

RESPIRATORY IRRITATION BY TRIMELLITIC ANHYDRIDE IN BROWN NORWAY AND WISTAR RATS

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Several acid anhydrides are known for their sensitizing and irritative properties. Since both irritation and respiratory allergy can cause changes of lung function, proper testing of allergen-dependent effects on the respiratory tract requires knowledge of the respiratory irritant effects. To study the latter effects, groups of female Brown Norway (BN) and Wistar rats were exposed for 30 min to a range of concentrations (10 to 300 mg/m³) of the well-known respiratory allergen trimellitic anhydride (TMA). Breathing pattern and frequency were monitored before, during, and after exposure. Animals were necropsied and lung weights were determined 1 day after exposure. In BN rats, changes in breathing pattern were seen at levels of 29 mg/m³ and higher and decreases in frequency at 60 mg/m³ and higher, whereas in Wistar rats changes in both pattern and frequency (increases followed by decreases) were seen at levels of 34 mg/m³ and higher. Changes in breathing pattern consisted of a spiked form instead of a wave form of the respiratory cycle, with a pause between breaths at the end of expiration. The length of the pause increased with increasing concentrations of TMA while the duration of the respiratory cycle decreased slightly, implying that breathing frequency was mainly determined by the magnitude of the increase in pause. These reversible changes in breathing pattern and frequency were considered to be suggestive of lower airway irritation, rather than upper airway irritation. No concentration-related changes in lung weights were observed. The highest level at which no acute airway irritation as based on both breathing pattern and frequency was observed in both rat strains was 14 mg/m³.

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Acid anhydrides are low-molecular-weight (LMW) chemicals that have been used in industry for more than 50 years as curing agents in the production of epoxy and alkyd resins and in the manufacture of the plasticizer dioctyl phthalate. Epoxy and alkyd resins have widespread applications in paints, plastics, and adhesives. Other applications are production of dyes, pesticides, pharmaceuticals, isolators, and thermostable polyvinyl chloride (PVC). The functional RCOOCOR' group endows acid anhydrides with the reactivity needed for these applications, but this reactivity is most probably also responsible for the irritative and sensitizing properties of these LMW chemicals (Bernstein et al., 1984; Topping et al., 1986; Durham et al., 1987; Nielsen et al., 1988; Grammer et al., 1992; Zeiss et al., 1992; Liss et al., 1993; Drexler et al., 1994; Yokota et al., 1997; Barker et al., 1998). Trimellitic anhydride (TMA) is a model compound among the acid anhydrides. This compound, besides inducing immunoglobulin E (IgE)-mediated asthma and rhinitis, is known to cause two other immune-mediated syndromes and a nonimmune syndrome. The latter syndrome is clinically characterized by airflow obstruction as a result of toxic and irritative effects on the airway epithelium and is designated as reactive airways dysfunction syndrome (RADS; Zeiss & Patterson, 1993; Bernstein et al., 1993; Brooks & Bernstein, 1993). Since airflow obstruction is a key symptom of IgE-mediated asthma and since irritant concentrations of chemical allergens can cause such functional airway reactions in unsensitized animals (Karol, 1991; Briatico-Vangosa et al., 1994; Pauluhn & Mohr, 1994), a lung function parameter able to distinguish toxic and immune-mediated effects is desired for the assessment and control of health risks.

Since sensitized rats of the high IgE-responding Brown Norway (BN) and low IgE-responding Wistar strain have shown different allergic airway reactions upon challenge with TMA (Arts et al., 1998), the purpose of the present study was to evaluate the acute airway irritating effects of TMA in naive rats of these two strains.

METHODS

Animals and Maintenance

Female, 7- to 8-wk-old, inbred Brown Norway (BN/SsNHsd) rats were purchased from Harlan UK Ltd. (Blackthorn, UK), and random-bred Wistar rats (CrI:[WI]WUBR) of the same age were purchased from Charles River Deutschland (Sulzfeld, Germany). The animals were acclimatized for at least 5 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 Commission of Animal Welfare.

Materials

Trimellitic anhydride (TMA; purity 97%) was obtained from Aldrich (Brussels, Belgium). TMA solutions were prepared shortly before use by dissolving it in pesticide-grade acetone (Merck, Darmstadt, Germany).

Atmosphere Generation and Analysis

An all-glass nebulizer designed at the institute was used to generate the test atmospheres from the solutions of TMA in acetone (Schaper & Brost, 1991) or acetone alone. The acetone concentration in air was kept between 3000 and 5000 ppm (~7–12 g/m³), levels that are considered to be far below a level inducing sensory irritation (Alarie, 1973; De Ceaurriz et al., 1981; Schaper & Brost, 1991). Atmospheric concentrations of TMA were determined gravimetrically by filter sampling and those of acetone by calculations based on the nominal concentration and the complete evaporation. The particle size distribution of TMA in the test atmospheres was determined using a 10-stage Andersen cascade impactor (Andersen, Atlanta, GA). Due to the low sampling airflow rate (2–5 L/min) and the large total volume required for analytical and particle size determinations (up to 500 L/sample), samples were not collected during exposure but prior to or after exposure. The mass median aerodynamic diameter (MMAD) of the TMA aerosol was between 0.5 and 2.2 μm (Table 1).

Study Conduct

Animals were weighed shortly before exposure and randomly assigned to a group ($n = 4$) receiving a given concentration of TMA. The rats were individually restrained in Battelle tubes, and each tube was then placed into one of four plethysmographs that were connected to a central exposure chamber for nose-only exposure to the test atmosphere. Each plethysmograph was provided with a pressure transducer that sensed changes created by inspiration and expiration and transmitted amplified signals to a polygraph recorder, allowing determination of respiratory frequency and pattern during challenge. Using this experimental setup, 4 rats at a time were subsequently exposed to fresh air for 30 min (preexposure period), to the appropriate TMA concentration or vehicle atmosphere for 30 min (exposure period), and again to fresh air for 30 min (recovery period). Respiration was monitored for 20-s periods in the preexposure period (at 1-min intervals

TABLE 1. Particle size distribution in TMA aerosols

Concentration (mg/m ³)	MMAD (μm)	GSD
10	0.50	2.0
30	0.90 1.00	2.8 2.6
50	0.90	1.9
100	2.00 1.70	1.9 2.0
200	2.00 2.20	1.9 1.7

Note. MMAD, mass median aerodynamic diameter; GSD, geometric standard deviation, (84%/16%)^{1/2}.

starting 6 min prior to the TMA exposure), during the exposure period (at 1-min intervals during the first 5 min and then at 2-min intervals), and during the recovery period (at 1-min intervals during the first 10 min and then at 3-min intervals). Respiratory frequency was expressed as the number of breaths per second.

At necropsy, the day after exposure, the animals were weighed, anesthetized, killed by exsanguination from the abdominal aorta, and examined grossly for pathological changes. Lungs with trachea and larynx were removed and absolute and relative (relative to body weight) lung weights were determined.

Data Handling and Statistics

For each animal, the maximum changes in breathing frequency during exposure—that is, at those points where the plateau value was reached in the time–response curve—were used to calculate the mean value. Mean pre- and postexposure (recovery) values were obtained by averaging the data obtained. Thus means of frequencies were calculated from 6 preexposure (PRE), 6–17 exposure (EXP), and 13 recovery (REC) values. To reduce variability, each animal was used as its own control. Hence, concentration effects were evaluated by a repeated-measures two-way analysis of variance (ANOVA). Significant effects were subsequently evaluated post hoc by one-way analysis of variance (ANOVA) of the relative changes, that is, $(PRE - EXP)/(PRE)$ and $(PRE - REC)/(PRE)$, followed by the Dunnett test to evaluate individual contrasts. The RD50 value, the statistically derived concentration at which animals are breathing at 50% of their original frequency, was assessed according to Alarie (1973). In addition, the RD50 value was calculated according to a method described by Bos et al. (1992), based on the reaction dynamics at the receptor sites and assuming that the maximal response is 100%, using the equation:

$$\log RD50 = \log C + \log [(100 - R)/R]$$

where C is the concentration (mg/m^3) and R the response (%). Lung weights were analyzed by one-way ANOVA followed by the Dunnett test.

RESULTS

Respiratory Changes

Control BN rats exposed to the vehicle (acetone) did not show changes in breathing frequency when compared to exposure to normal air. BN rats exposed to 14 or 29 mg/m^3 of TMA showed normal breathing frequency as well. At 60 mg/m^3 , 2 out of 4 animals had a decrease in breathing frequency of 24 and 29%, respectively, whereas 1 other rat breathed at a slightly increased rate. The mean breathing frequency of the group, however, was not different from that of the controls. At TMA levels of 100 mg/m^3

and higher, significant reductions in breathing frequency were observed in all animals (Figure 1). The changes in frequency started rapidly upon exposure to TMA, and plateau values were reached within 30 min of exposure.

Changes in breathing pattern of the BN rats were observed at TMA concentrations of 29 mg/m³ and higher. They were characterized by (1) a slight decrease in the duration of inspiration and expiration and (2) a spiked instead of a wave form of the respiratory cycle with, at the end of expiration (at peak expiratory pressure), a pause between breaths (Figure 2a). The length of these pauses increased with increasing exposure concentrations of TMA. Like the changes in frequency, changes in breathing pattern started rapidly upon exposure to TMA.

Breathing frequency and/or pattern almost completely returned to normal immediately after exposure at levels of 100 mg/m³ and below, whereas at levels of 100 mg/m³ and higher the whole recovery period was required to attain normal breathing, resulting in a significantly reduced frequency relative to preexposure values at these concentration levels (data not shown).

The RD50 value (Alarie, 1973) for BN rats was 260 mg/m³, with 95% confidence limits of 185 and 441 mg/m³ and a correlation coefficient of 0.905. Calculation of the RD50 value according to Bos et al. (1992) resulted in a slightly higher mean RD50 value (\pm SD) of 312 (\pm 109) mg/m³.

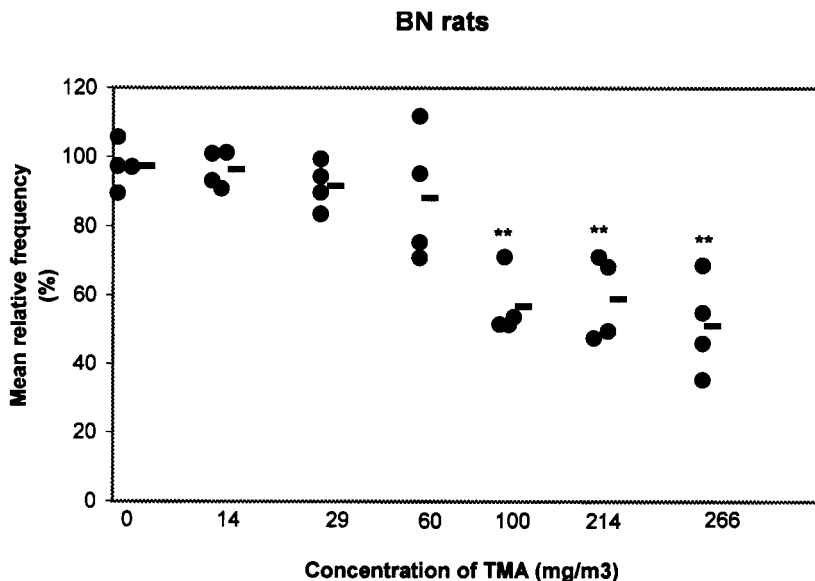


FIGURE 1. Mean relative changes in breathing frequency in groups of four BN rats during inhalation exposure to either vehicle or one of various concentrations of TMA. Dots indicate individual data expressed relative to preexposure frequency (set at 100%); horizontal bars indicate group means. Statistics: repeated-measures two-way ANOVA followed by one-way ANOVA/Dunnett on (PRE – EXP)/(PRE) values between vehicle and test groups; double asterisk indicates significance at $p < .01$.

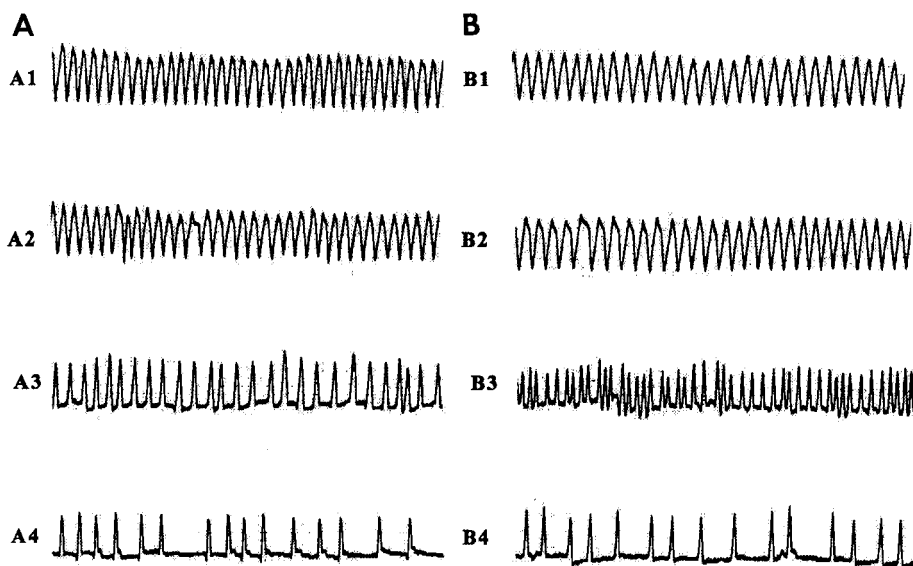


FIGURE 2. Representative breathing patterns of (A) BN and (B) Wistar rats before and during a 30-min inhalation exposure to vehicle (acetone) or TMA: (A1) preexposure, (A2) acetone exposure, (A3) 29 mg/m³ TMA, and (A4) 214 mg/m³ TMA; (B1) preexposure, (B2) acetone exposure, (B3) 34 mg/m³ TMA, and (B4) 215 mg/m³ TMA.

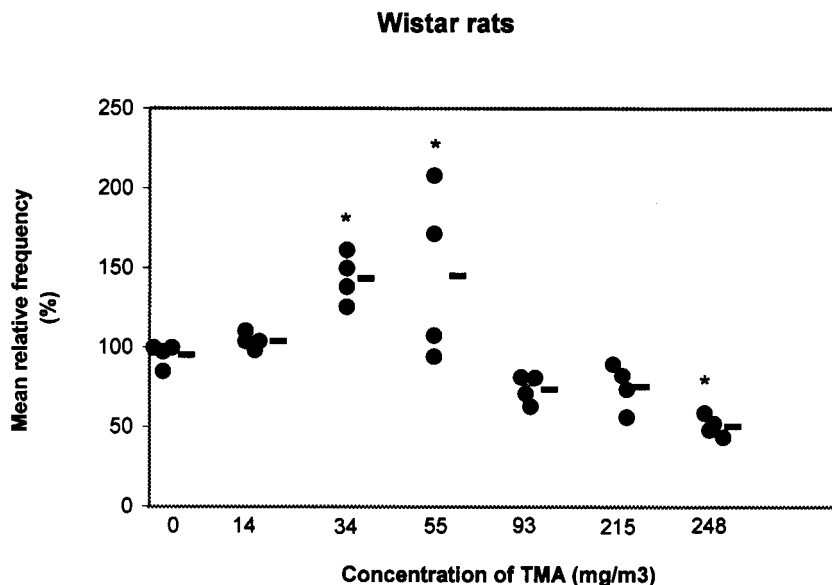


FIGURE 3. Mean relative changes in breathing frequency in groups of four Wistar rats during inhalation exposure to either vehicle or one of various concentrations of TMA. See further description in Figure 1. Statistics: repeated-measures two-way ANOVA followed by one-way ANOVA/Dunnett on (PRE - EXP)/(PRE) values between vehicle and test groups; asterisk indicates significance at $p < .05$.

Like BN rats, Wistar rats showed no changes of breathing frequencies during exposure to acetone or 14 mg/m³ TMA. Exposure to 34 or 55 mg/m³, however, resulted in a significant increase in breathing frequency. In contrast, exposure to 93 mg/m³ and higher reduced breathing frequency in all groups, although statistical significance was reached at the highest level only (Figure 3).

The breathing pattern of Wistar rats exposed to 14 mg/m³ was considered to be normal, whereas the changes in breathing pattern at levels of 34 mg/m³ and higher were comparable to those in BN rats (Figure 2b), that is, a spiked form with a slightly decreased inspiration and expiration time and a concentration-dependent increased pause between breaths.

Similarly, the changes in breathing pattern and frequency started rapidly upon exposure to TMA, and reached plateaus within 30 min of exposure. Mean postexposure breathing frequency was still higher at levels of 34 and 55 mg/m³. Breathing frequency and pattern almost completely returned to normal shortly after exposure to 93 mg/m³, whereas this required the full 30-min recovery period at levels of 215 and 248 mg/m³ (data not shown).

Since TMA caused no linear changes in the breathing frequency of Wistar rats, RD50 values could not be calculated. However, breathing frequency was reduced to about 50% at a concentration level of 248 mg/m³, which was comparable to the concentration resulting in a 50% decrease in BN rats.

Gross Pathology and Lung Weight

Gross observation of the lungs of BN rats, controls included, showed the presence of small hyaline areas and a few petechiae. TMA exposure did not increase the incidence and severity of these lesions. No macroscopic lung changes were observed in Wistar rats. No concentration-related changes in absolute and relative lung weights of BN and Wistar rats were observed (data not shown).

DISCUSSION

This study aimed to characterize the acute airway-irritating effects of the respiratory allergen TMA in naive rats in order to study whether respiratory irritant effects of TMA could be distinguished from immune-mediated reactions. A further question was whether BN and Wistar rats would display different respiratory irritant reactions to TMA, because the two strains showed quite different respiratory responses when challenged after sensitization (Arts et al., 1998).

Both in naive BN and Wistar rats, TMA aerosols induced reversible changes in breathing pattern and frequency. The wave-form breathing pattern became spiked, respiratory-cycle timing decreased slightly, and the pause between breaths lengthened. Since the increase in pause was concentration-dependent, the breathing frequency was mainly deter-

mined by the magnitude of the increase in pause (Schaper & Brost, 1991). Such changes are typical of pulmonary irritation, that is, stimulation of C-fiber vagal nerve endings that are located in the bronchial epithelium and alveolar walls (Alarie, 1981). Many airborne chemical irritants have been shown to elicit this type of stimulation, which typically results in a concentration-dependent lengthening of the pause after expiration and consequently a decreased breathing frequency. The finding of this typical pattern in naive rats exposed only once to a particular concentration of TMA indicates that TMA causes pulmonary irritation in two different rat strains at roughly the same concentrations. It also confirms that TMA aerosol particles with an MMAD between 0.5 and 2.2 μm , as obtained in this study, were small enough to reach the bronchi and/or alveoli. Further, although deposition of TMA aerosol particles in the nose and throat also must have occurred, signs of sensory irritation, namely, lengthening of the expiration time as a consequence of stimulation of the local trigeminal nerve endings (Alarie, 1973), were absent. Actually, the TMA-induced respiratory changes observed in this study were the same as seen in mice in which irritation of the higher airways was prevented by exposure to TMA via a trachea cannule (Schaper & Brost, 1991).

In addition to the absence of typical signs of sensory irritation, signs of allergic respiratory reactions were absent. The latter are characterized by a normal wave-form breathing pattern with irregularly lengthened pauses (apneas) between a varying number of breaths, also resulting in a decreased breathing frequency (Arts et al., 1998). While the absence of allergic respiratory reactions was expected because of the use of naive animals in this study, data on breathing pattern indicate that TMA-induced irritant effects were clearly distinguishable from TMA-induced allergic responses. If this is also true for other respiratory allergens, it may provide additional information for the distinction between irritant-induced reactions and IgE-mediated allergic reactions.

Although TMA caused respiratory changes characteristic of pulmonary irritation, it lacks many properties of classical pulmonary irritants. Notably, TMA effects on breathing frequency were prompt and recovery was fast, whereas classical pulmonary irritants gradually decrease the frequency to a nadir several hours later, whereafter recovery is generally poor (Weyel et al., 1982; Weyel & Schaffer, 1985; Ferguson et al., 1986). Moreover, irritating concentrations of TMA did not affect lung weight the day after exposure, while classical irritants increase lung weights 18–24 h after exposure by inducing edema (Weyel et al., 1982; Weyel & Schaffer, 1985; Ferguson et al., 1986). The presence of small hyaline areas and a few petechiae as observed macroscopically on the lungs of BN rats was not considered to be treatment related. Inflammatory abnormalities in lungs of healthy BN rats have been earlier observed by us and other groups, and attempts to identify an infectious agent have failed (Germann et al., 1998).

Although both BN and Wistar rats decreased breathing frequency in response to TMA concentrations of about 100 mg/m^3 and higher, Wistar, but not BN, rats increased breathing frequency upon exposure to TMA concentrations between 30 and 60 mg/m^3 . Such a biphasic irritant response was observed earlier in Swiss Webster mice at slightly different concentrations (Schaper & Brost, 1991). The observed increase in frequency was caused by a decreased respiratory cycle time and shortened pauses between breaths. Like the decrease in frequency, it is characteristic of pulmonary irritation. Although these data seem to indicate that Wistar rats are more susceptible to the irritant effects of TMA than BN rats are, changes in breathing pattern were seen at roughly the same concentrations.

In summary, TMA induced reversible airway reactions that were indicative of pulmonary irritation and consisted of clear changes in both breathing frequency and pattern when compared to effects of control (vehicle) exposure. The TMA-induced changes in breathing frequency were seen at higher levels than the levels that were needed to provoke respiratory reactions in sensitized animals in our previous studies. Furthermore, the changes in breathing patterns caused by irritant concentrations of TMA were clearly distinguishable from those elicited by the allergic reaction (Arts et al., 1998).

With respect to the current threshold limit value (TLV ceiling) of 0.04 mg/m^3 (ACGIH, 1999), the level at which no acute airway irritation was observed in rats in the present study, 14 mg/m^3 , suggests a reasonable margin of safety in sensitized humans. However, this may be different in sensitized subjects, since at concentrations as low as 0.04 mg/m^3 pulmonary hemorrhages have been observed in sensitized rats (Leach et al., 1987).

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