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Oxazolone (OXA) is a respiratory allergen in Brown Norway rats

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ABSTRACT

Oxazolone (OXA) is a potent contact allergen in man, and it is used as a model Th1-allergen to test (Q)SAR's and screening assays for allergenic potential of chemicals. However, it elevates serum IgE levels and Thelper2 cytokines at relatively low doses in test animals, suggesting that it has also respiratory allergenic potential. The lack of human data on respiratory allergenic potential of OXA may be due to lack of significant inhalation exposure. Here, female Brown Norway rats (BN) were sensitized by two or five dermal applications of OXA at the same total dose of 3.75 mg. Controls received vehicle. All animals were challenged by inhalation to 45 mg/m^3 OXA on day 21 and necropsy was performed on day 22. All sensitized animals had increased serum IgE. OXA challenge decreased breathing frequency, and induced apnoeic breathing in the sensitized animals – a hallmark of respiratory allergy in our model. An exudative, granulocytic inflammation was observed primarily in the larynx of the sensitized and challenged rats. Microarray analysis of lung tissue, sampled 24 h after challenge, revealed upregulation of several genes and activation of Gene Ontology (GO) pathways, which resembled more closely those found previously in lung tissue of rats sensitized and challenged by the respiratory allergen trimellitic anhydride than by the contact allergen dinitrochlorobenzene. The results indicate that the contact allergen OXA can also be a respiratory allergen, provided that it is inhaled. Its use as a model contact sensitizer must be reconsidered.

1. Introduction

Oxazolone (OXA), 4-ethoxymethylene-2-phenyl-2-oxazoline-5-one (CAS reg. no. 15646-46-5) is a heterocyclic compound and is a chemical intermediate in the production of drugs. It is a well-known, strong contact sensitizer in man, and is frequently used as a model contact allergen in in vivo and in vitro screening assays to examine the sensitizing potential of chemicals (Roggen et al., 2008). OXA has an EC3 of 0.01% in the mouse dermal LLNA (ECETOC, 2003; Van Och et al., 2002). Reports on respiratory allergenic properties of OXA in humans are absent, which may indicate that OXA is indeed no respiratory allergen or that significant exposures by inhalation do not take place. In contrast to acid anhydrides and diisocyanates, OXA is not a bulk chemical and it is mostly presented as relatively large flakes.

There are no validated tests to identify the respiratory allergenic potential of chemicals, but the IgE test and cytokine fingerprinting test have been used regularly (rat: Arts and Kuper, 2007; mouse: Dearman et al., 1992; Kimber et al., 1996). OXA was negative in a standard IgE test in mice with two topical applications (Dearman and Kimber, 1992), but induced pronounced increases in serum IgE, especially but not exclusively after 5 and 15 times application (mouse: Webb et al., 1998; rat: Mommers et al., 2006). Two skin applications followed by a single intranasal challenge increased also serum IgE levels (Vanoirbeek et al., 2003a,b). OXA induced the synthesis of both IFN-gamma (Th1-related) and Il-4 (Th2-related) cytokines in the lymph nodes draining the skin site of application (Thomson et al., 1993). Interestingly, the OXA-induced ulcerative colitis mouse model is considered to be a Th2-mediated process (Waldner and Neurath, 2009; Heller et al., 2002).

Although an increased total serum IgE level upon skin application (IgE test) has been proposed as being a predictive parameter of airway hypersensitivity caused by low molecular weight chemicals, it is unknown if total serum IgE translates directly to potential risk of inducing respiratory allergy (Farraj et al., 2007, 2004; Selgrade et al., 2006). Therefore, it is questionable if OXA is a respiratory

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Table 1
Experimental scheme.

Group designation[a]	Sensitization[b]	Day 21: challenge[c]	Day 22: necropsy
Veh2/OXA Unsensitized 2x/challenged	Vehicle 2x	45 mg/m^3 OXA	- Lung function[d] - Total IgE in serum - Liver, kidneys, left lung weights - BAL of right lung lobes - Formalin-fixation, paraffin-embedding of nasal passages, larynx, trachea (all animals) - Left lungs: 1. Formalin-fixation, paraffin-embedding (all groups; n = 3/group) 2. Immunohisto-chemistry on snap-frozen tissue (veh5/OXA and OXA5/OXA5; n = 3/group) 3. Transcriptomics on snap-frozen tissue (veh2/OXA, OXA2/OXA, OXA2/– and OXA5/–; n = 3/group)
Veh5/OXA Unsensitized 5x/challenged	Vehicle 5x	45 mg/m^3 OXA	
OXA2/OXA Sensitized 2x/challenged (allergic group)	Day 0: 300 μl on flanks 1% (w/v) OXA Day 7: 150 μl on ears 0.5% (w/v) OXA (total dose 3.75 mg)	45 mg/m^3 OXA	
OXA5/OXA Sensitized 5x/challenged (allergic group)	Days 0, 4, 11, 14 5 × 150 μl 0.5% (w/v) OXA, alternating on flanks and ear (total dose 3.75 mg)	45 mg/m^3 OXA	
OXA2/– Sensitized 2x/unchallenged	Day 0: 300 μl on flanks (1%, w/v, OXA) Day 7: 150 μl on ears (0.5% (w/v) OXA (total dose 3.75 mg)	–	
OXA5/– Sensitized 5x/unchallenged	Days 0, 4, 7, 11, 14 5 × 150 μl 0.5% (w/v) OXA, alternating on flanks and ear (total dose 3.75 mg)	–	

[a] Six female BN rats per group.
[b] Vehicle acetone–olive oil (AOO) 4:1 (v/v).
[c] OXA dissolved in acetone, concentration based on RF test (1–50 mg/m^3).
[d] Lung function parameters were determined before, during and after challenge.

allergen, based on increases in total serum IgE (and Th2 cytokine fingerprinting) alone.

Challenge tests in the skin and the respiratory tract are used to examine if an allergic response is really provoked in sensitized animals and if so, of what type and severity it is. Using a protocol that successfully identified respiratory allergens (rat: Arts and Kuper, 2007; Pauluhn et al., 2002; Zhang et al., 2004; mouse: Vanoirbeek et al., 2003a,b: Satoh et al., 1995) the respiratory allergen trimellitic anhydride (TMA) and the contact allergen/skin allergen dinitrochlorobenzene (DNCB) resulted in distinctly divergent responses (Arts et al., 1998; Kuper et al., 2008a,b). The divergence was also present in gene expression patterns in the lungs.

OXA was expected to behave like the respiratory allergen TMA in an inhalation challenge test as mentioned above, because of its potential to elevate IgE levels in serum. Breathing parameters, gene expression in the lungs and histopathology of the respiratory tract were examined and compared with the results found previously with the respiratory allergen TMA and the contact (skin) allergen DNCB.

2. Materials and methods

2.1. Animals and maintenance

Female, 7–8-week-old, inbred Brown Norway (BN) rats were purchased from Charles River Deutschland GmbH (Sulzfeld, Germany). The animals were acclimatized for 7 days before the start of the study. They were kept under conventional laboratory conditions and received the Institute's grain-based open-formula diet and unfluoridated tap water *ad libitum*. All animal procedures were approved by the TNO Committee of Animal Welfare.

2.2. Treatment schedules and groups

The study was conducted with 6 groups of rats (Table 1). Blood was collected one day before the start of the study. Female BN rats received 150 μl of oxazolone (OXA; purity 90%; Sigma–Aldrich Chemie GmbH Steinheim, Germany) in a 4:1 (v/v) mixture of acetone (Merck; Darmstadt, Germany) and refined olive oil (AOO) (Sigma Diagnostics, Inc. St. Louis, USA) as the vehicle on each flank (approximately 12 cm^2 each). Flanks had been shaved with an electrical razor at least 2–3 days earlier. The groups, that were sensitized twice, received 75 μl of OXA on the dorsum of both ears, seven days after the first sensitization. Groups that were sensitized five times received OXA alternating on the dorsum and the flanks (see Table 1). The sensitization concentrations were based on a preliminary study with OXA in which increased IgE levels were observed especially after 5 and 15 times application, but two times was effective also in most of the animals, all at the same total dose of 3.75 mg (Mommers et al., 2006). On day 20 or 21, basal breathing parameters (frequency, tidal volume and pattern) were assessed, followed by OXA inhalation challenge and assessment of the same breathing parameters during and immediately post challenge. Animals were challenged by inhalation of target concentration of 50 mg/m^3 of OXA for 15 min. Twenty-four hours later, breathing parameters were assessed again, whereafter necropsy was performed. The challenge concentration of OXA was based on a range-finding study with four reserve rats that were exposed to 1 mg/m^3 on the first day, 5 mg/m^3 on the second day, 15 mg/m^3 on the third day and 50 mg/m^3 on the fourth day. Only the concentration of 50 mg/m^3 turned out to be slightly irritating (slight decreased breathing frequency) and was therefore chosen as the target concentration.

2.3. Atmosphere generation and analysis

During inhalation challenge, an all glass nebulizer, Institute's design, was used to generate the test atmospheres from freshly prepared solutions of OXA in acetone. The acetone concentration was kept between 3000 and 5000 ppm (~7–12 g/m^3), which levels are far below the level inducing sensory irritation (Alarie, 1973). Atmospheric concentrations of OXA were determined gravimetrically by filter sampling and those of acetone by calculations based on the nominal concentration, assuming the usual 100% generation efficiency for this vapour. The particle size distribution of OXA in the test atmosphere was determined using a 10-stage cascade impactor (Andersen, Atlanta, GA). Due to the small sampling air flow rate and the large total volume required for analytical and particle size determinations, samples were not collected during the challenge itself but immediately prior to or after challenge. The mean concentration of OXA turned out to be 45 mg/m^3; the mass median aerodynamic diameter (MMAD) of the aerosolized OXA particles was 0.8 μm with a geometric standard deviation (gsd) of 2.7 and 3.7.

2.4. Inhalation challenge and measurements of breathing parameters

Rats were individually restrained in Battelle tubes and each tube was placed into one of four whole body plethysmographs connected to the central exposure unit. Using this experimental set up, two OXA-sensitized (OXA2/OXA or OXA5/OXA) and two vehicle-treated (veh2/OXA or veh5/OXA) rats at a time were first exposed to fresh air for at least 25 min (pre-challenge period) and then to the OXA atmosphere for exactly 15 min (challenge period), followed by a recovery (post challenge) period of 30 min as described previously (Arts et al., 1998; Kuper et al., 2008a,b).

As a surrogate for changes in lung function, breathing pattern (Arts and Kuper, 2007) was monitored by means of recording the pressure signal in the plethysmographs. Frequency and tidal volume were measured and respiratory flow (minute ventilation) was calculated from the breathing pattern. Before challenge, the respiration was monitored approximately 20 s each min starting 6 min prior to the actual challenge. During challenge respiration was monitored approximately 20 s during

each min; and after challenge approximately 20 s each for the first 10 min followed by 20 s each 4 min for the remaining period of 20 min. The day after challenge, the respiration was monitored again, approximately 20 s each min for about 6 min. This resulted for each rat in 6 values prior to challenge, 15 values during and immediately after challenge, and 6 values the day after challenge, for frequency, tidal volume and respiratory flow.

2.5. Clinical signs, serum collection, body and organ weights, necropsy

The animals were observed daily and weighed shortly before the OXA dermal applications, at weekly intervals thereafter, and just prior to necropsy. Individual serum samples were prepared from blood withdrawn via the orbital plexus prior to sensitization, and via the abdominal aorta at necropsy. For IgE analysis, the serum samples were stored at −20 °C until analysis by Enzyme-Linked-Immuno-Sorbent Assay (ELISA). At necropsy, animals were anaesthetised with pentobarbital and killed by exsanguination from the abdominal aorta. The liver, kidneys and the unlavaged left lungs were weighed. The left lung of half of the animals (see Table 1) was inflated with 50% Tissue Tek in saline, quick-frozen on dry ice and kept at −70 °C for microarray analysis or immunohistochemistry. The nasal tissues, trachea, larynx and left lung of the other half of the animals were collected and fixated in neutral, phosphate-buffered 4% (v/v) formaldehyde for histopathological evaluation.

2.6. Bronchoalveolar lavage

At necropsy, the right lung lobes were lavaged two times with a volume of 23 ml saline per kg bw, after binding of the left (main) lung. The procedure was as described previously (Arts et al., 1998). Total cell numbers were counted using an automated haematology analyser (K-800, Sysmex, Toa, Kobe, Japan). For differential cell counts, cytospins were prepared and stained with May-Grünwald Giemsa. At least 200 cells were counted per animal to determine absolute numbers and percentages of macrophages/monocytes, lymphocytes, neutrophils and eosinophils.

2.7. Serum IgE levels

Total IgE levels in serum were analyzed by means of an ELISA as described earlier (Arts et al., 1998). The concentration of IgE in the samples was calculated using a standard curve obtained with known quantities of monoclonal rat IgE and expressed as μg/ml serum.

2.8. Histopathology and immunohistochemistry

The formalin-fixed nasal tissues, larynx, trachea and left lung were embedded in paraffin wax and sectioned at 5 μm. Nasal tissues were cut at 6 levels according to Woutersen et al. (1994). The larynx was cut longitudinally through the epiglottis. The cranial part of the trachea was cut transversally, the caudal part longitudinally, together with the bifurcation and the two extrapulmonary bronchi. The formalin-fixed left lung was cut longitudinally through the main bronchus/bronchiolus. The sections were stained with haematoxylin and eosin.

Cryostat sections were made from the deep-frozen left lung of three animals of the veh5/OXA and the OXA5/OXA groups (the left lungs of the other four groups were used for microarray analysis). The lung sections were stained for CD4 (W3/25), CD8 (OX8), CD161 (10/78; marker for NK cells), CD68 (marker for macrophages), or CD11b (marker for conventional dendritic cells in the lung), all from Serotec Ltd., Oxford, UK, using a 2-step indirect immunolabeling, as described previously (Kuper et al., 2008a). Positive cells were counted in the lung parenchyma at 16 square fields (square grid of 0.3 mm^2) per left lung at 400× magnification.

2.9. Microarray analysis of OXA effects ('intrastudy comparison')

Total RNA was isolated from deep-frozen left lung tissue of three rats per group (in total 12 oxazolone treated samples; see Table 1), the samples were sent to ServiceXS BV (Leiden, The Netherlands) for cRNA synthesis and hybridization to Affymetrix Rat230.2 Chips, all as described previously (Kuper et al., 2008a,b). To pass filtering criteria, transcript was required to be detected as "present" based on MAS5 present calls in at least 1 of 12 experiments, resulting in 23283 probe sets that were considered expressed. Statistical analyses of differentially expressed genes were identified using the limma package (The Remote Analysis Computation for gene Expression data (RACE) suite at http://race.unil.ch). Threshold for statistical significance was set on p-value < 0.01 combined with absolute fold change >1.5. Gene expression under veh2/OXA, OXA2/− and OXA5/− treatments were compared to the OXA2/OXA treatment. To explore the variability in gene expression between the samples, principal component analysis (PCA) and hierarchical clustering were performed (GeneSpring GX 7.3.1 software; Agilent). The analysis of significantly up- or down-regulated pathways was performed using T-profiler as previously described (Kuper et al., 2008a,b). As input values, average log2 expression ratios (2OXA/OXA versus 2OXA/−, 2OXA/OXA versus 2veh/OXA and 2OXA/OXA versus 5OXA/−) of all 23283 probe sets were used. Gene Ontology (GO) (www.geneontology.org) pathway collection was used as source of queried gene sets. Pathways were considered significant if E-value (Bonferroni corrected) was smaller than 0.05.

2.10. Microarray analysis of OXA, TMA and DNCB effects ('interstudy' comparison)

The microarray data from the three OXA2/OXA BN rats were compared with those of rats treated similarly with TMA or DNCB (three TMA2/TMA and three DNCB2/DNCB rats, respectively), from two previously conducted studies (Kuper et al., 2008a,b).

To enable comparison between the effects on gene expression caused by the OXA treatments with the previously described effects observed upon TMA and DNCB treatments, data from all 36 experiments (the so-called allergic rats with their control groups, leading to 12 OXA, 12 TMA and 12 DNCB samples) were normalized together, according to the protocol for the intra-study. Accordingly, data was filtered based on MAS5 present calls to include only transcripts detected as "present" in at least 1 of 36 experiments. This resulted in 25267 probe sets that were considered expressed across oxazolone, TMA and DNCB conditions and were therefore taken into further analysis. Inter-study statistical and pathway analysis of microarray data were also performed according to the protocols for the intra-study.

2.11. Statistics and data analysis

Body weights were analyzed by one-way analysis of co-variance, followed by the two-sided Dunnett's multiple comparison test. Organ weights, IgE levels, BAL biochemical parameters and absolute cell numbers were determined by Anova-Dunnett's test. IgE levels were also analyzed with a 3-way ANOVA. Relative cell numbers in BAL were analyzed by Kruskall–Wallis non-parametric analysis of variance followed by a Mann/Whitney U-test. Breathing frequencies, tidal volume and respiratory flow were analyzed by a 3-way ANOVA. Time was the repeated factor and sensitization (versus non-sensitization) and numbers of sensitization (two or five times) were the between factors. Differences were considered statistically significant if $p < 0.05$. Analyses were performed by the usage of Graphpad Prism (Version 3.0, San Diego, CA, USA). Statistical analysis of the microarray data is described under Sections 2.9 and 2.10.

3. Results

3.1. Clinical signs, body and organ weights, and bronchoalveolar lavage

In all animals sensitized twice, dermal scaliness on the flanks was generally observed from day 4 onwards, later on (days 9–13) followed by dermal encrustations which remained till the end of the study. Dermal changes on the flanks of animals sensitized 5 time (receiving a lower dermal dose each time, see Table 1) were less in incidence and duration, as only 4 out of 12 animals showed dermal encrustations during a few days only. Gross changes were not observed after application of OXA to the ears. Body weights were not affected by the exposure to OXA. Relative liver and kidneys weights were significantly increased in sensitized rats when compared to controls (data not shown). Absolute and relative lung weights were statistically significantly increased in the OXA2/OXA group, but not in the OXA5/OXA group, when compared to their respective controls (Table 2). In the OXA2/OXA group, BAL contained an increased number of total cells, which was due to an increase in alveolar macrophages (Table 2). Other cell types were not affected (data not shown). In addition, levels of total protein and lactate dehydrogenase (LDH) were increased in the lavage fluid (data not shown). An increased number of total cells and alveolar macrophages, but without changes in protein and LDH, was also observed in the veh/OXA5 group.

3.2. Lung function and serum IgE levels

OXA challenge in sensitized rats induced apneic periods, i.e. irregularly lengthened pauses between varying numbers of breaths. Breathing frequency decreased to 88% of the original rate in the OXA2/OXA group and to 92% in the OXA5/OXA group, starting within 1 or 2 min of the challenge and returned to normal directly after the challenge, but increased at 24 h after challenge (Fig. 1).

Serum IgE levels (Table 2) were markedly increased in the four sensitized groups (OXA2/−; OXA2/OXA; OXA5/− and OXA5/OXA) when compared to the unsensitized groups (veh2/OXA and veh5/OXA) ($p < 0.0001$; 3-way ANOVA). The increase in IgE in the

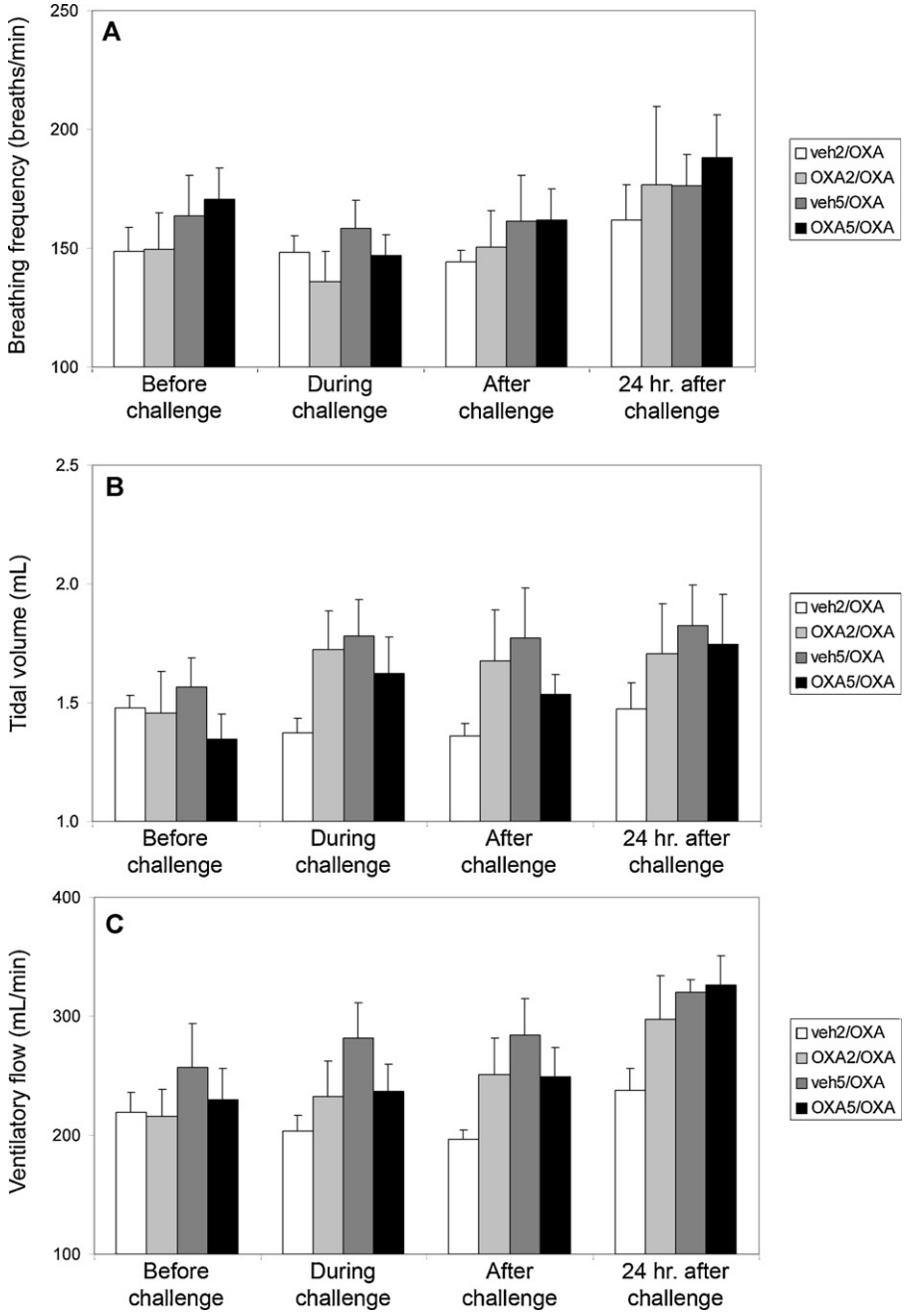

Fig. 1. Mean breathing frequency, tidal volume and respiratory flow ± SD of BN rats before, during challenge, and within 30 min and 24 h after the challenge. The main effect was a change in time in sensitized animals. Frequency: time $p < 0.001$; time × sensitization $p < 0.01$. Tidal volume: time $p < 0.001$; time × sensitization $p < 0.001$; time × sensitization × number of sensitizations $p < 0.001$; Respiratory flow: time $p < 0.001$; time × sensitization $p < 0.001$; time × sensitization × number of sensitizations $p < 0.01$, 3-way ANOVA analysis.

Table 2
Left lung weights, bronchoalveolar lavage parameters, and serum IgE levels in BN rats exposed to OXA.

Group[a]	Lung weight relative to body weight	BAL total cells ($\times 10^6$)	BAL macrophages ($\times 10^6$)	BAL total protein (mg/L)	BAL LDH (U/L)	Pre treatment total serum IgE (μg/ml)	After treatment total serum IgE (μg/ml)
Veh2/OXA	2.50 ± 0.07	0.88 ± 0.09	0.82 ± 0.09	174 ± 21	68 ± 5	0.40 ± 0.14	0.72 ± 0.19
Veh5/OXA	2.76 ± 0.08	1.37 ± 0.12**	1.30 ± 0.11*	234 ± 16	114 ± 24	0.36 ± 0.12	0.79 ± 0.21
OXA2/OXA	2.98 ± 0.07**	1.40 ± 0.11**	1.32 ± 0.11**	294 ± 21**	143 ± 25*	0.45 ± 0.13	1.78 ± 0.35**
OXA5/OXA	2.78 ± 0.11	1.08 ± 0.07	0.99 ± 0.06	258 ± 44	102 ± 12	0.30 ± 0.18	2.13 ± 0.86**
OXA2/–	2.73 ± 0.04	1.02 ± 0.09	0.96 ± 0.08	167 ± 15	65 ± 15	0.55 ± 0.32	2.01 ± 0.43**
OXA5/–	2.82 ± 0.08	1.12 ± 0.14	1.02 ± 0.12	211 ± 20	92 ± 5	0.32 ± 0.05	2.60 ± 0.69**

[a] Six female rats per group.
* $p < 0.05$; Anova + Dunnetts test's (two-sided).
** $p < 0.01$; Anova + Dunnetts test's (two-sided).

Table 3
Histopathological changes and their incidences in BN rats treated dermally with vehicle or OXA, left unchallenged or followed by an inhalation challenge with OXA.

	Veh2/OXA	Veh5/OXA	OXA2/OXA	OXA5/OXA	OXA2/–	OXA5/–
Nasal tissues, anterior levels	(6)[a]	(6)	(6)	(6)	(6)	(6)
Inflammatory cell infiltrates						
Very slight, focal	1	–	1	3	1	–
Very slight to slight, multifocal	–	–	5	3	–	–
Larynx						
Ulceration						
Slight/moderate	3	6	–	–	–	–
Severe	–	–	6	6	–	–
Mucopurulent exudate						
Slight	4	5	–	–	–	–
Severe	–	–	6	6	–	–
Inflammatory cells						
Very slight	–	1	–	–	1	1
Slight	2	2	–	–	–	1
Moderate/severe	–	–	6	6	–	–

[a] The number of animals examined is given in brackets.

groups sensitized twice was more than twofold, and in the groups sensitized five times it was about threefold (statistically significant difference between the groups sensitized twice and the groups sensitized five times, $p < 0.05$; 3-way ANOVA).

3.3. Histopathology and immunohistochemistry

The unchallenged (OXA2/– and OXA5/–) animals did not exhibit histopathological changes in the respiratory tract, except for a granulomatous inflammation in the lungs, which is common in naive BN rats of this age (Germann et al., 1998).

The most prominent effect of the challenge was observed in the larynx of the sensitized (OXA2/OXA and OXA5/OXA) animals: a dense, ulcerative inflammation with exudation at the ventral side of the epiglottis and the vocal cords. Numerous granulocytes, mainly neutrophils and a few eosinophils, lymphocytes, monocytes and macrophages were observed in the mucosa of almost the entire ventral side of the epiglottis (Table 3 and Fig. 2A–D). In addition, hemorrhages were present in almost all animals. Similar epithelial effects were observed in unsensitized/challenged rats (veh2/OXA), but the number of inflammatory cells was markedly less and hemorrhages were absent. In the nasal passages, the chal-

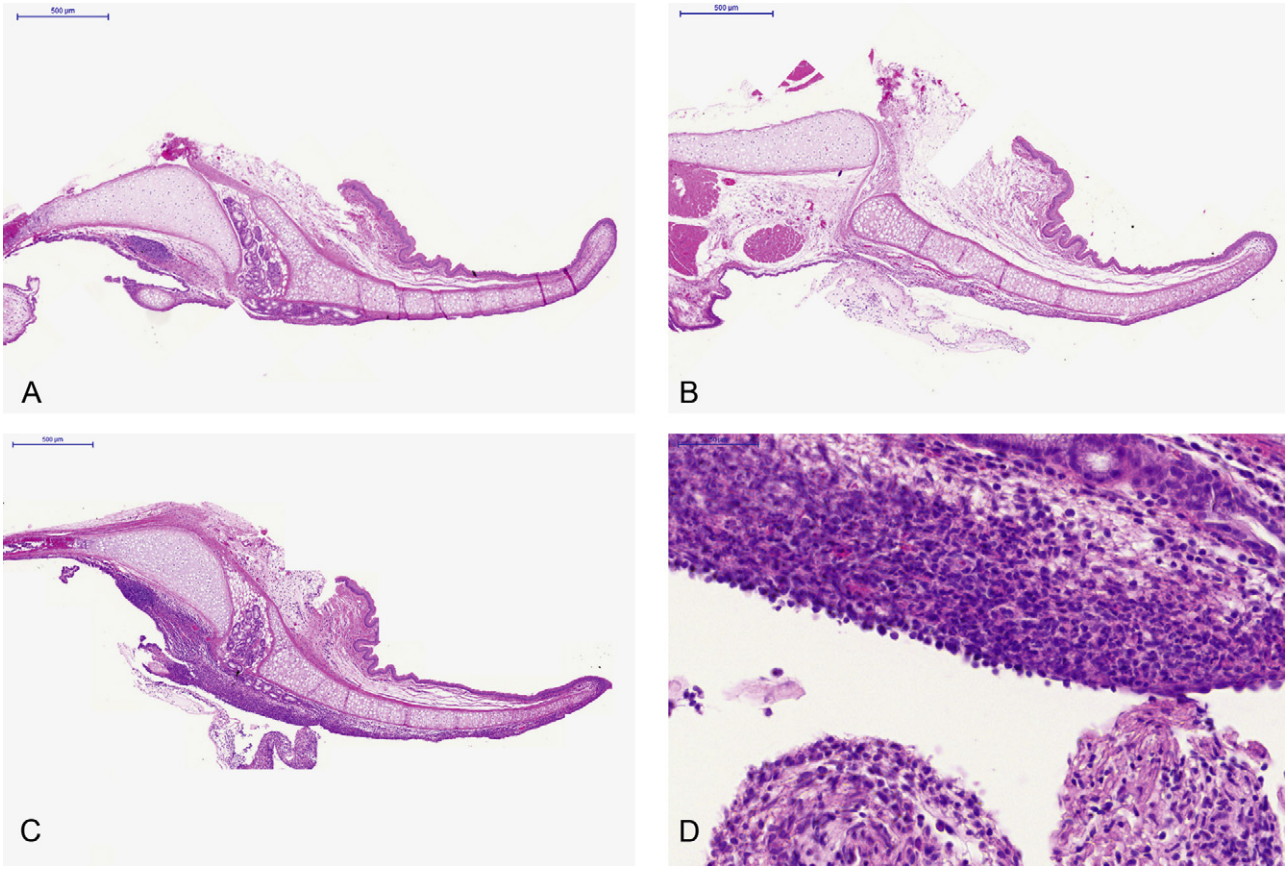

Fig. 2. Histopathology of the larynx of BN rats, 24 h after challenge. The larynx is sectioned longitudinally through the epiglottis. (A) Overview of twice-sensitized, unchallenged (OXA2/–) control animal; (B) overview of unsensitized, OXA-challenged (veh2/OXA) animal with epithelial ulceration and minimal neutrophilic cell involvement, primarily at the base of the epiglottis; (C) overview of twice-sensitized, OXA-challenged (OXA2/OXA) animal with an extensive inflammation along the entire ventral side of the epiglottis. (D) Detail of the extensive inflammation in the twice-sensitized, OXA-challenged (OXA2/OXA) animal.

Table 4
Differentially expressed genes in lung tissue of allergic (twice sensitized/challenged; OXA2/OXA) BN rats, 24 h after challenge.

Description	Gene name	Accession no.	Gene group/pathway (GO, KEGG)	Mean log 2 ratio > 1.00[a,b]
Cytokine (chemokine) 10	Cxcl10/IP-10	U22520	Positive regulation of leukocyte chemotaxis; chemotaxis; inflammatory response; immune response; signal transduction. Cytokine–cytokine receptor interaction; Chemokine signaling pathway; Toll-like receptor signaling pathway; RIG-I-like receptor signaling pathway;	5.14***
Cytokine (chemokine) 11	Cxcl11/I-TAC	BF281987	Chemotaxis; inflammatory response; chemokine activity; immune response; signal transduction; G-protein coupled receptor protein signaling pathway. Toll-like receptor signaling; cytokine–cytokine receptor interaction; Chemokine signaling pathway	5.30***
Cytokine (chemokine) 9	Cxcl9	Al044222	Chemotaxis; defense response; inflammatory response; immune response; cellular defense response. Cytokine–cytokine receptor interaction; chemokine signaling pathway; Toll-like receptor signaling pathway	3.96***
	Cxcl9	Al170387		3.80***
Ubiquitin D	Ubd	NM_053299	Antimicrobial humoral response; protein modification process; proteolysis	3.78***
Cytokine (chemokine) 7 – monocyte chemotactic protein 3	Ccl7/MCP3/SCYA7	BF419899	Cellular calcium ion homeostasis; chemotaxis; chemokine activity; immune response; inflammatory response; signal transduction. Cytokine–cytokine receptor interaction; Chemokine signaling pathway; NOD-like receptor signaling pathway	2.69*
Agrin	G1p2_predicted	BE096523	Plasma membrane organization; signal transduction; muscarinic acetylcholine receptor signaling; synapse assembly; neuromuscular junction development. ECM-receptor interaction	2.14***
Similar to Ac2-233	Igtp_predicted	Bl300770	–	−2.09**
Homologous to some human interferon-inducible proteins	MGC94037	AW531805	–	1.89***
Similar to interferon-inducible GTPase	MGC108823	Al408440	–	1.80***
	RGD1309362	AA955213	–	1.71***
Proteasome subunit, beta type 9	Psmb9	Al599350	Immune response; antigen processing and presentation; anaphase-promoting complex-dependent proteasomal ubiquitin-dependent protein catabolic process; interspecies interaction between organisms; negative regulation of ubiquitin-protein ligase activity during mitotic cell cycle.	1.68***
Granzyme B	Gzmb	M34097	Apoptosis; extracellular space; proteolysis; cleavage of lamin; serine-type (endo)peptidase activity; natural killer cell mediated cytotoxicity; type I diabetes mellitus; autoimmune thyroid disease; allograft rejection	1.60***
Interferon regulatory factor 7 (predicted)	Irf7_predicted	BF411036	Negative regulation of transcription from RNA polymerase II promoter; regulation of transcription, DNA-dependent; response to virus; immunoglobulin mediated immune response; interspecies interaction between organisms. Toll-like receptor signaling pathway; RIG-I-like receptor signaling pathway; Cytosolic DNA-sensing pathway	1.44***
Granzyme B	Gzmb	Al029386	See above under 'Granzyme B M34097'	1.36***
Signal transducer and activator of transcription 1	Stat1	AW434718	Regulation of transcription, DNA-dependent; transcription from RNA polymerase II promoter; induction of apoptosis; activation of caspase activity; signal transduction. Chemokine signaling pathway; Toll-like receptor signaling pathway; Jak-STAT signaling pathway; pathways in cancer	1.27***
Ubiquitin-activating enzyme E1-like (predicted)	Ube1l_predicted	Al177396	–	1.21***
Granzyme M	Gzmm	L05175	Cytolysis; proteolysis; extracellular space	1.20**
Signal transducer and activator of transcription 1	Stat1	BM386875	See above under 'Stat1 AW434718'	1.20**
Interferon regulatory factor 1	Irf1	NM_012591	Immune response; negative regulation of progression through cell cycle; positive regulation of IL-12 biosynthesis; regulation of transcription, DNA-dependent; transcription from RNA polymerase II promoter; CD8-positive, alpha-beta T cell differentiation	1.19***
Guanylate nucleotide binding protein 2	Gbp2	NM_133624	Immune response	1.19***
Similar to hypothetical protein MGC29390	RGD1310490	BF284106	–	1.18***
Natural killer cell group 7 sequence	Nkg7	NM_133540	Extracellular space; integral to membrane	1.17**
Uridine phosphorylase 1 (predicted)	Upp1_predicted	Bl292558	Nucleobase, nucleoside, nucleotide and nucleic acid metabolic process; pyrimidine nucleotide metabolic process; nucleoside metabolic process; nucleotide catabolic process; uridine metabolic process. Pyrimidine metabolism; Drug metabolism - other enzymes; Metabolic pathways	1.12**
Similar to 5830458K16Rik protein (predicted)	RGD1306974_predicted	AA819788	–	1.12***
Putative ISG12(b) protein	Isg12(b)/Ifl27l2	AA819034	Membrane; integral to membrane	1.05***

[a] OXA2/OXA versus veh2/OXA.
[b] Significances $^{***}p < 0.001$; $^{**}p < 0.01$ or $^{*}p < 0.05$ OXA2/OXA versus either of all intrastudy control groups (veh2/OXA, OXA2/− and OXA5/−).

Table 5
T-values of differentially expressed groups of genes (Gene Ontology) in left lung tissue of BN rats, sensitized twice and subsequently challenged with OXA (OXA2/OXA).

GO ontology pathway	OXA2/OXA versus OXA2/–	OXA2/OXA versus OXA5/–	OXA2/OXA versus veh2/OXA
Chemokine activity	14.41***	8.36***	14.91***
Chemokine receptor binding	14.16***	7.88***	14.59***
Immune response	10.57***	4.75*	17.00***
Cytokine activity	8.09***	5.12*	8.02***
Defense response	7.18***	0.05	10.67***
Inflammatory response	6.18***	−0.46	8.89***
Extracellular matrix	−5.28**	−8.52***	−3.80
Blood vessel development	−5.71***	−5.48***	3.44
Anatomical structure development	−6.49***	−8.42***	−1.08
Muscle contraction	−3.91	−9.63***	−6.53***

** *E*-value < 0.001.
*** *E*-value < 0.0001.

lenge in the sensitized animals caused a very slight to slight mixed granulocytic–monocytic inflammatory cell infiltrate, whereas in the unsensitized animals (veh2/OXA and veh5/OXA) the inflammation was only very slight (Table 3). Moreover, the inflammatory cell infiltrates in the unsensitized animals were only focal, whereas the infiltrates in about half of the sensitized animals were multifocal. The infiltrates in the sensitized animals did not show a distinct predilection site, other than being located in the anterior part of the nasal passages (Levels 1–3): they were observed ventrally on the septum, the lateral wall and/or the maxilla- and nasal turbinates. Challenge-related effects were not observed in the H&E-stained sections of the trachea and lungs.

The number of immunohistochemically stained $CD4^+$, $CD8^+$, $CD161^+$, and $CD68^+$ cells in the alveolar septa of the left lungs did not differ between the sensitized (OXA5/OXA) and unsensitized (veh5/OXA) animals. The number of $CD11b^+$ cells was slightly lower in the sensitized animals (OXA5/OXA: 70.7 ± 2.1 and veh5/OXA: 81.7 ± 2.9 per total surface area of 1.4 mm^2).

3.4. Effect of OXA on lung tissue transcriptome

3.4.1. Intrastudy comparison: single genes analysis

Comparison between the groups resulted in total of 1150 differentially expressed genes in at least one of the veh2/OXA, OXA2/– and OXA5/– treatments compared to the sensitized/challenged group (OXA2/OXA). The genes affected most are listed in Table 4. The mean fold changes were highest for the cytokines Cxcl9, Cxcl10 and Cxcl11 and for ubiquitin.

3.4.2. Gene group analysis

The GO Ontology groups/pathways of genes 'Chemokine activity', 'Chemokine receptor binding', 'Immune response' and 'Cytokine activity' were upregulated considerably, and highly significantly in the OXA2/OXA group, compared to the other three groups (Table 5). The pathways 'extracellular matrix', 'blood vessel development', 'anatomical structure development' and 'muscle contraction' were downregulated significantly compared to two of the groups (Table 5).

3.4.3. Interstudy comparison

Single gene comparison between OXA, TMA and DNCB (groups sensitized/challenged versus unsensitized/challenged rats) revealed an overlap of 112 genes between OXA and TMA, and 6 of these genes were shared by OXA, DNCB and TMA (Fig. 3 and Table 6). The overall gene expression profile of OXA resembled more that of TMA than of DNCB.

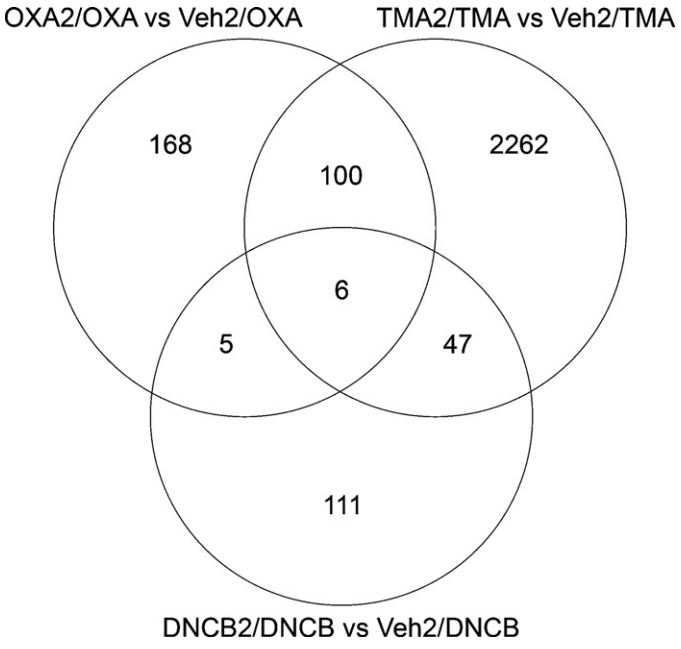

Fig. 3. The number of genes differentially expressed by OXA, TMA and DNCB in the twice-sensitized and challenged (compound 2x/compound) groups compared to the unsensitized, but challenged (veh2x/compound) groups, and the number of genes shared by OXA, TMA and/or DNCB.

4. Discussion

The breathing pattern, IgE levels and inflammation induced by OXA in this study indicated that OXA behaved comparable to the respiratory allergen TMA in previous studies, using the same protocol (Arts et al., 1998, 2003, 2004; Kuper et al., 2008a). Animals sensitized five times had higher IgE levels and tended to have a more decreased breathing frequency during challenge than animals sensitized twice, but the type and degree of inflammation was unaffected by the number of sensitizations.

The allergic inflammation induced by the inhalation challenge of OXA in the OXA-sensitized animals was localized primarily in the larynx. Allergic laryngitis was observed also in TMA-allergic Brown Norway rats and DNCB-allergic Wistar rats. Allergic laryngitis is not diagnosed often in man, perhaps because particle impaction patterns in the respiratory tract differ from those of rats. However, it is more plausible that the reported low incidence in man is due to underdiagnosis of allergic laryngitis (Hellings and Fokkens, 2006; Krouse and Altman, 2010; Leino et al., 1998; Randhawa et al., 2010).

Table 6
Overlap of genes in lung tissue of allergic (twice sensitized/challenged) BN rats: comparison OXA, TMA and/or DNCB.[a]

Description[b]	Gene name	Accession no.	Description
The most significant differentially expressed genes shared by OXA and TMA, but not DNCB[c]			
	Cxcl9	AI170387	
	Cxcl10	U22520	
	Cxcl11	BF281987	
Amiloride binding protein 1	Abp1	NM_022935	Amine metabolic process; oxidation reduction, arginine and proline metabolism; histidine metabolism; tryptophan metabolism
	G1p2-predicted	BE096523	
	Gbp2	NM_133624	
	Irf1	NM_012591	
	Irf7-predicted	BF411036	
	Isg12(b)/Ifl27l2	AA819034	
Interferon inducible protein 1 (predicted)	If1-predicted	AI407339	Autophagy; inflammatory response; innate immune response
Oligoadenylate synthetase-like 1	Oasl1-predicted	BF419319	
Proteasome (prosome, macropain) subunit, beta type, 8	Psmb8	NM_080767	Immune response; antigen processing and presentation, anaphase-promoting complex-dependent proteasomal ubiquitin-dependent protein catabolic process; interspecies interaction between organisms; negative regulation of ubiquitin-protein ligase activity during mitotic cell cycle
	Psmb9	AI599350	
–	Psmb10-predicted	BG373505	–
Member RAS oncogene family	Rab20-predicted	BI293985	Intracellular protein transport; nucleocytoplasmic transport; signal transduction; small GTPase mediated signal transduction; protein transport
Signal transducer and activator of transcription 2	Stat2-predicted	BG666368	Regulation of transcription, DNA-dependent; regulation of transcription from RNA polymerase II promoter; signal transduction; JAK-STAT cascade; chemokine signaling pathway; Jak-STAT signaling pathway
Selectin P	Selp	BI296054	Positive regulation of leukocyte migration; regulation of cellular extravasation; inflammatory response; cell adhesion; heterophilic cell–cell adhesion.
Transporter 1	Tap1	X57523	Protein complex assembly; transport; intracellular protein transport; defense response; immune response. ABC transporters; antigen processing and presentation; primary immunodeficiency
Transporter 2	Tap2	NM_032056	positive regulation of T cell mediated cytotoxicity; response to molecule of bacterial origin; antigen processing and presentation of exogenous protein antigen via MHC class Ib, TAP-dependent; protein complex assembly; positive regulation of antigen processing and presentation of peptide antigen via MHC class I. ABC transporters; Antigen processing and presentation; Primary immunodeficiency
TNF receptor superfamily member; B-cell surface antigen CD40	Tnfrsf5-predicted/CD40	AW433947	Immune response-regulating cell surface receptor signaling pathway; protein complex assembly; inflammatory response; platelet activation; positive regulation of B cell proliferation. Cytokine–cytokine receptor interaction; Cell adhesion molecules; Toll-like receptor signaling pathway; Intestinal immune network for IgA production; asthma
	Upp1-predicted	BI292558	
Superoxide dismutase 3, extracellular	Sod3 (down-regulated)	NM_012880	Response to hypoxia; superoxide metabolic process; response to copper ion; oxidation reduction
Engulfment adaptor PTB	Gulp1-predicted (down-regulated)	BM390519	Lipid transport; phagocytosis, engulfment; apoptosis; metabolic process; oxidation reduction
Flavin containing monooxygenase 2 (non-functional); pulmonary flavin-containing monooxygenase	Fmo2 (down-regulated)	BM389350	Organic acid metabolic process; NADP metabolic process; oxygen and reactive oxygen species metabolic process; xenobiotic metabolic process; toxin metabolic process; Drug metabolism – cytochrome P450
The six differentially expressed genes shared by OXA, TMA and DNCB			
	Ubd	NM_053299	
	Gzmb	AI029386	
	Ccl7/MCP3	BF419899	
Runt-related transcription factor	Runx3	AI709792	Regulation of transcription, DNA-dependent; transcription from RNA polymerase II promoter; induction of apoptosis; axon guidance; cell proliferation
–	RGD1309596_predicted	BG377140	–
–	NA	AW525366	–

[a] For all three allergens, the same protocol was used. The genes were measured in lung tissue, 24 h after challenge with the allergen.
[b] Only for the genes not included in Table 4.
[c] Of the 106 genes expressed by both OXA and TMA, 24 genes were selected on the basis of (i) being significantly expressed in the twice sensitized/challenged groups (OXA- and TMA-allergic rats) compared to all other groups, (ii) expressed in the same direction (up- or down-regulated for both OXA and TMA), and (iii) at $p < 0.01$.

The histopathology of the laryngitis pointed to an intriguing aspect of respiratory allergy. The challenge with OXA induced large numbers of neutrophils in the larynx of the sensitized animals, but some neutrophils were observed in the unsensitized animals as well (Fig. 2), suggesting that the sensitization made the animals exceptionally sensitive for the irritating properties of OXA. This sensitization-aggravated irritation is in line with the concept proposed by Noble (2009) that there may be memory for danger signals as well as for specific antigens. The concept of Noble (2009) could explain the presence of the large numbers of neutrophils in the OXA-induced allergic laryngitis as well as the neutrophils in moderate to severe asthma and the aspecific hyperreactivity (AHR) in asthmatic individuals.

The development of respiratory allergy is considered to be regulated predominantly by Th2-polarized immune reactions in which IgE plays a dominant role. The OXA-induced increased total IgE

levels in serum and the breathing pattern were in line with a Th2-polarized mechanism. Neutrophils in the lungs are associated with certain types of Th2-mediated asthma, but also with Th1-mediated hypersensitivity pneumonitis/allergic alveolitis (HP/AA; De Vooght et al., 2011; Fireman, 2006; Matsuoka et al., 2010). The observed hemorrhages in the OXA-induced laryngitis are more in line with the hemorrhages found in the lungs of acute and subchronic HP/AA patients (Bogaert et al., 2009; Jancar and Crespo, 2005). This complex picture is in accordance with the increasing awareness that lung allergies like asthma and HP/AA have considerable overlap in pathogenesis and histopathology, depending on the severity and chronicity of the diseases and this may be the case with laryngeal allergies as well.

In the present study, allergic inflammation was not observed in the lungs of the OXA-sensitized and -challenged rats, but microarray analysis of lung tissue revealed strong upregulation of several genes (Table 4). There was considerable overlap between OXA- and TMA-induced differentially expression of genes (Table 6), although TMA induced more strongly Th2-associated genes and OXA Th1-associated genes, namely Cxcl9, Cxcl10 and Cxcl11 (Table 4). These genes are regulated by STAT1 (Chen and Hershey, 2007; Fulkerson et al., 2004; Mikhak et al., 2006). Interestingly, these genes were not upregulated by the contact allergen DNCB (Table 6). This in accordance to the findings of Ku et al. (2009), who found Cxcl9 and Cxcl10 upregulation in the skin by OXA and TDI but not by DNCB. Cxcl10 has been found in a murine asthma model (Fulkerson et al., 2004) and in the late-phase reaction in human asthmatic lung (Lai et al., 2008). The Th1-related chemokines may reflect a negative feedback mechanism by which Th1 counterbalances Th2 to self-limit the allergic inflammation (Dharajiva et al., 2009; Wells et al., 2007). OXA and TMA induced upregulation of Ccl7 (Table 6), which is consistently associated with respiratory allergy (Bloemen et al., 2007; Fulkerson et al., 2004; Johnson et al., 2004), but so did DNCB. In contrast to TMA, OXA did not activate the known pathways for remodeling, which is considered a significant health aspect of asthma (Davies et al., 2003). However, shared genes like SELP and GZMB suggest that OXA, like TMA, interfered with vascular permeability and leukocyte extravasation and maybe even lung remodeling via platelet activation (Pitchford et al., 2008).

Although the GO Ontology pathways related to chemokines were strongly upregulated in the OXA-sensitized and -challenged rats, no genes were identified for those chemokines that are being used in current cytokine profiling tests for distinguishing typical respiratory from typical contact allergens. In addition, the genes and pathways were dissimilar to those found by Verstraelen et al. (2009a,b,c) in in vitro studies, exposing macrophages and epithelial cells to chemical respiratory allergens. Microarray analyses have not provided us yet with markers to be applied in a robust test to identify respiratory allergens, but they clearly add in our understanding of the complex issue of respiratory allergy.

In summary, the sensitizer OXA behaved like the respiratory allergen TMA in our inhalation challenge model in rats, with respect to the induction of elevated serum IgE levels and apneas, and the type of allergic laryngeal inflammation. However, microarray analysis of the lung suggested that OXA acts through different mechanisms than TMA, although the overlap in genes and pathways between OXA and TMA was larger than with the contact allergen DNCB. It is possible that the variability in balance between Th1- and Th2-associated genes reflects different subtypes of respiratory allergies (Bogaert et al., 2009; Truyen et al., 2006). It is concluded that, although the mechanisms may differ from those induced by the recognized respiratory allergen TMA, OXA has respiratory allergenic potential and should therefore not be used as a model contact allergen to develop and validate (Q)SAR models and in vivo and in vitro screening tests to identify sensitization potential of chemicals.

Conflict of interest

All authors declare that there is no conflict of interest.

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