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Development of a respiratory sensitization/elicitation protocol of toluene diisocyanate (TDI) in Brown Norway rats to derive an elicitation-based occupational exposure level

Jürgen Pauluhn*

Bayer Pharma AG, Wuppertal, Germany

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ABSTRACT

Toluene diisocyanate (TDI), a known human asthmagen, was investigated in skin-sensitized Brown Norway rats for its concentration × time ($C \times t$)-response relationship on elicitation-based endpoints. The major goal of study was to determine the elicitation inhalation threshold dose in sensitized, re-challenged Brown Norway rats, including the associated variables affecting the dosimetry of inhaled TDI-vapor in rats and as to how these differences can be translated to humans. Attempts were made to duplicate at least some traits of human asthma by using skin-sensitized rats which were subjected to single or multiple inhalation-escalation challenge exposures. Two types of dose-escalation protocols were used to determine the elicitation-threshold $C \times t$; one used a variable C (C_{var}) and constant t (t_{const}), the other a constant C (C_{const}) and variable t (t_{var}). The selection of the "minimal irritant" C was based an ancillary pre-studies. Neutrophilic granulocytes (PMNs) in bronchoalveolar lavage fluid (BAL) were considered as the endpoint of choice to integrate the allergic pulmonary inflammation. These were supplemented by physiological measurements characterizing nocturnal asthma-like responses and increased nitric oxide in exhaled breath (eNO). The $C_{const} \times t_{var}$ regimen yielded the most conclusive dose–response relationship as long C was high enough to overcome the scrubbing capacity of the upper airways. Based on ancillary pre-studies in naïve rats, the related human-equivalent respiratory tract irritant threshold concentration was estimated to be 0.09 ppm. The respective 8-h time-adjusted asthma-related human-equivalent threshold $C \times t$-product (dose), in 'asthmatic' rats, was estimated to be 0.003 ppm. Both thresholds are in agreement of the current ACGIH TLV® of TDI and published human evidence. In summary, the findings from this animal model suggest that TDI-induced respiratory allergy is likely to be contingent on two interlinked, sequentially occurring mechanisms: first, dermal sensitizing encounters high enough to cause systemic sensitization. Second, when followed by inhalation exposure(s) high enough to initiate and amplify an allergic airway inflammation, then a progression into asthma may occur. This bioassay requires an in-depth knowledge on respiratory tract dosimetry and irritation of the involved test substance to clearly understand the dosimetry causing C- and/or $C \times t$-dependent respiratory tract irritation and eventually asthma.

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1. Introduction

Exposure to diisocyanates can cause contact dermatitis, skin and respiratory tract irritation, immune sensitization, and less commonly hypersensitivity pneumonitis. Diisocyanates have been reported to be a cause of occupational asthma (OA) (Baur, 2007; Baur et al., 1994; Bello et al., 2004, 2007; Malo et al., 1999; Redlich and Herrick, 2008; Redlich, 2010; Vandenplas et al., 1993). The exposure-response information in most epidemiological and clinical studies to date is limited and the exposure scenarios too variable to make any robust conclusions regarding relative potency differences between diisocyanates (Bello et al., 2004). Airborne isocyanate exposures have been reduced through improved controls and use of less-volatile isocyanates. Yet isocyanate asthma continues to occur, not uncommonly in work settings where isocyanate respiratory exposures are very low or non-detectable, but where there is opportunity for skin exposure (Bello et al., 2007). Thus,

* Correspondence to: Experimental Toxicology, Bayer Pharma AG, 42096 Wuppertal, Germany. Tel.: +49 202 363909; fax: +49 202 364589.
E-mail addresses: juergen.pauluhn@bayer.com, juergen-pauluhn@t-online.de

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the currently applied methodologies cannot unequivocally distinguish the respective contribution of any specific exposure route to the events initiating and exacerbating diisocyanate-induced OA. This issue is complicated further because seemingly less potent diisocyanates may be affected by volatility which also influences the potential of inhalation and dermal exposure intensities. Although specific inhalation challenges are used as diagnostic tool, the bronchial response to inhaled isocyanates is dependent on multiple factors, these include the inhaled dose, defined as a concentration × exposure duration ($C \times t$) relationship, the degree of immunological sensitization of the subject, and the level of non-specific bronchial responsiveness (Vandenplas et al., 1993). Such relationships may also have a C-dependent component that determines the magnitude of sensory irritation as well as the depth of penetration of TDI-vapor into the distal airways of the lung. This means for toxicologists, who routinely consider the precautionary principle, that the $C \times t$-relationship priming the respiratory tract to become asthmatic needs to be identified as starting point for risk assessment, including how this endpoint compares with the threshold of the site-specific respiratory tract irritation. For isocyanates, the latter is the dose-limiting factor of pulmonary toxicity in repeated dose inhalation toxicity studies which commonly serve as starting point to derive safe occupational exposure levels.

The hazard identification and risk assessment of chemicals that cause sensitization of the respiratory tract and its progression to multiple phenotypes of asthma may pose a significant problem to industry and regulators alike, not at least because, as yet, there are no available validated or even widely accepted predictive test methods (Basketter and Kimber, 2011; Kimber et al., 2007, 2010, 2011; Kimber and Dearman, 1997, 2005; Vandebriel et al., 2011). Asthma is the most elusive of all common chronic disorders of the lung. Similarly elusive are the perceptions about the interactions of immunological and irritant mechanisms through which chemicals may cause sensitization of the respiratory tract. Regardless of whether occupational asthma results from an immune response or an irritant reaction to a substance, the symptoms are virtually the same: airway hypersensitivity characterized by episodes of cough, wheezing, chest tightness and breathlessness. It can result in severe and potentially fatal breathing problems. The absence of any clear consensus within the scientific community which of the many putative mechanisms studied in human-surrogate species are most important for workplace or consumer risk assessment has been considered to be the reason for the lack of standard methods for toxicological evaluations on respiratory sensitization (Kimber et al., 2011). The approach described in this paper is broadly reflective of at least some traits of occupational asthma and is independent on any pre-conceived specific immunological mechanisms that are confounded by non-specific acute pulmonary irritation (Pauluhn, 2008a).

The objective of study was to determine the elicitation threshold dose in skin-sensitized, repeatedly inhalation-challenged Brown Norway rats with emphasis on tolerance and amplification of responsiveness. At the first place, an integrated physiological approach chosen was chosen to appraise the dichotomous outcome 'respiratory tract sensitizer following dermal sensitization' utilizing a highly rationalized inhalation-challenge protocol (for details see Section 2.3). If positive, this protocol is then supplemented by a dose-escalation challenge in order to derive an occupational exposure level (OEL) for the asthma endpoint. One prerequisite of this type of translational toxicology is a robust control of physiological factors that influence inhalation dosimetry. Therefore, particular emphasis was directed toward the analysis of variables affecting the $C \times t$-dose inhaled to further minimize the uncertainty in extrapolating data from rats to humans.

2. Methods

2.1. Test material and chemicals

TDI, is a mixture of 2,4/2,6-toluene diisocyanate (DESMODUR® T 80; Bayer Material Science AG, Leverkusen, Germany). The content of 2,4: 2,6 isomers was 80% and 20%, respectively (analytically confirmed). The free isocyanate (NCO) content was 48%. During handling and storage the headspace of TDI containers was purged with dry nitrogen to remove air and humidity to prevent its decomposition.

2.2. Animals, diet, and housing conditions

Male Brown Norway (BN) rats of the strain BN/Crl BR were from Charles River, Sulzfeld, Germany. The Wistar rats, strain Hsd Cpb:WU (SPF), used for the sensory irritation measurements were from the experimental animal breeder Harlan-Nederland (NL), AD Horst. Animals were maintained in polycarbonate cages with one rat per cage, which contained bedding material (low-dust wood shavings), and were provided with a standard fixed-formula diet (ssniff® R/M-H pellets maintenance diet for rats and mice; ssniff Spezialdiäten GmbH, http://www.ssniff.de) and municipality tap water in drinking bottles. Both feed and water were given ad libitum except during inhalation exposures. At the commencement of study, the mean body weights were approximately 240 g. For this study, only male rats were used and they were approximately three months old. Animals were quarantined for at least 5 days prior to being placed on study. Animal rooms were maintained at approximately 22 °C with relative humidity at 40–60% and a 12-h light cycle beginning at 0600 h.

The principle execution of studies described in this paper was in accordance with contemporary, internationally harmonized testing standards/guidelines (OECD, 2009) and Good Laboratory Practice standards (OECD, 1998). The experiments were performed in an animal care-approved laboratory in accordance with the German Animal Welfare Act and European Council Directive 2010/63/EU as of 22 September 2010.

2.3. Experimental protocol and mechanistic rationale

Dermal penetration studies in rats indicate that the essentially non-volatile aromatic diisocyanate MDI (4,4′-methylene diphenyl diisocyanate) and the volatile TDI are absorbed (as adducted peptides/proteins) on the skin of rats only to a very small fraction (<1%). Considerable amounts of the applied radioactivity were found at the application site which could not be washed off. These findings suggest that the majority of the administered dose remains in the upper skin layers and is subject to protein-binding and/or polymerization with slow clearance (Hoffmann et al., 2010). Hence, topically administered diisocyanates may generate in situ stationary high local concentrations of haptenated potentially antigenic proteins in the skin. As conceptualized in Fig. 1, these antigens may be drained to the local lymph nodes as such or via translocation after uptake by Langerhans dendritic cells. Especially in experimental models, these antigens get access to the immunological network in a microenvironment of coexisting local inflammation. The activation of dendritic cells with subsequent T-lymphocyte transformation in the lymph nodes draining the skin produce activated T-lymphocytes effector- or memory cells in the systemic circulation (Condon et al., 2011; Lira, 2005). Also in the lung resident macrophages (AM), as a part of Langerhans dendritic cell network, may be involved in immune inflammatory reactions. However, while macrophages have antigen-presenting properties in the skin, lung macrophages preferentially inhibit T-lymphocyte activation and their activation may favor proteolysis over

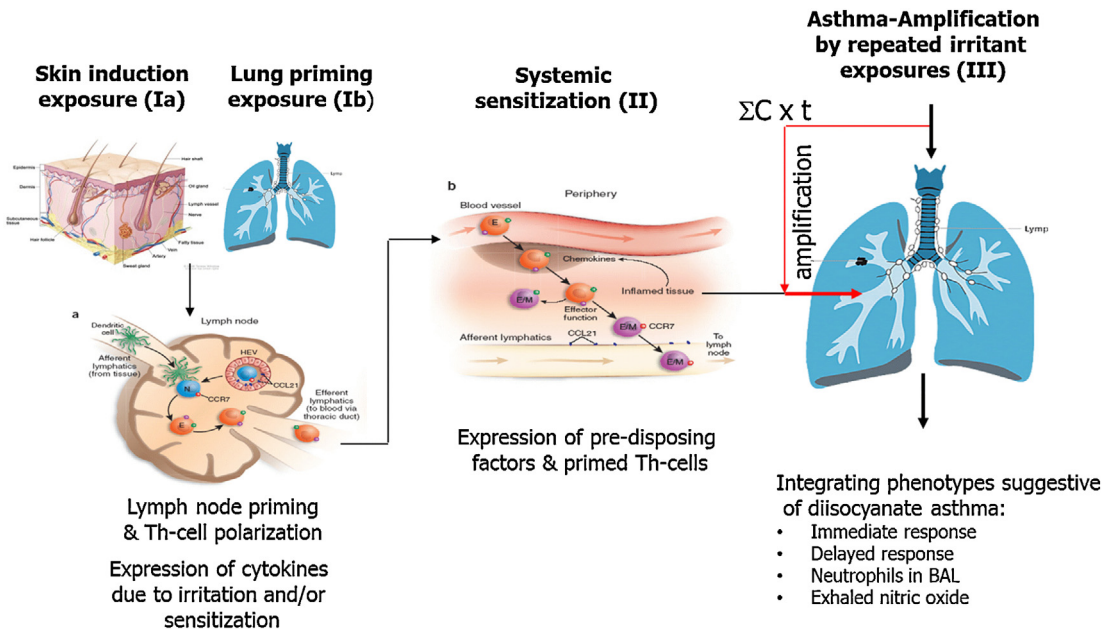


Fig. 1. This illustration conceptualizes the potential dermal (Ia) and inhalation (Ib) routes for sensitization and the immunological factors involved for T-cell migration and entry into lymph nodes (figure modified from Lira, 2005; abbreviations and additional mechanistic explanations are given by this author). The circulation of T-cells involves their entry into lymph nodes (a) from blood, in a process dependent on CCR7 and adhesion molecules. Recognition of the appropriate antigen by incoming T-cells leads to their differentiation into effector cells (E). Exit through efferent lymphatics will bring the T-cells into the systemic circulation (II) and into areas where inflammatory chemokines may be produced (b), especially following successive repeated inhalation encounters (III). Once in the tissue (lung), effector T-cells will produce cytokines and chemokines or undertake cytotoxic functions. Repeated inhalation exposures to irritant inhalation doses may synergistically amplify this allergic inflammation and asthma.

antigen-presentation. The suppressive effect on the dendritic cells seems to be directed to maintain lung function rather than stimulating overshooting immunologically mediated inflammatory reactions (Condon et al., 2011; Holt et al., 1993). Adducted peptides (soluble) in the lining fluids of the lung (surfactant) are rapidly cleared from the lung either by absorption or AM-mediated proteolytic degradation. The isocyanate-specific localized irritation and antigenicity could, in contrast to the skin, seem to be conducive to tolerogenesis over aggravation of an allergic inflammation within the lung. The morphology and physiology of the respiratory tract as well as the much lower retained dose with shorter residence time may result in substantially lower levels of hapten-conjugates/antigens in the lung as compared to the skin. Therefore, it is believed that skin-sensitized rats may become more readily pre-disposed and susceptible to subsequent inhalation exposures following skin exposure rather than inhalation exposures.

Intermediate concentrations of hapten-conjugates/antigens may attract primed Th-cells from blood into the lung where they initiate and amplify an allergic inflammation and eventually asthma. Any repeated inhalation elicitation challenge protocol needs to be designed to employ adequately spaced challenge intervals to prevent carry-over of an irritant-related response. This time-period is required to wane off the irritation-related inflammatory response with amplification of the immunological sequence of events in the lung. Dose ($C \times t$)-selection is critical to achieve this objective and to prevent the occurrence of tolerogenesis. Similarly, especially for a volatile, reactive diisocyanate such as TDI, the concentration selected must be high enough to overcome the scrubbing capacity of the upper airways. The prerequisite of the induction of any asthma-like phenotype is that sufficiently high doses of TDI reach the lower airways; that site of the tract where asthma is initiated and amplified.

The protocol for sensitization and elicitation used in this study was adapted from previous studies with the non-volatile diisocyanate MDI (Pauluhn, 2005, 2006, 2008b; Pauluhn et al., 2005; Pauluhn and Poole, 2011). While MDI can technically be aerosolized to be deposited within the lower airways, the dosimetry of TDI-vapor is profoundly more complex. Reactive TDI-vapor is retained concentration-dependently throughout the entire respiratory tract and too low concentrations may be scrubbed to an appreciable extent within the upper airways of obligate nasal breathing rats. Additionally, any predominating upper respiratory tract irritation prompts a reflex-induced, concentration-dependent depression of ventilation which may further affect the inhaled dose and depth of vapour-penetration into the lung. For MDI-aerosol a variable concentration (C_{var}) × constant exposure duration (t_{const}) protocol was used for challenge due to negligible upper respiratory tract irritation (Pauluhn, 2000). This protocol was duplicated in a pilot study but was abandoned due to the reasons detailed in Section 3. In the steady dealt with in this paper, a fixed concentration (C_{const}) × variable exposure duration (t_{var}) protocol was devised to achieve reproducible challenge exposure conditions. The outcome using the $C_{var} \times t_{const}$ approach is cursory compared with the $C_{const} \times t_{var}$ protocol in Section 3.

A sequence of steps considered in the protocol is summarized in Fig. 2. Step I is based either on existing evidence or specialized sensory irritation ancillary studies in both Wistar and Brown Norway rats. The previous strain is commonly used in inhalation studies for upperrespiratory tract irritation-based endpoints. The comparative sensory irritation study with Brown Norway and Wistar rats served the purpose to evaluate potential strain differences to inhaled TDI-vapor at identical concentrations. The Wistar rat is considered to be the reference strain for the irritant response. To make challenge-exposure reproducible in the $C_{const} \times t_{var}$ protocol, the time to attain the maximum depression must be negligible relative to the duration of challenge. As detailed above, C_{const} of TDI must be high enough to dose the lower airways. Either objective was achieved in the $C_{const} \times t_{var}$ protocol at ≈85 mg TDI/m^3 as evidenced by the contrariwise changes of tidal volumes at this concentration in BN rats (see Section 3). This assumption was substantiated in the preceding proof-of-concept feasibility step II (see Table 1 group 1/2-pre). The objectives of step I and II was to impose a reversible, minimally

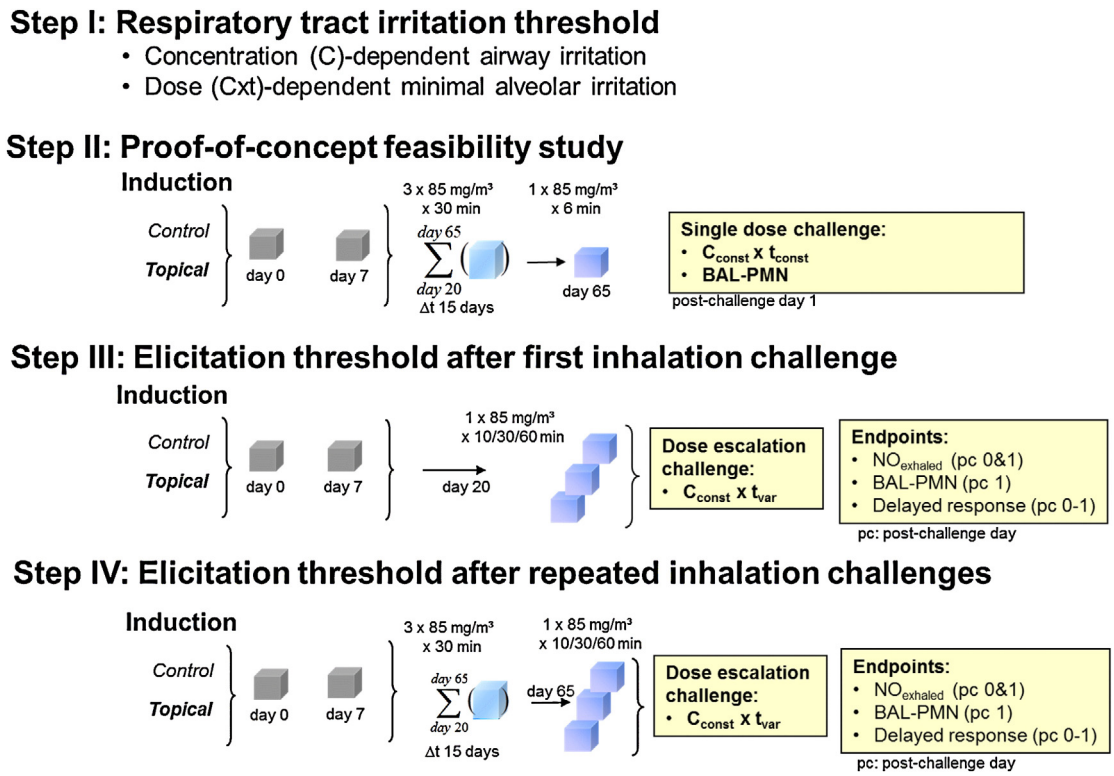


Fig. 2. Sequence of steps required to test for respiratory allergy in a topical-induction and repeated inhalation priming/elicitation Brown Norway rat model. Step I examines the sites of upper/lower respiratory tract irritation (dependent on whether the test substance is an aerosol or vapor) and defines the inhalation dose (expressed as concentration (C) × exposure duration (t)) required to elicit a minimal lower respiratory tract irritation. Step II is a verification step at which rats are exposed by inhalation to this $C \times t$ by 3 successive priming exposures of t = 30-min followed by an elicitation exposure (same C but shorter t). This step is expected to show evidence of reversible alveolar irritation in the absence of undue stress to animals. Steps III and IV use an escalation challenge at one concentration (C_{const}) with variable escalating exposure durations (t_{var}) without (step III) and with three time-spaced priming inhalation challenges (step IV). Naïve controls and TDI-sensitized rats (eight per escalation challenge subgroup) are challenged simultaneously to the same $C_{const} \times t_{var}$ dose. The asthma phenotype is probed by PMN in bronchoalveolar lavage fluid (BAL) one day after the respective escalation inhalation challenge. This key endpoint was supplemented by measurements of nitric oxide in exhaled air and lung function to better qualify the etiology of elevated BAL-PMN (irritant vs. allergic).

Table 1
Study protocol of respiratory sensitization in skin-sensitized Brown Norway rats. Groups of rats were subjected to constant concentration × variable time escalation protocol without (I) and with (IV) 3 priming inhalation exposure. During all challenges the respective vehicle/TDI and TDI/TDI groups were exposed simultaneously to identical atmospheres.

Group	Topical induction	Chal-I[d] (min)	Chal-II (min)	Chal-III (min)	Chal-IV (min)	Endpoints
1-pre[a]	Vehicle	30	30	30	6	BAL-PMN
2-pre	TDI	30	30	30	6	“
I-1a[b]	Vehicle	10[e]	–	–	–	LF, eNO, BAL[f]
I-1b	Vehicle	30[e]	–	–	–	“
I-1c	Vehicle	60[e]	–	–	–	“
I-2a[c]	TDI	10[e]	–	–	–	“
I-2b	TDI	30[e]	–	–	–	“
I-2c	TDI	60[e]	–	–	–	“
IV-3a[b]	Vehicle	30	30	30	10[e]	LF, eNO, BAL[f]
IV-3b	Vehicle	30	30	30	30	“
IV-3c	Vehicle	30[g]	30[g]	30[g]	60[g]	“
IV-4a[c]	TDI	30	30	30	10	“
IV-4b	TDI	30	30	30	30	“
IV-4c	TDI	30[g]	30[g]	30[g]	60[g]	“

In each group eight male BN rats were used.

[a] Pre-study(pre): Consistent with the stepwise procedure illustrated in Fig. 2/step II the following feasibility study was performed: sensitization 2% TDI (w/v) in 150 μL AOO (acetone:olive oil 4:1) on days 0 and 7 followed by 3 inhalation challenge at 86 mg TDI/m^3 × 30 min. Challenge IV was with the same concentration for 6 min.

[b] Application volume: 100 μL AOO on days 0 and 7.

[c] Application volume: 2% TDI (w/v) in 100 μL AOO on days 0 and 7. Concentration and stability of TDI in AOO analytically verified.

[d] Inhalation challenge at 85 mg TDI/m^3 × 30 min; challenge days were spaced by approximately 2 weeks to minimize carry-over of irritation-related effects from one challenge to another.

[e] Inhalation challenge with approximately 85 mg TDI/m^3 of all groups.

[f] LF: whole-body plethysmography (overnight), eNO: exhaled Nitric Oxide, BAL: bronchoalveolar lavage.

[g] These subgroups were repetitively examined by whole-body plethysmography.

alveolar irritant response after recurrent challenges in the absence of imposing any undue stress to rats. Steps III and IV served the purpose to examine the outcome of escalation challenge in the absence (III) and presence (IV) of prior lung priming inhalation exposures (step III: see Table 1 group I-1a-c/I-2a-c; step IV: see Table 1 group IV-3a-c/IV-4a-c). The groups shown in Table I are those of $C_{const} \times t_{var}$ protocol.

The duration of challenge utilized the following rationale: work-related diisocyanate asthma is commonly examined in specific human bronchoprovocation tests using a stepped 4×30 min cumulative challenge protocol per day (with breaks in between) with a $C \times t$ ranging from 0.15 to 0.9 ppmV × min and a cumulative challenge $C \times t$ of ≈2 ppmV × min (≈14 mg TDI/m^3 × min) (Raulf-Heimsoth et al., 2013). A dose-escalation challenge in Brown Norway rats at 85 mg/m^3 at 30 min translates to 2550 mg/m^3 × min × 1/100 = 26 mg/m^3 × min (the rationale for the applied assessment factor 1/100 is given in the discussion and considers the physiological differences of nasal breathing non-asthmatic but pre-disposed rats and oronasally breathing asthmatic humans). Thus, the lung priming dose of rats was in the range of ≈0.5 to 4-times the maximum human-equivalent challenge dose within a t_{var} of 10 to 60 min.

2.4. Experimental procedures

The sensory irritation study utilized both Wistar and Brown Norway rats with simultaneous exposure of four rats/strain/group in head-out volume displacement plethysmographs. Exposure to TDI-vapor was for 30 min, an exposure duration similar to that used for the lung-priming inhalation exposures. The procedures used in the sensitization study with Brown Norway rats were consistent with those described previously in lung sensitization studies with MDI-aerosol (Pauluhn, 2008a,b; Pauluhn and Poole, 2011). In brief, as conceptualized in Table 1 and Fig. 2, eight rats per escalation challenge subgroup were dermally sensitized on contralateral flanks on days 0 and 7. TDI was administered in the vehicle acetone (from Merck; further desiccated using a molecular sieve Fluka 69839) and olive oil ('highly refined' from Sigma Chemicals, cat. no. 01514). The volume of the vehicle acetone:olive oil (4:1 v/v) was 100 μl vehicle/administration; 2% TDI w/v. This concentration was selected based on pre-studies using different concentrations (1% and 5% TDI in the same vehicle) and administration volumes (see footnote of Table 1). The content and stability of TDI in the vehicle was analytically verified. Rats were prevented from grooming or scratching by wearing an Elizabethan collar up to the morning the day following administration (Buster Birdcollars; Kruuse, DK, Cat no.: 273375). The respective control groups received the vehicle only under otherwise identical conditions. For priming respiratory tract allergy, rats were exposed 3-times by inhalation for a duration of 30-min at concentrations of approximately 80–85 mg TDI/m^3 (Fig. 2). Rats from the non-sensitized control and TDI-induction groups were exposed simultaneously in the same chamber. At the dose-escalation challenge, rats of the control (vehicle/TDI) and TDI-induction (TDI/TDI) groups were challenged as detailed in Table 1 and Fig. 2. Nitric oxide in exhaled air (eNO) was determined after each provocation challenge before and after the respiratory function measurements. eNO has been shown to be a sensitive, non-invasive biomarker of airway inflammation of rats (Liu et al., 2013) and is generally considered as a diagnostic biomarker in OA (Ewald-Kleimeier et al., 2013). This non-invasive measurement was made twice to be able to diagnose changes attributable to effects early and delayed in onset. Breathing patterns were recorded overnight for approximately 20 h using barometric plethysmography. One day after the escalation challenge all rats were subjected to bronchoalveolar lavage (BAL). Lung and lung-associated lymph nodes (LALN) weights were determined in exsanguinated rats.

2.5. Generation and characterization of exposure atmospheres

Atmospheres of TDI for inhalation exposures were generated under dynamic conditions. A directed-flow nose-only exposure inhalation chamber was used. Air flow devices were calibrated (Bios DryCal Defender 510; http://www.smglink.com/bios/drycal_defender/drycal_defender.html). The glass bubbler containing the TDI was maintained at 45 °C using a thermostat (JULABO UC, Julabo, Seelbach, Germany). Targeted concentrations were achieved by pull/push dilution cascades. The chamber equilibrium concentration (t_{95}) was attained in less than 1 min with air flow rates of 0.75 L/min at each exposure port. The inhalation chamber was operated in a well-ventilated chemical fume hood. Temperature and humidity measurements utilized a computerized Data Acquisition and Control System equipped with HC-S3 sensors (Rotronic, http://www.rotronic-usa.com/prod_oem/hc2%20probes/hc2_main.htm). The temporal stability of TDI-vapor in exposure atmospheres were measured using a Gasmet Dx-4000 FT-IR (Fourier Transform Infrared spectroscopy; http://www.ansyco.de) gas analysis system using CalcmetTM-Software. Throughout all measurements the same resolution of 8 cm^{-1} with 10 scans/s were used. Sampling tubes (all made from Swagelog stainless steel flexible tubes ¼ in. in diameter) were isotherm to the inhalation chamber inner plenum (breathing zone area). For analysis, the peak height of the most prominent spectral band of TDI (wavenumber 2277.4 cm^{-1}) was used for calibration and measurements. The reference curves were based on the nominal and nitro-reagent-based analyses (see below). Potential interferences with exhaled gases, such as humidity or CO_2, can be excluded either due to the use of dry air and/or minimization of re-breathing of exhaled test atmospheres (Pauluhn and Thiel, 2007). The O=C=N–R–N=C=O-specific nitro-reagent-method was also used for the determination of the 2,4/2,6-isomers in exposure atmospheres. Adsorption tubes containing glass powder coated N-4-nitrobenzyl-N-n-propylamine (nitro reagent) were eluted with acetonitrile (1 L acidified with 1 mL formic acid). The resultant 2,4-TDI and 2,6-TDI urea derivatives were quantified by high-performance liquid chromatography (HPLC; with DAD-detection wave length: 276 nm). Standard solutions of the reference substance 2,4 TDI and 2,6 TDI were used for calibration. Details of the validation of this exposure system have been published elsewhere (Pauluhn and Thiel, 2007; Pauluhn, 2008a,b). The exposure methodology used is consistent with that called for by OECD (2009).

2.5.1. General observations

Body weights of animals were recorded once per week. The appearance and behavior of all animals were examined systematically in an ordinal (categorical) manner.

2.5.2. Analysis of upper respiratory sensory irritation

Pulmonary function tests were simultaneously performed on eight restrained, spontaneously breathing rats in head-out volume displacement plethysmographs. Fluctuations of thoracic air flows were measured with a differential pressure transducer (MP 45 ± 2 cm H_2O, Validyne) fitted onto the plethysmograph (for details see Pauluhn, 2013).

2.5.3. Analysis of delayed-onset respiratory response

Measurements were simultaneously made on 4 vehicle/TDI and 4 TDI/TDI unrestrained, spontaneously breathing rats in the subgroups IV-3c/IV-4c (Table 1) at all priming challenges time points. At the escalation challenge I-1a-c/I-2a-c and IV-3a-c/IV-4a-c (Table 1) the same number of rats from all groups was examined

in calibrated barometric whole-body plethysmographs. Data collection commenced shortly after challenge by placing the animals into the pre-calibrated barometric whole-body plethysmographs (for further details see Li et al., 2011; Pauluhn, 2008a,b; Pauluhn and Poole, 2011).

2.5.4. Measurement of NO in exhaled breath (eNO)

This measurement utilized 4 vehicle/TDI and 4 TDI/TDI rats per subgroup at the escalation challenge I-1a-c/I-2a-c and IV-3a-c/IV-4a-c (Table 1). NO was analyzed real-time using a chemi-luminescence analyzer (Sievers 280B NOA; Sievers Instrument, Inc., Denver, CO) (for details see Liu et al., 2013).

2.5.5. Bronchoalveolar lavage

After exsanguination, the excised lung was weighed and then lavaged via a tracheal cannula with two volumes of 5-ml of physiological saline (kept at 37 °C), each withdrawn, re-instilled once. In the supernatant, BAL-fluid was analyzed for total protein and lactate dehydrogenase (LDH). Further details were published in detail previously (see Pauluhn and Poole, 2011).

2.6. Data analysis

Body weights, organ weights, and BAL data were analyzed using a one-way analysis of variance (ANOVA) followed by a Tukey-Kramer post hoc test. The time-related changes in respiratory minute volume were analyzed using the following sigmoidal non-linear regression function: $y = y_0 + a/(1 + \exp(-(x - x_0)/b)$ where y is the respiratory minute volume relative to the pre-exposure period (=100%) and x is the time point of data collection during the 15-min pre-exposure period followed by the 30-min TDI exposure period. The parameter 'a' reflects the plateau of the maximum decrease in respiration and was used to calculate the 'respiratory decrease fifty percent' by linear regression. Parameters were estimated using SigmaPlot 11.0 software (Systat Software Inc., Point Richmond, CA). For all tests the criterion for statistical significance was set at $P < 0.05$.

3. Results

3.1. Characterization of provocation atmospheres

The analytical characterization of the test atmospheres by FT-IR from breathing zone samples indicated that the exposure conditions were temporally stable over the respective exposure periods. Due to the instant and less complex series of analytical steps involved, the results from the FT-IR method were given preference to the derivatization method. Up to approximately 110 mg/m^3 there was a consistent relationship of actual analytical to nominal concentrations which was at this exposure level 111 mg/m^3. Substantially lower recovery was found at the maximum exposure level examined (nominal concentration: 155 mg/m^3, actual breathing zone concentration: 113 mg/m^3), possibly due to the presence of supersaturated TDI-vapor/condensation aerosol atmospheres due to the temperature drop from 45 °C (gas bubbler) to 22 °C (inhalation chamber after dilution) and limited subsequent dilution with air at this exposure level. Therefore, challenge concentrations within the range of 80–90 mg TDI/m^3 were considered most appropriate from a technical and dosimetry perspective.

3.2. Upper respiratory tract sensory irritation

Naïve Wistar and Brown Norway rats that were nose-only exposed to TDI vapour for 30 min displayed an instant concentration-dependent decrease of the respiratory frequency and minute volume as shown in Fig. 3. Up to 81 mg/m^3 these changes recovered to the level of the pre-exposure period within almost 15 min post-TDI-exposure. The maximum and stable depression of ventilation was attained at exposure concentrations of 40–60 mg/m^3 (Fig. 4). While a minimal, if any, concentration-related decrease in tidal volumes occurred in Wistar rats, a more pronounced depression were observed in Brown Norway rats (Fig. 5). The first reversal of the tidal volumes from shallower breathing patterns occurred in Brown Norway rats at 81 mg/m^3. This is taken as evidence that alveolar irritant receptors (Paintal reflex) were stimulated. Based on these measurements a challenge concentration of about 85 mg TDI/m^3 was considered to reach the objective of study.

3.3. Pilot bronchoprovocation escalation challenge $C_{var} \times t_{const}$

Skin-sensitized rats receiving three prior lung-priming challenges at about 80–85 mg/m^3 × 30 min displayed transient signs of upper respiratory tract irritation (nasal discharge, bradypnea, and labored breathing patterns). Unlike the naïve but challenged control rats, labored breathing patterns occurred in sensitized rats only from the second challenge onwards. Body weights of equally challenged naïve and sensitized animals were indistinguishable (data not shown).

While the rats from the vehicle/TDI groups did not elaborate any consistent changes in Penh over 20 h (Penh-AUC$_{20\,h}$), only few rats in the TDI/TDI groups elaborated a somewhat increased Penh at challenges 2 to 4 of unclear toxicological significance (data not shown). BAL-PMN obtained one day after the bronchoprovocation-escalation challenge were different from the control at 85 mg/m^3 × 30 min (2550 mg/m^3 × min) only (Fig. 6). Challenge concentrations in the range of 15–50 mg/m^3 × 30 min did not reveal any conclusive dose–response relationship. The 5-fold difference of TDI in the concentrations used for the dermal induction (see Fig. 6) had no apparent effect on the elicitation-endpoints examined. This MDI-based $C_{var} \times t_{const}$ protocol showed differences between equally challenged naïve and sensitized rats at the maximum examined challenge dose only in the absence of any robust $C \times t$-dependence. Therefore, the $C_{var} \times t_{const}$ protocol was abandoned.

3.4. Bronchoprovocation escalation challenge $C_{const} \times t_{var}$

Rats dermally induced with 2%-TDI received three lung-priming challenges at about 85 mg/m^3 × 30 min. At the 4th escalation challenge, the rats from all groups were challenged to the same concentration for 6 min in the pre-study and 10, 30, and 60 min in the main-study (for details see Table 1). The possible occurrence of tolerance was evaluated in subgroups of rats receiving the escalation challenge in the absence of any prior priming challenges already at the 1st challenge time point. The challenge dose PMN%-relationships of the respective protocol are depicted in Figs. 7 and 8. The absolute counts of BAL-PMN are compared in Fig. 9. In the early stage of study, eosinophilic granulocytes were observed in all groups, independent on whether the rats received additional priming challenges or not (Fig. 10). Group or time-related changes of lymphocytes were not apparent. Significantly increased lung weights and BAL-protein after the 60-min escalation challenge were observed only in groups receiving the three priming inhalation challenges (Fig. 11). These elevations coincided with increased concentrations of nitric oxide (adjusted) in exhaled breath without appreciable difference of measurements made shortly after the 4th challenge and one day thereafter (Fig. 12). Only one out of the four measured rats primed by three prior inhalation challenges exposed for 60 min elaborated typical delayed-onset response (Fig. 13). This rat had the highest concentration of BAL-PMN (53%).

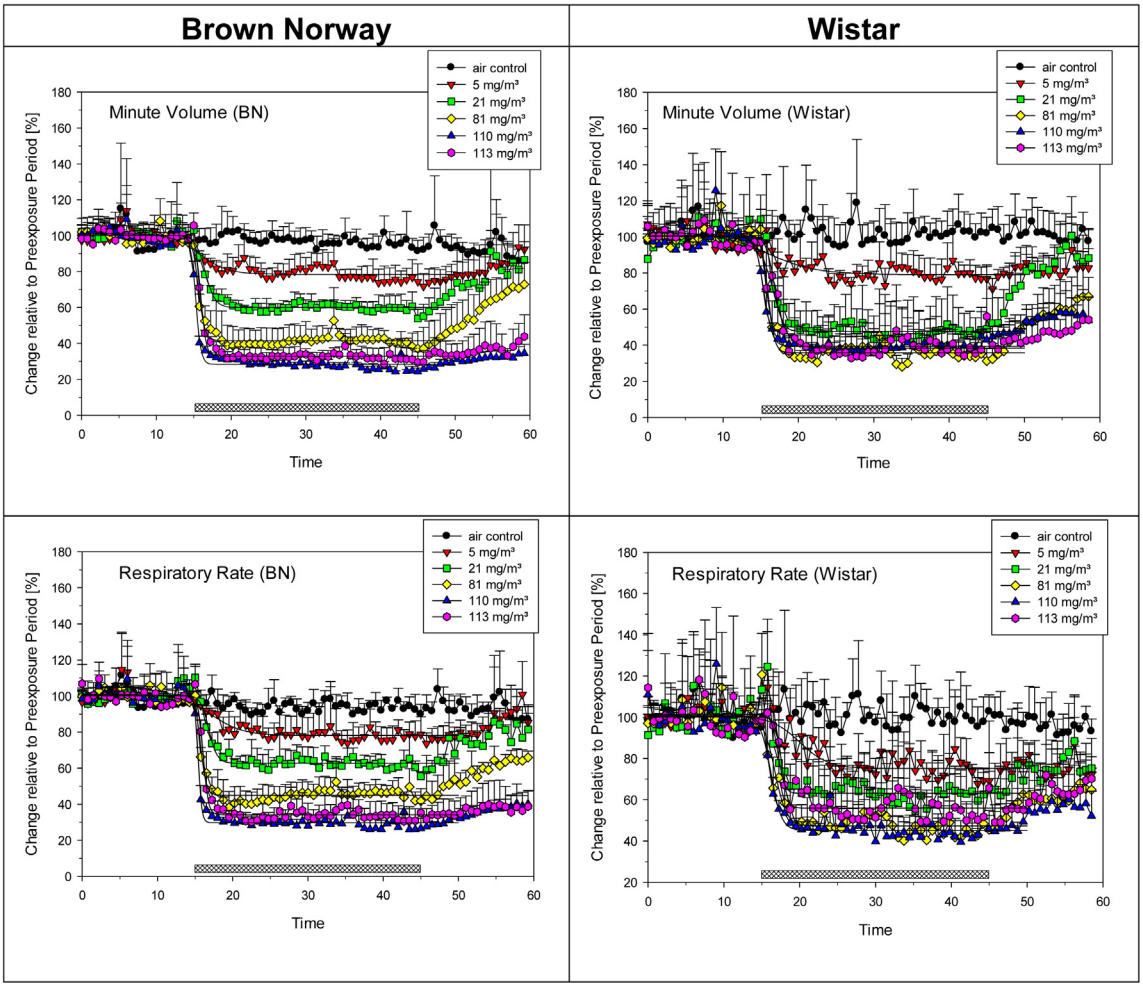


Fig. 3. Analysis of the time- and concentration-dependence of respiratory minute volumes and frequencies in Brown Norway and Wistar rats (4 animals of each strain/group/measurement) were simultaneously exposed for 15-min to air, 30 min to TDI-vapor followed by a 15-min recovery period. Measurements were made in head-out volume-displacement plethysmographs attached to a directed-flow nose-only inhalation chamber (Pauluhn and Thiel, 2007). The solid lines were derived using a sigmoid model fitted to measurements from the pre-exposure and exposure periods to estimate the degree of respiratory depression. All data were normalized to the pre-exposure period (=100%). The average body weights of both strains were 240 g. The average measured minute volume ± SD of Brown Norway and Wistar rats exposed to air only were 276 ± 14 and 244 ± 13 mL/rat/min (approximately 1 L/kg min).

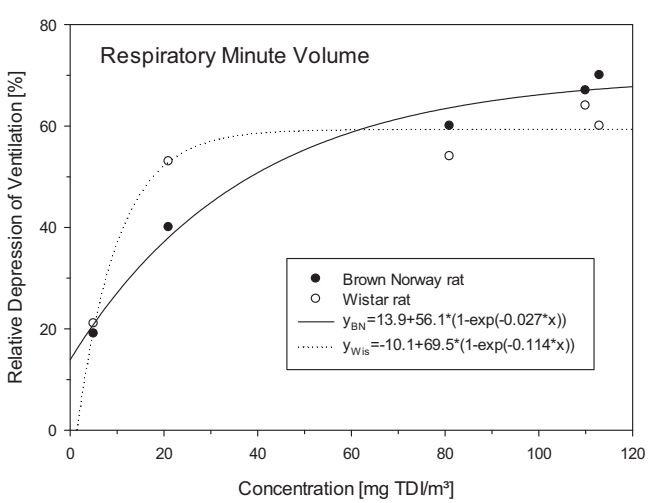


Fig. 4. Comparison of the concentration-dependence of the relative depression of respiratory minute volumes of naïve Brown Norway and Wistar rats nose-only exposed to variable concentrations of TDI (for details see Fig. 3). All data were normalized to the pre-exposure period (=100%).

The results from these analyses suggest that differences in PMNs between equally challenged controls and sensitized group could not be observed at the 10-min challenges whereas distinct differences occurred at the longer escalation challenge durations. Equally challenged control groups receiving no (Fig. 7) and three priming-challenges (Fig. 8) elaborated comparable, minimally $C \times t$-dependent elevations of PMN. This shows that the regimen used for priming and elicitation did not cause any marked irritation-related effects that could interfere with the interpretation of study. Consistent with the results reported for MDI-aerosol, repeated priming exposure caused an amplification of response. Overall, the outcome of the $C_{const} \times t_{var}$-protocol supports the conclusion that C-dependent changes in inhalation dosimetry may confound any elicitation-based dose–response analysis due to upper respiratory tract sensory irritation. These shortcomings were aptly resolved by the change from the $C_{var} \times t_{const}$-protocol (pre-study) to a $C_{const} \times t_{var}$-protocol utilizing a concentration at which a concentration-independent ventilation and vapour penetration into the deep lung can be anticipated. Evidence of any tolerance did not exist under the conditions applied. Based on the analysis given in Fig. 8, 1000 mg TDI/m^3 × min are considered to be the NOAEL for elicitation.

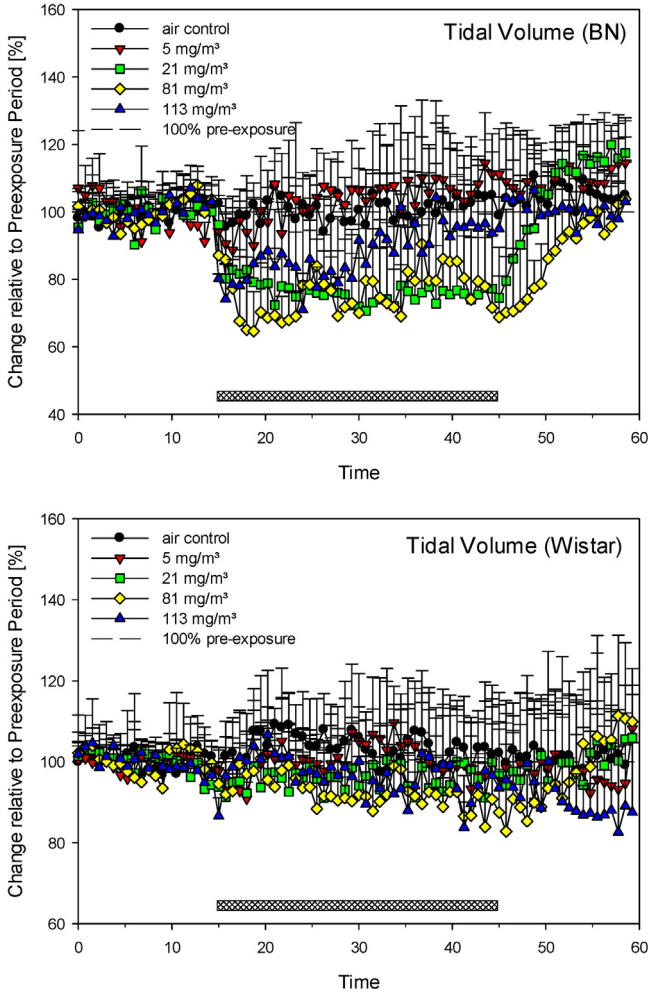


Fig. 5. Analysis of the time- and concentration-dependence of respiratory tidal volumes in Brown Norway (top) and Wistar rats (bottom). Further details are given in Fig. 3.

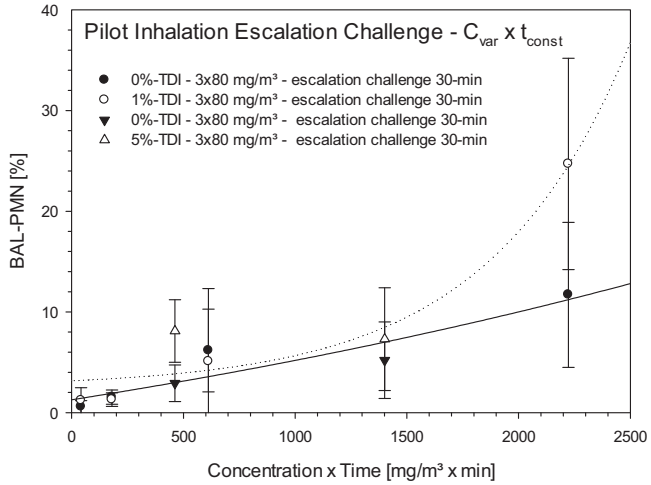


Fig. 6. Ancillary pre-study using a variable concentration × fixed exposure duration protocol ($C_{var} \times t_{const}$) for the elicitation of TDI-induced lung sensitization approximately 9-weeks after the first topical induction (100 μL of either 1% or 5% TDI in AOO on days 0 and 7). These animals were primed by three prior inhalation exposures of approximately 80–85 mg TDI/m³ for 30 min. The escalation challenge utilized concentrations of 1.4, 6, 15′, 20, 47′, and 74 mg TDI/m³ for 30 min (apostrophes denote groups sensitized with 5% TDI). PMNs in bronchoalveolar lavage 1-day post-challenge. Data are presented as means ± SD (8 animals/subgroup).

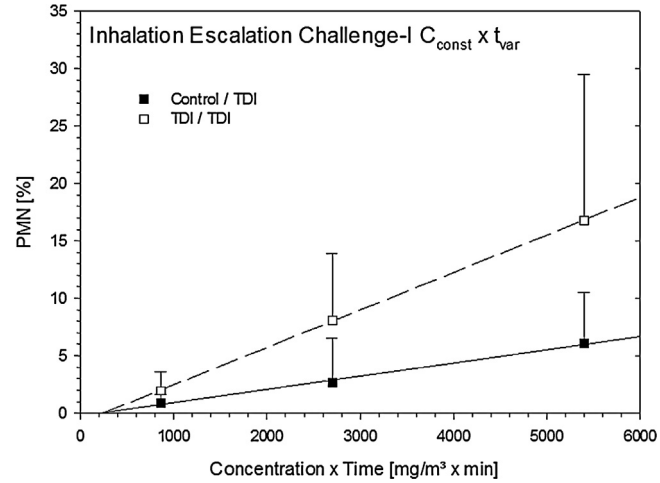


Fig. 7. Fixed concentration × variable exposure duration protocol ($C_{const} \times t_{var}$) used for the elicitation of TDI-induced lung sensitization 3-weeks after the first topical induction (escalation challenge I; for details see Table 1). These animals were not primed by prior inhalation exposures to TDI. PMNs in bronchoalveolar lavage 1-day post-challenge. Data are presented as means ± SD (8 animals/sub-group).

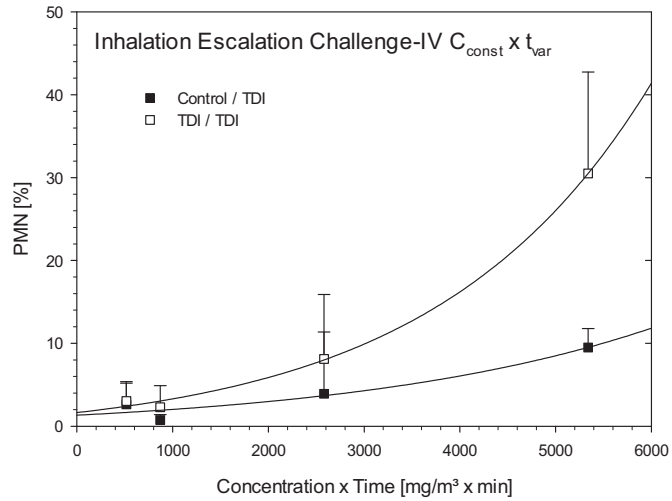


Fig. 8. Fixed concentration × variable exposure duration protocol ($C_{const} \times t_{var}$) used for the elicitation of TDI-induced lung sensitization in rats primed by three prior exposures to 85 mg TDI/m³ for 30 min each (escalation challenge IV; for details see Table 1). PMNs in bronchoalveolar lavage 1-day post-challenge. Data are presented as means ± SD (8 animals/sub-group). The regression models to fit the data were $y_{vehicle/TDI} = -0.56 + 1.90(3.1E{-}4x)$ and $y_{TDI/TDI} = 1.29 + 2.94(4.5E{-}4x)$ for the vehicle/TDI and TDI/TDI groups, respectively.

4. Discussion

Today we are seeing a movement toward focusing less on categorizing asthma as an immune or irritant response, and more on classifying a chemical that can produce such a reaction as a *respiratory sensitizer*. This represents a significant change in thinking, since the term 'respiratory sensitizer' has traditionally been interpreted to imply an underlying immunological mechanism for the production of asthma. Volatile chemicals may be differently deposited and retained within the respiratory tract depending on their concentration and phase. Typically, vapors such as TDI, produce a characteristic anterior–posterior gradient of airway irritation and inflammation and inhalation-challenge exposure must use the optimal gradient of inhaled dose to potentially elicit the critical lower airway response. The difficulties in dose-selection have partially been overcome by utilizing bolus instillation techniques (intratracheal, intranasal or pharyngeal aspiration) to administer

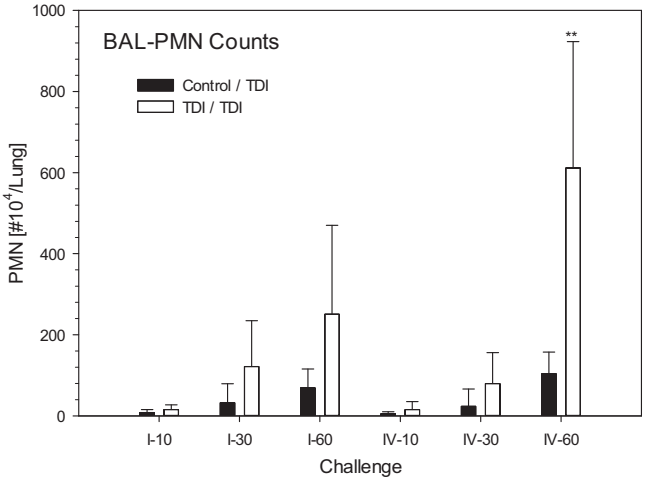
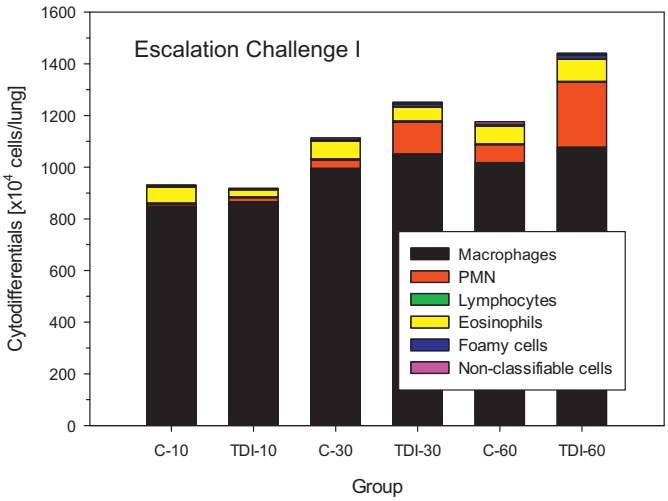


Fig. 9. Absolute counts of PMN in bronchoalveolar lavage using the $C_{const} \times t_{var}$-protocol (escalation challenge I and IV; for details see Table 1). PMNs in bronchoalveolar lavage 1-day post-challenge. Data are presented as means ± SD (8 animals/subgroup). Asterisks denote significant difference to the similarly challenged naïve control group (*P < 0.05, **P < 0.01).



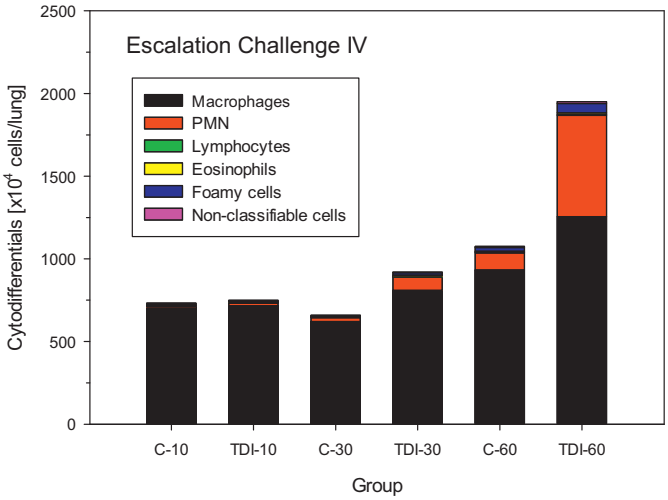
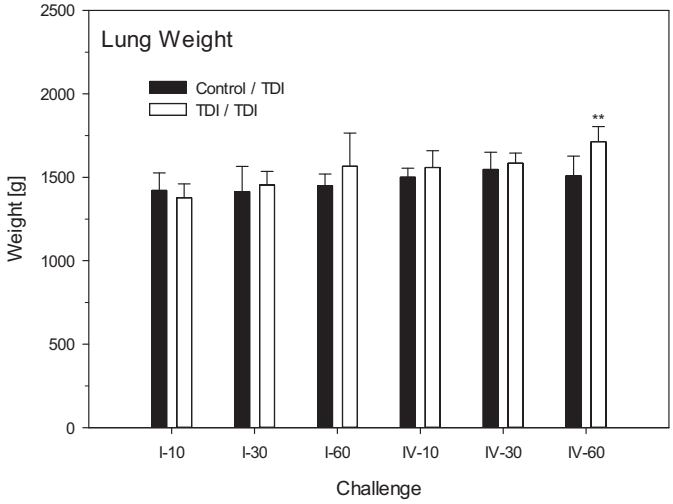


Fig. 10. Absolute cytodifferentiation of bronchoalveolar lavage cells using the $C_{const} \times t_{var}$-protocol (escalation challenge I and IV; for details see Table 1). PMNs in bronchoalveolar lavage 1-day post-challenge.



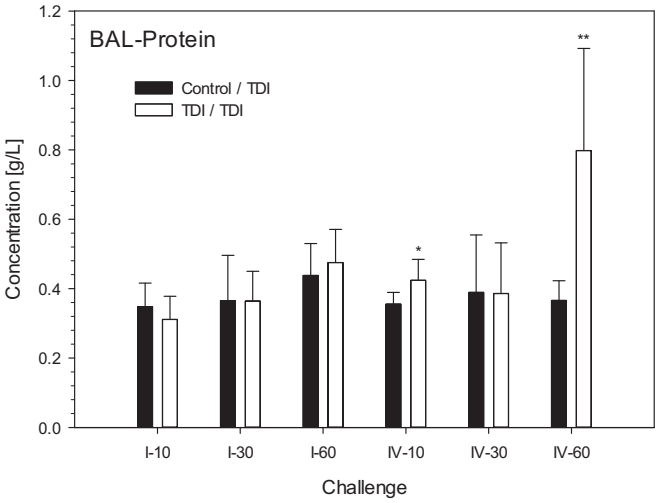


Fig. 11. Lung weights (wet) and protein in bronchoalveolar lavage using the $C_{const} \times t_{var}$-protocol (escalation challenge I and IV; for details see Table 1). Data are presented as means ± SD (8 animals/subgroup). Asterisks denote significant difference to the similarly challenged naïve control group (*P < 0.05, **P < 0.01).

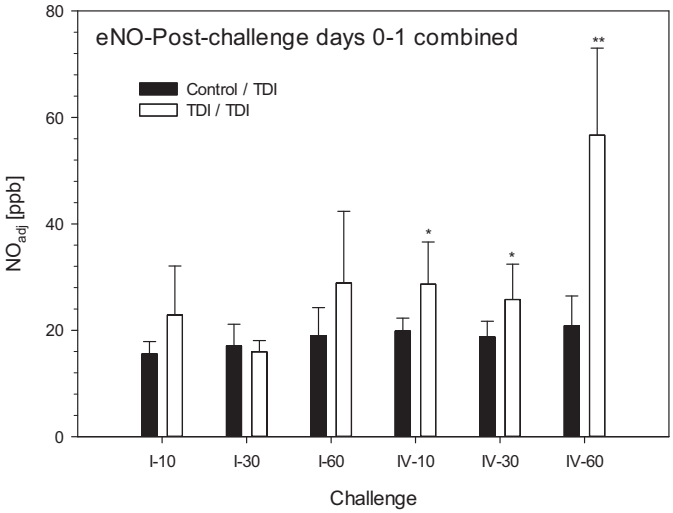


Fig. 12. Exhaled nitric oxide (adjusted) in rats challenged by the $C_{const} \times t_{var}$-protocol (escalation challenge I and IV; for details see Table 1). Measurements were made both on the challenge and first post-challenge days (data combined). Data are presented as means ± SD (4 animals/subgroup). Asterisks denote significant difference to the similarly challenged naïve control group (*P < 0.05, **P < 0.01).

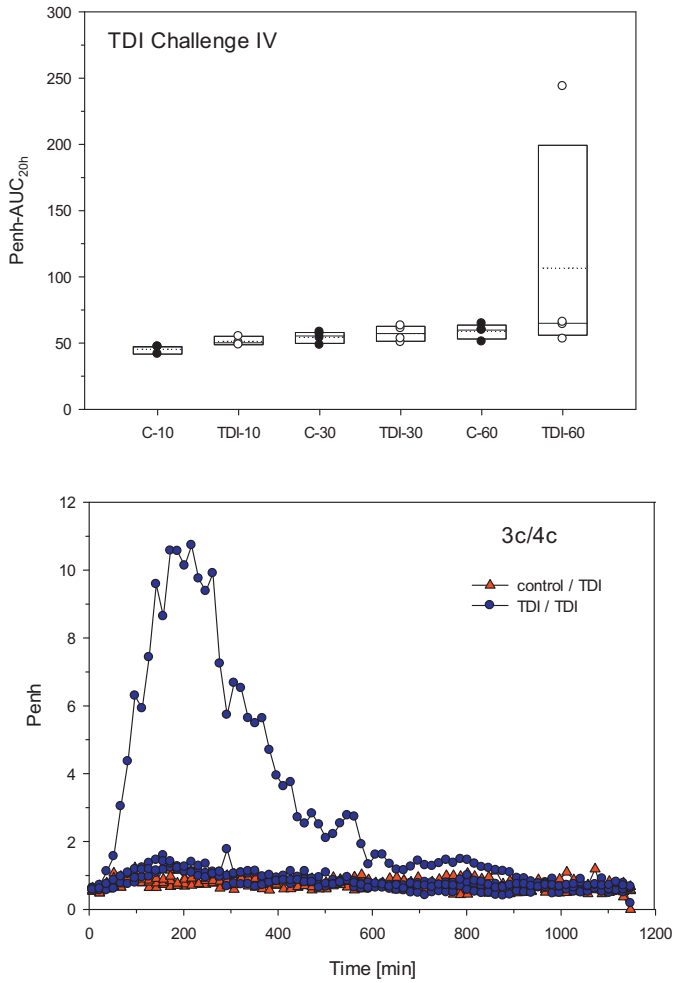


Fig. 13. Top: Measurement of enhanced pause (Penh) in naïve but challenged (C) and skin-sensitized and challenged (TDI) rats in whole body barometric plethysmographs after escalation challenge IV in subgroups 3c and 4c (for details see Table 1). Data represent records from individual rats (4 rats/group) after challenge over a time period of approximately 20 h (Penh-$AUC_{20\,h}$). Boxes represent Tukey Box Plots (dotted lines: mean, solid lines: median). Bottom: Typical delayed-onset respiratory response indicated by a transient increase of Penh.

the chemical (hapten) to the lung (Ban et al., 2006; Vanoirbeek et al., 2004, 2008, 2009a,b). Alternatively, appropriate hapten-homologous protein conjugate have been used to achieve this objective in the absence of irritation (Day et al., 1996; Lange et al., 1999; Herrick et al., 2002; Pauluhn, 1997, 2006; Pauluhn and Mohr, 1998; Pauluhn et al., 2002; Wisnewski et al., 2011). Although there is reason to believe that the effectiveness of protein conjugates vary with the molecular substitution ratio, it can be argued that reactive, multifunctional chemicals that may react in many ways with numerous proteins and epitope structures may further be compounded by cross-linking. Instillation techniques have gained popularity because they are relatively inexpensive and can be used without the technically demanding inhalation technology.

The acquisition and the subsequent elicitation of an allergic reaction of the lung similar to occupational diisocyanate asthma were examined in a Brown Norway rat bioassay. The primary focus of this assay was to duplicate at least some phenotypes typical of diisocyanate-asthma using two cutaneous-exposures to induce and boost systemic sensitization via a non-inhalation route (Fig. 1). Previous studies with liquid MDI using this bioassay demonstrated that repeated inhalation encounters to concentrations of aerosol up to 10,000 mg/m^3 × min resulted in a less pronounced response upon dose-escalation challenge as compared to dermally sensitized rats, despite the putative synergistic effect of respiratory tract sensitization and irritation (Pauluhn and Poole, 2011). Cytokine patterns varied substantially depending on whether BAL-fluid, BAL-cells or LALNs were used for analysis (Pauluhn and Vohr, 2006; Pauluhn, 2008a). While previous dermal sensitization studies in Brown Norway rats with MDI showed increased total serum IgE (Pauluhn, 2005), specific IgE antibodies to diisocyanates in the serum of occupationally exposed asthmatic subjects were not found at all (Scheidler et al., 2013; Vandenplas, 2011). This discordance of any conclusive exposure–response (IgE vs. respiratory symptoms)-relationship seems to be poorly suited as basis for establishing maximum workplace concentrations. From these findings it was deduced not to pursue any further analyses on induction-related endpoints as local irritation and the associated systemic immunological sequelae are difficult to disentangle. Moreover, the residence time of tissue-conjugated diisocyanate and the degree of inflammation-related kinetics of tissue-turn-over may differ from one possible induction-site to another. This may further hamper any robust retrospective analysis of the most critical sites of exposure, including their intensity profile. Based on the hypothesized sequence of events required to initiate and propagate diisocyanate asthma (Fig. 1), pre-disposition precipitated by dermal exposures seems to be difficult to put into any quantitative perspective. Therefore, this protocol focuses solely on prevention of the elicitation response to occur via the respiratory tract in predisposed 'asthmatic' rats.

As demonstrated systematically by the seminal mechanistic work in mice from Vanoirbeek et al. (2007, 2008) and De Vooght et al. (2011, 2013), neutrophilic granulocytes (probed by BAL) play a key role in TDI-induced asthma. Similar observations have been made in humans inflicted with isocyanate-induced occupational asthma (Jatakanon et al., 1999; Lemiere et al., 2002). Park et al. (1999) found that activated PMN may contribute to TDI-induced bronchoconstriction. As explained by Raulf-Heimsoth et al. (2013), the differences (neutrophilic vs. eosinophilic airway inflammation) may depend on multiple methodological factors, the severity of response and the associated kinetics of cell influx into the fluids lining the airways. In this animal model it was shown that BAL-PMN was the most salient endpoint characterizing both MDI-aerosol induced asthma (Pauluhn, 2008a,b) and TDI-vapor-induced asthma. Systemic sensitization does not necessarily mean that these reactive isocyanates as such gain access to the systemic circulation suggesting putative bioavailability. In this context, systemic sensitization is attained when activated T-lymphocyte effector-/memory cells from the lymph nodes draining the sites of sensitization enter the systemic circulation (Fig. 1). This supposition was experimentally verified by De Vooght et al. (2011).

Dermal sensitization may render the rat potentially hypersusceptible to subsequent repeated inhalation encounters which then prime the respiratory tract to develop asthma, as conceptualized in Fig. 1. Hypothetically, due to the anatomy and physiology of the lung, both the priming response as well as the elicitation response ought to be linked to irritation (inflammation) as a reflection of local tissue injury. However, the airway surface area dose must be sufficiently high to overcome the anterior-posterior gradient of concentration to reach the susceptible lung structures to initiate this location-specific response. The airways are protected by lining fluids conducive to adduct and conjugate nucleophilic peptides and biopolymers. Thus, a threshold dose must be exceeded to allow highly reactive electrophilic isocyanates to gain access to these susceptible structures of the lung. Notably, such threshold of acute-to-chronic irritation was shown to exist for MDI (Pauluhn, 2011).

This sequence of events was modeled by three equally spaced inhalation priming/amplification exposures to mildly alveolar irritant concentrations (C) using exposure durations long enough to deliver a dose ($C \times t$) of TDI-vapor to the distal airways of the lung which are the prerequisites to initiate and amplify the localized asthma-like response. The intensity of the emerging allergic response depends on the $C \times t$-related degree of local injury embedded in a complex network of nociceptive neurogenic and signal-transduction factors that will further modulate the release of pro-inflammatory neuropeptides, cytokines, and chemokines. This complex interrelationship makes it difficult to unequivocally identify the key mechanisms involved in the initiation and progression of diisocyanate-asthma. Keeping this complexity in mind, an animal model was developed to define the threshold $C \times t$-relationship upon elicitation in dermally sensitized rats and subsequent inhalation encounters to make these rats 'asthmatic'. While the resultant bronchial hypersensitivity from inhaled isocyanates is dependent on numerous factors, the inhaled $C \times t$-provocation escalation dose was shown to be amongst the most important experimental variables both in rats (this study) and humans (Vandenplas et al., 1993).

Most irritation-related data stem from toxicological studies on Wistar rather than the IgE-mediated Brown Norway rat. Therefore, the sensitization studies described in this paper were preceded by ancillary sensory irritation studies on both strains. The data summarized in Fig. 3 demonstrate typical reflexively-induced changes in respiration almost instant in occurrence, clearly concentration-dependent, and rapidly reversible. This type of nociceptive reflex depresses ventilation by 60–70% (Figs. 3 and 4) with tremendous impact on the inhaled dose (which is the physiological purpose of this reflex). The time-course of attaining the maximal response was not appreciably different between these two strains. However, Brown Norway rats displayed a minimally more pronounced depression of ventilation with lesser fluctuations than Wistar rats. Upper respiratory tract trigeminal reflexes seem to be superimposed by the C-fiber-related alveolar Paintal reflex at concentrations exceeding 81 mg/m^3. This trend is taken as indirect evidence that the lower airways of the tract were adequately exposed to TDI. Thus, the objective of study, which is to dose the distal airways of the lung with TDI-vapor at concentrations high enough to overcome the scrubbing capacity of the airways of the upper respiratory tract of the obligate nasal breathing rat, has been achieved.

Although thoroughly rationalized, this high concentration may call into question the human relevance of the course taken already at the outset of study. From the data compiled in Fig. 4, the concentration causing a "Respiratory Decrease fifty percent" (RD_{50}) can be estimated to be in the range of 20 mg TDI/m^3 without appreciable strain differences. The respective human irritant threshold concentration and RD_{50} are interrelated (Alarie, 1981a,b). Commonly $RD_{50} \times 0.03$ (in mice), which is 0.6 mg/m^3 (0.09 ppmV), is equal to the non-irritant threshold concentration in humans. Human volunteers exposed to TDI-vapor at concentrations of 0.08 ppmV and above for 30-min developed symptoms of irritation. Smell and subjective irritation of the eyes and nose were reported to occur at 0.05 ppmV (Henschler et al., 1962). A concentration of 0.01 ppmV led in one of 15 asthmatic persons to an asthma attack, while in healthy persons even 0.02 ppmV did not have effects on the airways (Baur et al., 1994). Exposure of healthy or asthmatic persons not occupationally exposed to isocyanates TDI a concentration of 0.02 ppmV for 1–2 h (average taken for the calculations below 90 min) did not cause bronchial obstruction in healthy subjects whereas in 5/15 asthmatic subjects an increase in the airway resistance occurred (Fruhmann et al., 1987).

By comparison, the average $C \times t$-dose of 85 mg/m^3 × 30 min (=2550 mg/m^3 × min) used to challenge Brown Norway rats can be translated to the human exposure scenario detailed above by applying the following adjustments for differences in the averaged exposure duration {2550 mg/m^3 × min adjusted the human challenge duration of 90 min equals 28 mg/m^3 or 4 ppmV for the 90 min challenge duration of asthmatic workers} and 10-old differences in intra-human susceptibility {4 ppmV × 1/10 = 0.4 ppmV}. To adjust from obligate nasal breathers to oronasally breathing humans an additional dosimetric adjustment for factor of 3 is called for based on the findings from Schroeter et al. (2013). These authors estimated that the airway wall flux of inhaled hexamethylene diisocyanate vapor in rats is about 1/3 of the respective human lung airways. Based on the findings presented in Figs. 3 and 4, it is conceived that the inhaled dose of rats exposed to TDI-vapor at concentrations penetrating the lower respiratory tract is 1/3 of normally breathing rats {0.4 ppmV × 1/3 × 1/3 = 0.04 ppmV}. From these terms the human equivalent concentration (HEC) can be calculated as follows: HEC = 4 ppmV × 1/(10 × 3 × 3) which is 0.04 ppmV × 90 min. Thus, the average $C \times t$-dose of 85 mg/m^3 × 30 min used to challenge Brown Norway rats compares favorably with the calculated challenge duration-adjusted HEC of 0.04 ppmV × 90 min and the actually used 0.02 ppmV × 90 min that resulted in 33% responsiveness in workers showing TDI-induced asthma.

The dose-escalation challenge at 85 mg/m^3 utilized exposure-durations from 6 to 60 min which translates to 2550 mg/m^3 × min × 1/7 × 1/100 = 0.7 ppmV × min to 7 ppmV × min. Interestingly, this range is somewhat similar to the more recent recommendations as to how work-related diisocyanate asthma should be revealed in specific human bronchoprovocation tests (Raulf-Heimsoth et al., 2013). These authors describe a stepped 4 × 30 min cumulative challenge protocol per day (with breaks in between) ranging from 0.15 to 0.9 ppmV × min resulting a cumulative challenge $C \times t$ of 2 ppmV × min. Thus, when applying the established principles of translational inhalation dosimetry, the challenge $C \times t$ used in Brown Norway rats seems to be aptly placed within the respective dose–response curve of humans inflicted with occupational diisocyanate asthma. This similarity gives definite credence to the factors chosen to adjust target organ dose, differences in sensitivity, and $C \times t$ adjustments. The indispensable adjustment factor for upper respiratory tract sensory irritation-related depression in ventilation can be abandoned for pulmonary irritants that do not stimulate sensory nerve endings in the upper airways to any appreciable extent as exemplified for MDI-aerosol (Pauluhn, 2008a,b).

In full concordance with the mechanistic studies of TDI in BALB/c mice (dermal sensitization and bronchoprovocation challenge via intranasal instillation; for details see publications from Vanoirbeek and coworkers) also Brown Norway rats elaborated marked elevations of neutrophilic granulocytes in BAL. Opposite to previous studies with MDI-aerosol, repeated challenges with TDI-vapor did not clearly amplify the physiological response with increasing number of challenges. Similar observations were reported from studies on BALB/c mice (Vanoirbeek et al., 2004, 2009a,b). The $C_{const} \times t_{var}$ – escalation challenge protocol was executed with two modifications (Fig. 2), either as single escalation provocation challenge (Fig. 7) or as escalation provocation challenge forgone by three inhalation priming exposures (Fig. 8). Notably, the elicitation-thresholds of ≈1000 mg/m^3 × min were essentially identical after single inhalation challenge (lung not yet primed by prior inhalation challenges) and repeated inhalation challenges of putatively 'asthmatic rats'. At the maximum escalation challenge $C \times t$ of 2550 mg/m^3 × min BAL-PMN% were 17% and 30%, without and with inhalation priming exposure, respectively. This outcome is concordant with the threshold hypothesis articulated above, namely that the 'buffering capacity' of airway fluids must be exceeded by high $C \times t$ dose-equivalents to cause deleterious, progressive events within the airways.

Due to the similarity of findings from this dose–response study in Brown Norway rats with the findings from the Vanoirbek-studies on BALB/c mice, a dosimetric comparison of both approaches was attempted. The inhaled dose of TDI at the provocation level 85 mg/m^3 at 30 min is equal ≈0.8 mg/kg-rat {minute ventilation: 1 L/(kg-rat × min) × 30/100 drop in ventilation × 30 min × 85 μg TDI/L ≈0.8 mg TDI/kg-rat}. The ventilation of rats measured in air-exposed rats (see legend of Fig. 3) was similar to that reported by Mauderly (1986). Interestingly, almost the same dose was administered by Vanoirbeek et al. (2004) using a mouse model and intranasal challenge {10 μL of 0.1% TDI in the vehicle AOO into each nostril of a 20 g mouse ≈1 mg TDI/kg-mouse). However, as already indicated above, the regionally retained dose within the airways of the lung is likely to be different following intranasal aspiration of an instilled bolus of TDI dissolved in AOO as compared to vapor inhalation. Most liquid bolus instillation protocols have in common that dosing is preferentially more toward the alveolar region rather than the bronchial airways (Pauluhn and Mohr, 2005). The particular advantage of inhalation challenge exposure is that interferences by re-challenge exposures with TDI-vapor dissolved in the same vehicle can be prevented. Despite differences in species, procedures, and dosimetry, the outcome and conclusions from both bioassays seem to converge remarkably well.

From a hazard assessment perspective, a high-dose systemic sensitization via the skin followed by single and repeated inhalation provocation exposures to a well-defined supra-threshold irritation inhalation priming dose appears to deliver the most conservative experimental condition for this type of animal bioassay. When observing the assessment factors rationalized above, the operational elicitation NOAEL of 1000 mg TDI/m^3 × min can be converted to an 8-h workday HEC (OEL). This time-adjusted $NOAEL_{adj}$ is estimated to be 0.02 mg/m^3-day or 0.003 ppmV {1000 mg/m^3 × min × 1/480 min × 1/(10 × 3 × 3); conversion from mass to volume: × 1/7; see above for the justification of assessment factors}. Interestingly, this estimate happens to be essentially identical with the current TLV-TWA of 0.005 ppmV of TDI (TLV, 2011), and DFG-MAK of 0.01 ppmV (DFG, 2000) for non-sensitized workers.

In summary, the conceptual design of this bioassay is to determine the $C \times t$-dose that does not cause any asthma-like response in dermally sensitized rats upon repeated inhalation challenge exposures. Under the given circumstances, this Brown Norway rat TDI respiratory allergy bioassay demonstrates the existence of a threshold dose for the elicitation of respiratory sensitization in skin-sensitized rats. It duplicates multiple hallmarks of occupational diisocyanate asthma (Ewald-Kleimeier et al., 2013; Jung and Park, 1999; Jatakanon et al., 1999; Lindén and Adachi, 2002; Lemière et al., 2002; Persson and Uller, 2001), that included increased nitric oxide in exhaled air, neutrophils in bronchoalveolar lavage, and lung function measurements identifying nocturnal changes in lung function. Only 50% of rats were subjected to this measurement at each escalation challenge with the objective to qualify the onset of changes in respiratory function. Apart from one rat of group IV-4c (Fig. 13) conclusive responses delayed in onset TDI could not be identified whereas following MDI-aerosol unequivocal delayed-onset respiratory responses occurred with increasing numbers of successive inhalation exposures (Pauluhn et al., 2005; Pauluhn, 2005). Whether this inconsistency is related to the lower alveolar dose of TDI-vapor relative to MDI-aerosol needs to be elucidated by future research. Overall, occupational exposure may result from both the skin and respiratory tract. However, to acquire diisocyanate asthma, skin-sensitization followed by successive alveolar irritant inhalation encounters seems to be the key for the initiation and propagation of this occupational disease. The protocol devised seems to deliver a NOAEL on the elicitation-response to derive a safe OEL to prevent this vicious cycle to occur.

Conflict of interest

There is no financial interest or any involvement of the sponsor that would have influenced the design, conduct or interpretation of study. This manuscript reflects solely the opinion of the author and not of any other funding source. There are no conflicts of interests.

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