were used 1-3 h after induction. Recordings were performed in the cell-attached patch-clamp configuration. During these recordings CHO cells were maintained in an extracellular recording solution containing (in mM): 150 KCl, 5 $CaCl_2$, 10 glucose and 10 HEPES, buffered at pH 7.4 with KOH. The pipette solution contained (in mM) 150 NaCl, 5 $CaCl_2$, 10 glucose and 10 HEPES, buffered at pH 7.4 with NaOH. Agonists were added to the extracellular recording solution. Patch-clamp electrodes were pulled from Vitrex capillary tubes (Modulohm, Herlev, Denmark) on a DMZ-Universal puller (Zeitz Instruments,

Augsburg, Germany). When filled with pipette solution, they showed a resistance between 1.8 and 2.4 MΩ. An Ag-AgCl wire was used as reference electrode. The holding potential was set to -50 mV. Membrane currents were recorded using an EPC-7 patch-clamp amplifier (List Electronic, Lambrecht/Pfalz, Germany). For control of voltage-clamp protocols and data acquisition, we used the pCLAMP 9 software (Molecular Devices, Foster City, CA) run on an IBM-compatible PC, which was connected to the amplifier via a TL-1 DMA interface (Molecular Devices). Experiments were performed at room temperature (20-25 °C).

Experimental protocols of mouse experiments

Model of immune-mediated chemical-induced AHR

Our model of chemical-induced asthma [2-4], initially developed in Balb/c mice, was applied to C57Bl/6 WT mice, *Trpa1* KO, *Trpv1* KO, *Rag2* KO and mast cell KO ($Kit^{Wsh/Wsh}$) mice. Briefly, on days 1 and 8, mice were dermally treated with 1% toluene-2,4-diisocyanate (TDI) or vehicle (acetone/olive oil, AOO, 2:3) on the dorsum of both ears (20 µl/ear). On day 15, mice received an intranasal instillation with 30 µl of 0.1% TDI or vehicle (AOO, 2:3). We did not anesthetize the mice for this challenges, because anesthetics are known to activate TRPA1 channels [5]. Mice were sacrificed 24 h after the last challenge. We refer to our experimental treatment groups as AOO/AOO, AOO/TDI and TDI/TDI, with the first abbreviation identifying the agent used for dermal applications on days 1 and 8 (sensitization) and the second abbreviation identifying the agent administered via intranasal instillation on day 15 (challenge).

Pharmacological treatments

In this set of experiments we dosed 200 µl of the TRPA1 antagonist HC030031 (R&D systems, Abingdon, UK); the substance P (SP) receptor (NK1R) antagonist RP67580 ((3a*R*,7a*R*)-Octahydro-2-[1-imino-2- (2 methoxyphenyl) ethyl]-7, 7-diphenyl-4*H*-isoindol; R&D systems, Abingdon, UK) or the mast cell stabilizer ketotifen (Sigma Chemical Co., St. Louis, MO, USA) intraperitoneally (IP), 30 min prior to the intranasal challenge. The TRPA1 antagonist was dissolved in 0.5% methylcellulose in dH_2O and administered IP at 160 mg/kg [6]. NK1R antagonist was dissolved in 20% DMSO in PBS and administered IP at 1µg/µl [7]. Ketotifen was dissolved in 0.5% methylcellulose solution in dH_2O and administered IP at 2 mg/kg [7]. Control mice received IP injections of 200 µl vehicle.

The experimental treatment groups are AOO/Veh/AOO, AOO/HC030031/AOO, AOO/RP67580/AOO, AOO/Keto/AOO, TDI/Veh/TDI, TDI/HC030031/TDI, TDI/RP67580/TDI and TDI/Keto/TDI, with the first abbreviation identifying the agent used for dermal applications on days 1 and 8, the second abbreviation identifying the agent injected prior to the challenge and the third identifies the agent used for the airway challenge on day 15.

In a next experimental set-up, the TDI challenge on day 15 was replaced by a single intranasal instillation (30 µl) of allyl isothiocyanate (AITC; Sigma; 400 µM) or capsaicin (CAP; Sigma, 100 µM). In an extension of this experiment, AITC-challenged mice received a second intranasal instillation with TDI or AITC one week later, i.e. on day 22. The experimental treatment groups are AOO/AITC/AOO, AOO/AITC/TDI, TDI/AITC/AITC and TDI/AITC/TDI, with the first abbreviation identifying the agent used for dermal applications on days 1 and 8, the second abbreviation identifying the agent administered via intranasal instillation on day 15 and the third abbreviation identifying the agent administered via intranasal instillation on day 22.

Early ventilatory response

Using the double chamber plethysmograph (EMKA Technologies, Paris, France), the early ventilatory response, i.e. breathing frequency, was assessed 5 min before (baseline measurement) and up to one hour after the intranasal challenge. All mice showed a profound, but temporary decrease in breathing frequency, typical of respiratory sensory irritation, immediately after a challenge with TDI (data not shown).

Non-specific airway hyperreactivity measurements

Twenty-four hours after the challenge, airway reactivity to methacholine was measured using a forced oscillation technique (FlexiVent, SCIREQ, Montreal, Canada) [8]. Mice were anesthetized by an IP injection of pentobarbital (70 mg/kg body weight, Nembutal®, Sanofi Santé Animale, CEVA, Brussels, Belgium). The trachea was exposed and tracheotomized to insert a 19-gauge cannula. Mice were quasi-sinusoidally ventilated with a tidal volume of 10 ml/kg at a frequency of 150 breaths/min and a positive end-expiratory pressure of 3 cm of H_2O, to mimic the characteristics of spontaneous breathing. Airway resistance (Rn) was measured using the 'quick-prime 3' protocol, which induces oscillations of 1 to 20.5 Hz during 3 s. After baseline measurements, each mouse was exposed for 5 s to an aerosol of methacholine using increasing concentrations (0, 1.25, 2.5, 5, 10, 20 mg/ml). After aerosol exposure, 5 consecutive 3-s oscillations are induced, resulting in 5 measurements for one concentration of methacholine. For each mouse, Rn was plotted against methacholine concentration and the AUC was calculated to perform statistical analysis.

Serum IgE and bronchoalveolar lavage (BAL)

After the assessment of airway hyperreactivity to methacholine, blood was taken from the retro-orbital plexus, centrifuged (14000 g, 4 °C, 10 min) and total serum IgE (diluted 1/70) levels were measured using the OptEIA set from Pharmingen (BD Biosiences, Erembodegem, Belgium). The lungs were then lavaged, in situ, three times with 0.7 ml sterile saline (0.9 % NaCl), and the recovered fluid was pooled. Cells were counted using a Bürker hemocytometer (total cell count) and the bronchoalveolar lavage (BAL) fluid was centrifuged (1000 g, 4 °C, 10 min). For differential cell counts, 250 µl of the resuspended cells (100,000 cells/ml) were spun (300 g, 4 °C, 6 min) (Cytospin 3, Shandon, TechGen, Zellik, Belgium) onto microscope slides, air-dried and stained (Diff-Quik® method, Medical Diagnostics, Düdingen, Germany). For each sample, 200 cells were counted to determine the number of macrophages, eosinophils, neutrophils and lymphocytes.

Lymph node analysis

Auricular lymph node cells were counted using a Bürker hemocytometer and resuspended (10^7 cells/ml) in complete tissue culture medium (RPMI-1640 supplemented with 10% heat-inactivated fetal bovine serum, 10 mg/ml streptomycin/penicillin). Five-hundred thousand cells were stained with anti-$CD3^+$ (APC), anti-$CD4^+$ (APC-Cy7), anti-$CD8^+$ (PerCP-Cy5.5) and anti-$CD25^+$ (PE), or received a single staining with anti-$CD19^+$ (PE) labeled antibodies, according to standard procedures (BD Biosciences, Erembodegem, Belgium). Percentages of labeled cells were determined by flow cytometry (Facsarray, BD Biosciences, Erembodegem, Belgium) on at least 10^5 cells.

Auricular lymphocytes were seeded into 48-well culture plates at a density of 10^6 cells/ml and incubated in complete RPMI-1640 medium for 42 h with 2.5 µg/ml of concanavalin A (ConA) (Sigma–Aldrich, Bornem, Belgium). Cell suspensions were then centrifuged (1000 *g*,

10 min) and supernatant was stored at −80 °C. Levels of IL-13, IL-10 and IFN-γ were measured via Cytometric Bead Array and analyzed with the FCAP Array Software (BD Biosciences, Erembodegem, Belgium) on the LSR Fortessa (BD Biosciences, Erembodegem, Belgium).

Statistics

Electrophysiological measurement data were analyzed using the WinASCD software package (Guy Droogmans, KU Leuven, Leuven, Belgium; ftp://ftp.cc.kuleuven.ac.be/pub/droogmans/winascd.zip). Graphical presentations were performed using the Origin version 9.0 software (OriginLab, Northampton, MA). All data are presented as mean with standard error of the mean (SEM).

Dose-response curves of the AHR measurements, presented as group means, were analyzed using two-way parametric ANOVA, followed by a Bonferroni multiple comparison *post hoc* test. Treatment groups were compared with corresponding control groups for each individual methacholine concentration.

All other data are presented as means with standard deviation (SD). In case of normality of distribution, assessed by the Kolmogorov-Smirnov test, data were analyzed using one-way parametric ANOVA, followed by a Bonferroni multiple comparison *post hoc* test. Data with non-normal distribution were analyzed with non-parametric Kruskal-Wallis test and Dunn's multiple comparison *post hoc* test (Graph Pad Prism 5.01. Graphpad Software Inc, San Diego, USA). A level of $p < 0.05$ (two tailed) was considered significant.

References

1 Story GM, Peier AM, Reeve AJ, Eid SR, Mosbacher J, Hricik TR, Earley TJ, Hergarden AC, Andersson DA, Hwang SW, McIntyre P, Jegla T, Bevan S, Patapoutian A: ANKTM1, a TRP-like channel expressed in nociceptive neurons, is activated by cold temperatures. Cell 2003;112:819-829.

2 Vanoirbeek JA, De Vooght V, Vanhooren HM, Nawrot TS, Nemery B, Hoet PH: How long do the systemic and ventilatory responses to toluene diisocyanate persist in dermally sensitized mice? J Allergy Clin Immunol 2008;121:456-463.

3 Tarkowski M, Vanoirbeek JAJ, Vanhooren HM, De Vooght V, Mercier C, Ceuppens JL, Nemery B, Hoet PMH: Immunological determinants of ventilatory changes induced in mice by dermal sensitization and respiratory challenge with toluene diisocyanate. Am J Physiol Lung Cell Mol Physiol 2007;292:L207-L214.

4 De Vooght V, Vanoirbeek JA, Luyts K, Haenen S, Nemery B, Hoet PH: Choice of mouse strain influences the outcome in a mouse model of chemical-induced asthma. PLoS One 2010;5:e12581.

5 Eilers H, Cattaruzza F, Nassini R, Materazzi S, Andre E, Chu C, Cottrell GS, Schumacher M, Geppetti P, Bunnett NW: Pungent general anesthetics activate transient receptor potential-A1 to produce hyperalgesia and neurogenic bronchoconstriction. Anesthesiology 2010;112:1452-1463.

6 Caceres AI, Brackmann M, Elia MD, Bessac BF, del CD, D'Amours M, Witek JS, Fanger CM, Chong JA, Hayward NJ, Homer RJ, Cohn L, Huang X, Moran MM, Jordt SE: A sensory neuronal ion channel essential for airway inflammation and hyperreactivity in asthma. Proc Natl Acad Sci U S A 2009;106:9099-9104.

7 Hox V, Vanoirbeek JA, Alpizar YA, Voedisch S, Callebaut I, Bobic S, Sharify A, De Vooght V, Van Gerven L, Devos F, Liston A, Voets T, Vennekens R, Bullens DM, De VA, Hoet P, Braun A, Ceuppens JL, Talavera K, Nemery B, Hellings PW: Crucial role of transient receptor potential ankyrin 1 and mast cells in induction of nonallergic airway hyperreactivity in mice. Am J Respir Crit Care Med 2013;187:486-493.

8 Vanoirbeek JA, Rinaldi M, De Vooght V, Haenen S, Bobic S, Gayan-Ramirez G, Hoet PH, Verbeken E, Decramer M, Nemery B, Janssens W: Noninvasive and Invasive Pulmonary Function in Mouse Models of Obstructive and Restrictive Respiratory Diseases. Am J Respir Cell Mol Biol 2010;42:96-104.