ORGAN TOXICITY AND MECHANISMS

Acute airway irritation of methyl formate in mice

Søren T. Larsen · Gunnar D. Nielsen

Abstract Methyl formate (MF) is a volatile solvent with several industrial applications. The acute airway effects of MF were evaluated in a mouse bioassay, allowing the assessment of sensory irritation of the upper airways, airflow limitation of the conducting airways and deep lung (pulmonary) irritation. MF was studied at vapour concentrations of 202–1,168 ppm. Sensory irritation was the only effect observed, which developed slowly over the 30-min exposure period. The potency at steady state was at least 10-fold higher than expected from a hypothetically similar, but non-reactive compound. Methyl formate may be hydrolysed in vivo to formic acid, a potent sensory irritant, and methanol, a low-potent sensory irritant. Hydrolysis may be catalysed by carboxyesterases, and therefore, the role of the esterases was studied using the esterase inhibitor tri-ortho-cresyl phosphate (TOCP). TOCP pre-treatment reduced the irritation response of MF, suggesting that carboxyesterase-mediated hydrolysis plays a role in the irritative effect. However, even after administration of TOCP, MF was considerably more irritating than expected from a quantitative structure–activity relationship (QSAR) model. The slope of the concentration–effect relationship for formic acid was lower than that for the MF in the low-dose range, suggesting that different receptor activation mechanisms may occur, which may include an effect of MF itself, in addition to an effect of formic acid and potentially an effect from formaldehyde.

Keywords Methyl formate · Airway irritation · BALB/c mice · Mechanisms of action

S. T. Larsen (✉) · G. D. Nielsen
National Research Centre for the Working Environment,
Lersø Parkallé 105, 2100 Copenhagen, Denmark
e-mail: stl@nrcwe.dk

Introduction

Methyl formate (MF), the methyl ester of formic acid, is a colourless liquid with a molecular weight of 60.05 (ACGIH 2001). At 25°C, the water solubility is 230 g/l and the vapour pressure is 586 mmHg (http://www.chem.sis.nlm.nih.gov/chemidplus/, accessed 24 June 2011). It is used as a solvent, chemical intermediate, fumigant, and as a larvicide (ACGIH 2001). Under the trademark Ecomate, it is used as a blowing agent for foam insulation, replacing CFC, HCFC or HFC gasses, since MF does not deplete the ozone layer.

MF has a pleasant ethereal odour, but at high concentrations, it may induce airway irritation (ACGIH 2001). MF is taken up through the lungs (Schrenk et al. 1936; Nihlen and Droz 2000) and the skin (Gettler 1940; DFG 2010). The odour threshold of MF is about 600 ppm (Amoore and Hautala 1983). Foundry workers exposed to 36 ppm MF and 44 ppm isopropanol showed no deterioration in neurobehavioural tests (Sethre et al. 2000b). In another study (Sethre et al. 2000a), volunteers were exposed to 100 ppm MF for 8 h and compared to an air-exposed control group; comprehensive neurobehavioural and physiological testings were performed. An increased feeling of fatigue was reported in the MF group. It was also reported that MF could not be smelled. As low level of sensory irritation and smell may be confused, it follows that no overt sensory irritation was apparent at 100 ppm. Sensory irritation has been reported to occur at 3,500 ppm (Ruth 1986), but the validity of the value is not clear as data were collected from various unspecific sources. No other information about sensory irritation in humans has been retrieved. The occupational exposure limit (OEL) in Germany is 50 ppm (8-h time-weighted average; TWA) with a peak limit of 100 ppm due to systemic effects (DFG 2010). In the US,

the OEL (TLV) is 100 ppm with an addition short-term exposure limit (STEL) of 150 ppm (ACGIH 2001).

The nasal mucosa contains several enzymes, including carboxyesterases and cytochrome P450, which may degrade esters through hydrolysis and oxidation, respectively (Nihlen and Droz 2000; Peng et al. 1995). In this way, MF may be metabolised to methanol, a weak sensory irritant (Schaper 1993), and two potent airway irritants, formic acid and formaldehyde cf (Nielsen et al. 1999; Wilkins et al. 2001). The metabolism has been substantiated from increased methanol and formic acid in urine of MF-exposed subjects (Nihlen and Droz 2000; Sethre et al. 2000a, b). Increased airway irritation of organic compounds due to enzymatic degradation of the parent compound has recently been shown for styrene and naphthalene (Lanosa et al. 2010).

Due to the limited data on the sensory irritating effect, we evaluated the airway irritating effects of MF and its mechanisms in a mouse bioassay. The role of enzymatic hydrolysis of MF was studied by pre-treatment with the carboxylesterase inhibitor, tri-ortho-cresyl phosphate (TOCP) prior to MF exposure.

Materials and methods

Animals

Male BALB/c mice were used in the present study, as they have a similar sensitivity as male Swiss-Webster mice (Nielsen et al. 1999), which is the recommended laboratory animal according to the standard method (ASTM 1984). No relevant difference was observed between male and female mice in the sensory irritation response to sulphur dioxide (Alarie et al. 1980), which is also observed in humans (van Thriel et al. 2010) suggesting a similar receptor sensitivity in the two gender. Inbred mice were purchased from Taconic, Denmark. At the initiation of the study, the mean weight and SD of the mice was 25.8 ± 1.3 g. Mice were housed in polypropylene cages ($380 \times 220 \times 150$ mm) with pinewood sawdust bedding (Lignocel S8, Brogaarden, Denmark). The photoperiod was from 6 a.m. to 6 p.m., and the temperature and relative humidity in the animal room were $22 \pm 2°C$ and $50 \pm 5\%$, respectively. Food (Altromin no. 1324, Altromin, Lage, Germany) and tap water were available ad libitum.

Treatment of the animals followed procedures approved by The Animal Experiment Inspectorate, Denmark.

Chemicals

Methyl formate (CAS 107-31-3, purity $\sim 97\%$) was purchased from Merck Schuchardt OHG, Hohenbrunn, Germany, whereas formic acid (CAS 64-18-6, purissima)

was from Fluka Chemicals, Buchs, Switzerland. Tri-ortho-cresyl phosphate (TOCP, CAS 78-30-8, purity >96%) was from Acros Organics, Geel, Belgium.

Generation of test atmospheres

The MF was evaporated, diluted with room air and fed to a 24-l exposure chamber. The airflow rates in the chamber were set between 18.9 and 23.8 l/min. Each nominal concentration was calculated from the amount of evaporated chemical and the total gas-air flow. The chamber concentrations were monitored continuously by infrared spectroscopy (Larsen et al. 2000). The differences between the nominal and the monitored exposure concentrations were in all cases less than 10%. Air concentrations are given in ppm, i.e. ml gas per m^3 gas-air mixture, where 1 ppm $= 2.46$ mg/m^3 at 25°C and one atmosphere pressure. The vapour concentrations studied were from 202 to 1,168 ppm.

Formic acid was studied in a similar manner at concentrations of 55 and 191 ppm. The exposure–effect relationship from these two points complied with the results from a previous comprehensive study (Nielsen et al. 1996) where a single straight line on the log concentration–effect relationship was observed in the range 29–656 ppm in CF-1 mice. Due to compliance with previous results (Fig. 2; taking into account strain and time differences), the two data points from the present study were considered sufficient for the description of the formic acid exposure–effect relationship in BALB/c mice.

Bioassay

Inhalation of chemicals can affect the airways at three levels, the upper respiratory tract, the conducting airways and the alveolar level. These effects can be investigated by the analyses of the breathing patterns in mice (Alarie 1973; Nielsen et al. 1999). Briefly, the breathing pattern analysis consists in recognition and quantification of specific deviations from the normal breathing pattern. Thus, after end of inhalation, a short break occurs before the exhalation is initiated, termed time of break (TB, ms). An increase in TB leads to a decrease in the respiratory frequency (f, breaths/min). TB is a specific marker of sensory irritation, and it increases with increasing exposure levels of sensory irritants due to a stimulation of the trigeminal nerves. Formaldehyde, ammonia and methacrolein are examples of compounds being sensory irritants (Larsen and Nielsen 2000; Nielsen et al. 1999, 2007). Additionally studied parameters were time of inspiration (ms), time of expiration (ms) and expiratory flow rate (ml/s), which was used to evaluate airflow limitations of the conducting airways. Other parameters were tidal volume (VT, ml) and the time of pause (TP, ms), which is the period after end of expiration and until the

beginning of the next inspiration. An elongation of TP is used as a marker of pulmonary irritation.

The Notocord Hem (Notocord System SA, France) data acquisition software was used to collect respiratory parameters. Modules and settings for data acquisition and calculations were as previously described as were the head-out body plethysmographs, pneumotachographs, transducers and transducer signal amplifiers (Larsen et al. 2004).

Groups of 5–10 mice were inserted into plethysmographs that were connected to the exposure chamber and simultaneously exposed at the same concentration. Each exposure was preceded by a period (∼10 min) that allowed the mice to adapt to the plethysmograph to reach a stable level of breathing. Then, a 15-min period was used to establish the baseline values of the respiratory parameters for each mouse. The baseline period preceded a 30-min exposure period, which was followed by a 15-min recovery period. The baseline, exposure and recovery periods are indicated in Fig. 1. Concentration–effect relationship for MF was established from exposure levels of 202, 238, 314, 328, 375, 397, 412, 662, 765 and 1,168 ppm.

Inhibition of upper respiratory tract carboxyesterase activity

Intraperitoneal administration of 125–1,000 mg/kg b.w. tri-ortho-cresyl phosphate (TOCP) has been shown to inhibit the upper respiratory tract carboxyesterase activity in rats more than 21-h post-treatment (Silver et al. 1981; Stott and McKenna 1984). In the study with 125 mg/kg, the vehicle was corn oil. No effect was observed on the respiration, and no toxic effect was observed. In the present study, mice were treated with 50 mg/kg TOCP intraperitoneally 20 h prior to the MF exposure. Before administration, TOCP was dissolved in polyethylene glycol 400, which has previously been used as vehicle for i.p. administration without complications (Larsen and Nielsen 2007). An injection volume of 50 µl was applied. A pilot study showed that 50 mg/kg TOCP was the highest dose that did not depress respiration and did not cause clinical signs of adverse effects as assessed from the body weight gain and appearance of the mice (data not shown). From effects on respiration and clinical signs, it is suggested that our bioavailable dose of TOCP is at least as high as that used in the rat studies.

The concentration–effect relationship for MP with TOCP pre-treatment was established from exposures at 238, 314 and 412 ppm MF.

Estimation of potency of non-reactive sensory irritants

Sensory irritants activate pain sensitive receptors in the trigeminal nerves (e.g. Alarie et al. 1996; Bessac and Jordt 2008; Julius and Basbaum 2001). The receptor activation can be caused either by a physical adsorption to the receptor ("non-reactive irritants") or by a physical absorption and a chemical reaction with the receptor ("reactive irritants"), where the last mentioned mechanism gives rise to the most potent irritants (Alarie et al. 1998). Based on experimental data from a large number of chemicals, quantitative structure–activity relationships (QSARs) were established (Alarie et al. 1996), which allows the prediction of the sensory irritating potency of non-reactive volatile organic chemicals based on their physicochemical parameters. For the non-reactive compounds, the relationship between the saturated vapour pressure (P, mmHg at 25°C) was log RD50 (ppm) $= 0.844 \times$ log P $+ 2.634$ ($r^2 = 0.89$), which predicts the RD50 to be 93,348 ppm if MF is an entirely non-reactive compound. The RD50 can also be obtained from the molecular weight (MW), but this relationship is less accurate. From this relationship log RD50 $= -0.02 \times$ [MW] $+ 5.359$ ($r^2 = 0.61$), the RD50 is predicted to be about 14,388 ppm. Overall, if MF is a non-irritating compound, the RD50 is expected to be in the range >10,000–100,000 ppm, presumably closest to 100,000 ppm.

Data analyses

Time–response relationships for the decrease in respiratory rate and the increase in time of break were plotted as 1-min mean values of the groups. Linear regression analyses were used to establish concentration–effect relationships for the sensory irritating effects by means of MS Excel; the decrease in respiratory rate (RD) as percentage of the pre-exposure baseline respiratory rate was used as endpoint. A regression line was obtained using group mean value of decrease in respiratory rate from the last 10 min of the exposure period where steady state was observed. The regression line was used for estimating the concentration depressing the respiratory rate by 50% (RD50) and the extrapolated threshold (RD0), which is also considered the no-observed-adverse-effect level (NOAEL) of the reflex-mediated response in mice. The 95% confidence intervals (95% CI) for each of the RD levels were used to detect differences between the TOCP-treated and control groups.

To increase precision and accuracy of estimates, we increased the number of mice from four per data point as recommended by ASTM (1984) to 5–10 mice. Thus, our estimated average effects rely on an increased number of animals as well as on established exposure–effect relationships, which increase precision and accuracy beyond that from a NOAEL and a lowest-observed-adverse-effect level (LOAEL) from a single exposure concentration. The established average effects in male mice are used to predict average effects in the occupational population (Alarie et al. 1980). Inter-individual variations in humans are apparent from experimental studies (e.g. Kendal-Reed et al. 2001;
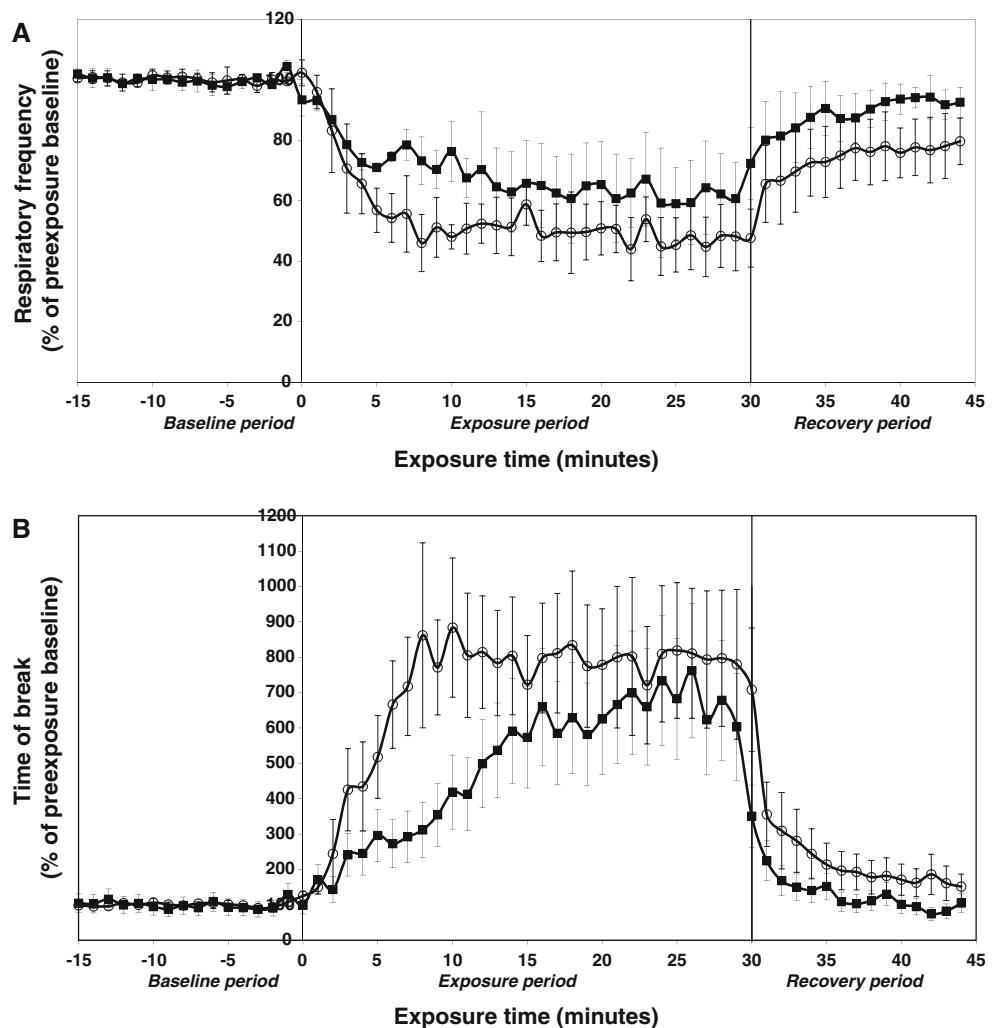
van Thriel et al. 2005). Our extrapolation to the occupational population takes into account the variation in sensitivity in the occupational population. For example, an occupational exposure limit (OEL) based on irritation can be predicted from OEL (irritation) $\approx 0.2 \times$ RD0 (Nielsen 1991; Nielsen et al. 2007), which indicates that extrapolation from the mouse NOAEL to an OEL includes an additional assessment factor of five. An assessment factor is also included in the relationship OEL $\approx 0.03 \times$ RD50 as this relationship forms the basis for the first relationship (Nielsen 1991; Nielsen et al. 2007; Schaper 1993). The assessment factors rely on the regression constituting the relationship between a high number of RD50 values and their OELs (Schaper 1993).

Result

Airway irritation of methyl formate

Exposure to MF caused a slowly progressing decrease in the respiratory rate (Fig. 1a), which occurred simultaneous with

an elongation of the specific marker of sensory irritation, i.e. time of break (Fig. 1b). In contrast to the 412 ppm exposure level, the sensory irritation response at the highest exposure concentration (1,168 ppm) did not fully resolve during the 15-min post-exposure recovery period (Fig. 1). Neither air-flow limitation in the conducting airways nor pulmonary irritation was observed (data not shown). For pure sensory irritants, the risk assessment is commonly performed from the decrease in respiratory rate (Nielsen et al. 2007), which in the present study decreased in a concentration-dependent manner (Fig. 2). However, the concentration–response relationship did not follow a single linear relation over the whole concentration range studied (202–1,168 ppm). In consequence, two linear regression lines were used to describe the data, one covering the 202–397 ppm MF concentration interval and one covering the 412–1,168 ppm interval (cf. Fig. 2). The 202–397 ppm regression line was used to extrapolate the RD0 value, whereas the 412–1,168 ppm regression line was used to interpolate the RD50 value.

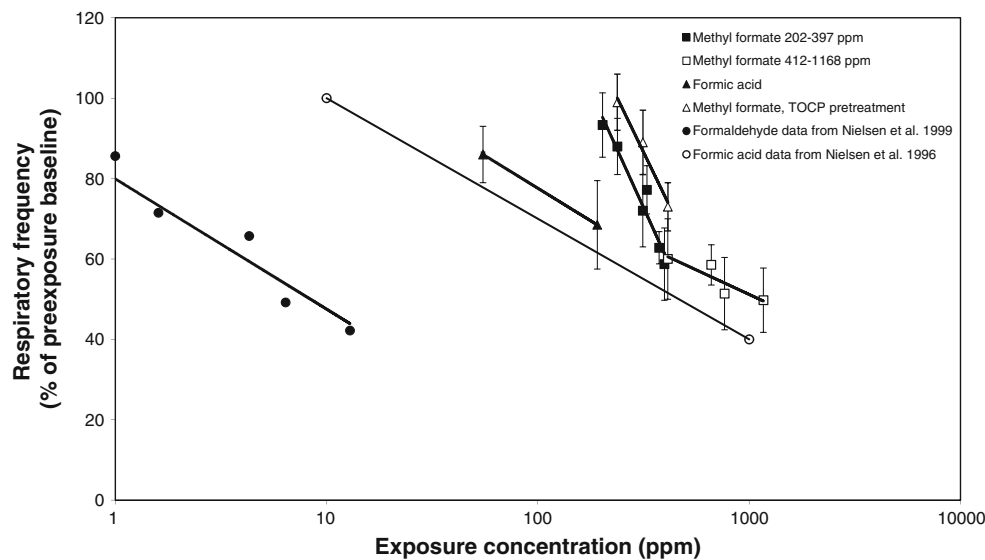The regression line for the 202–397 ppm concentration range was RD $= -115.8 \times \log$ C (ppm) $+ 362.3$; the

Fig. 2 Effects of exposures to methyl formate or formic acid on the respiratory frequency. Mice ($n = 5$–10/group) were exposed 30 min to either methyl formate or formic acid. Three groups of mice were pre-treated with the esterase inhibitor tri-ortho-cresyl phosphate (TOCP) before methyl formate exposure to investigate the role of mucosal esterase activity for the irritation effect of methyl formate. The mean respiratory frequency for each mouse during the last 10 min of the exposure period was used to establish the concentration–effect relationship by means of linear regression analysis. The regression line was used for estimating the concentration depressing the respiratory frequency by 50% (RD50) and to extrapolate the threshold value (RD0). The responses are presented as mean value with 95% confidence interval for each concentration

95% CI for the slope was -92.9 to -138.7. For the 412–1,168 ppm concentration range, the regression line was RD $= -24.4 \times \log C$ (ppm) $+ 124.3$; the 95% CI for the slope was -12.2 to -36.6. Thus, the RD0 and RD50 values are 184 (95–357) and 1,109 (680–1,808) ppm, respectively, with 95% confidence limits in brackets.

Inhibition of carboxyesterase activity by TOCP

The potency of MF as a sensory irritant was significantly decreased in mice treated with TOCP as indicated from the regression line (RD $= -108.6 \times \log C$ (ppm) $+ 358.2$); the 95% CI for the slope was -91.5 to -125.8. The 95% CI for the RD at each exposure concentration at each time point is indicated in Fig. 2. Treatment with TOCP did not alter the base line levels of any of the respiratory parameters assessed (data not shown). To investigate whether TOCP changed the general responsiveness of the animals to airway irritants, TOCP-treated mice were exposed to formic acid, a hydrolysis product of MF and a potent sensory irritant (Nielsen et al. 2007) and confirmed in this study. As apparent from Fig. 3, TOCP pre-treatment did not alter the responsiveness of the mice to formic acid exposure. Also, the irritation effect of formic acid occurred rapidly after onset of exposure and reached a plateau within the first 10 min of exposure (Fig. 3).

Airway irritation of formic acid and formaldehyde

The airway irritation of formic acid was studied at the concentrations 55 and 191 ppm. In this range, the only airway effect observed was sensory irritation. The regression line for the irritation-induced reduction in respiratory rate was given by the equation:

RD $= -32.4 \times \log C$ (ppm) $+ 142.3$;

the 95% CI for the slope was -27.2 to -37.5. The RD50 and the RD0 were 706 and 20.2 ppm, respectively. For comparison, data from previous studies of formic acid (Nielsen et al. 1996) and formaldehyde (Nielsen et al. 1999) are shown in Fig. 2. The regression line for form-aldehyde-induced decrease in respiratory rate was RD $= -31.5 \times \log C$ (ppm) $+ 79.9$.

Discussion

Inhalational exposure to MF vapour induced acute irritation of the upper airways in mice. No effect was observed on the conducting airways and at the alveolar level since the effect was seen only as an elongation of TB. This is overall in agreement with the very high water solubility of the compound, which suggests a high deposition in the upper airways (Medinsky and Bond 2001). The theoretical

Fig. 3 Airway response to 191 ppm formic acid exposure in mice pre-treated with tri-ortho-cresyl phosphate (TOCP) or vehicle (control)
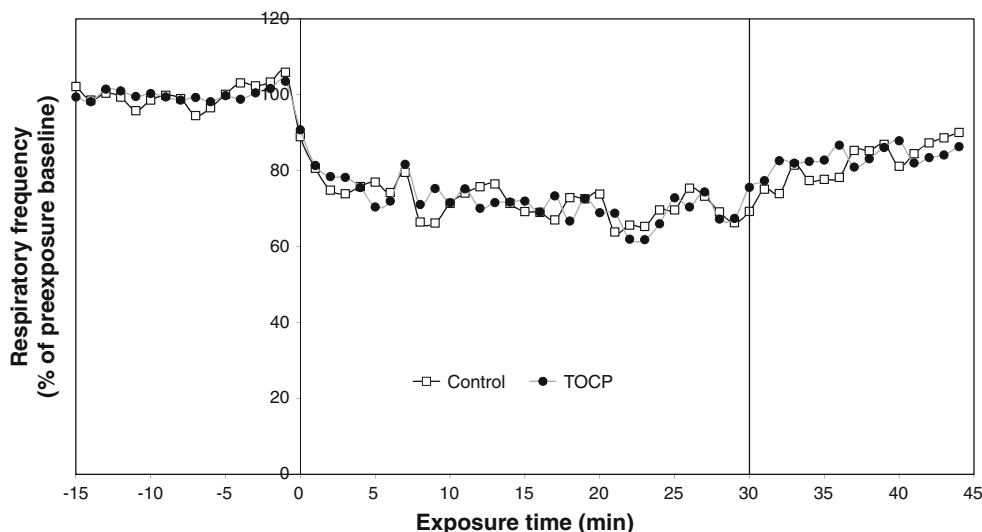
RD50 is expected to be in the range >10,000 to about 100,000 ppm, if MF is a non-reactive sensory irritant. The observed RD50 was 1,109 ppm, and thus, the potency of MF is at least 10-fold higher than for the corresponding hypothetically non-reactive compound. This suggests that MF is a reactive sensory irritant where the response is driven either by a reaction between a trigeminal receptor and the parent compound or, in addition, a reaction by one or more of the reactive metabolites.

Comparing the iso-irritating concentrations, 412 ppm MF and 191 ppm, formic acid indicates that the irritation response of MF developed gradually over the 30-min exposure time (Fig. 1a), compared with effects of formic acid where the maximum irritation effect was reached within the first 10 min of exposure (Fig. 3). The slowly developing response of MF may be due to a slow reaction between MF and a receptor in the trigeminal nerve. Alternatively, the lag in response might be due to bio-transformation of the MF to one or more potent irritants. In the nasal mucosa, MF may be degraded to formic acid by enzyme-catalysed hydrolysis by, e.g., carboxyesterases (Nihlen and Droz 2000). A direct hydrolysis results in the formation of the potent sensory irritant, formic acid, and methanol, a very low potent irritant (Schaper 1993); methanol is not converted to the formaldehyde in the nasal mucosa in any significant extent since it in this case would have been a strong irritant. Thus, if hydrolysis is the only pathway causing sensory irritation, the response would be driven exclusively by the liberated formic acid. However, the methanol part of MF may be oxidised to formyl formate (an aldol compound), which may be hydrolysed to the two potent irritants formaldehyde and formic acid. Oxidation may be caused by cytochrome P450 enzymes (Peng et al. 1995). Both carboxylesterases and cytochrome P450 are present in the nasal mucosa (Bogdanffy et al. 1987; Dahl

et al. 1987; Hadley and Dahl 1983). Vinyl acetate, an aldol acyl compound from acetaldehyde and acetic acid, is hydrolysed by the nasal carboxylesterases (Bogdanffy et al. 1999), suggesting that this may also be the case by other aldol acyl compounds.

A role of carboxyesterases in the formation of the more potent airway irritant formic acid is suggested from the right-shift of the concentration–response curve in mice pre-treated with the carboxylase inhibitor TOCP; the right-shift should only be considered qualitative as suboptimal enzyme inhibition cannot be ruled out. However, even after administration of TOCP, the potency of MF was considerably higher than the theoretical irritation effect for a similar but potentially non-reactive counterpart to MF. The lower MF response in mice treated with TOCP is not likely due to a lower responsiveness to airway irritants in the mice in general, since our control study revealed that TOCP treatment did not change the sensitivity to formic acid. This is similar to the sensory irritation effect of acrylic acid, which was not affected by pre-treatment with TOCP (Silver et al. 1981), whereas the effects of methyl and ethyl acrylate were enhanced, indicating that the parent compounds and not the hydrolysis products were the cause of the sensory irritation responses by these compounds. In contrast to these compounds, MF metabolites seem to play a role in MF-induced irritation.

MF may be hydrolysed to formic acid and methanol as the nasal mucosa contains esterases (Nihlen and Droz 2000; Peng et al. 1995). Methanol is a weak sensory irritant (Schaper 1993) and is consequently not expected to add to the sensory irritation response. Also, oxidation of the generated methanol to the potent airway irritant formaldehyde can be excluded since methanol elsewise would induce a strong irritation response in mice. This pathway leaves unhydrolysed MF and formic acid as the potential

sensory irritants. Furthermore, cytochrome P450-induced oxidation of MF followed by hydrolysis to formic acid and formaldehyde is another potential route of generating strong sensory irritants. The exposure–response relationship for MF reveals two remarkable points. First, the slope in the low-dose range is unusually steep compared to the two reactive compounds formaldehyde and formic acid. Secondly, it is remarkable that the log concentration–response relationship is broken and constitutes two lines, one in the range 202–397 ppm and one in the 412–1,168 ppm range. If an unlimited amount of formaldehyde was generated, the respiratory rate can be decreased by at least 70–80% and constitute one straight line in the log concentration–effect plot as shown (Kane and Alarie 1977; Nielsen et al. 1999). Similarly, the formic acid exposure–effect relationship constituted a straight line in the log concentration–effect plot at least to a 60% decrease in the respiratory rate (Nielsen et al. 1996). Our interpretation is that MF is converted to one or more metabolites, which all activate receptors in the trigeminal nerve, explaining the steep exposure–response relationship in the low exposure range. In the high exposure range, the metabolic pathways may be saturated and in this way the parent compound could drive the response. This hypothesis is supported from the fact that metabolism plays a role for the sensory irritation response and that saturation of metabolism has been observed above 300 ppm for ethyl acetate in the nasal mucosa (Stott and McKenna 1984).

The mouse bioassay used in the present study can predict sensory irritation in humans (e.g. Kuwabara et al. 2007; Nielsen and Alarie 1982), where the OELs for sensory irritants have been estimated from the relations OEL $\approx 0.03 \times$ RD50 and OEL $\approx 0.2 \times$ RD0 (Nielsen et al. 2007; Schaper 1993), suggesting that no sensory irritation would appear at 30–40 ppm. The RD0 is the threshold for the reflexively induced decrease in respiratory rate in the mice and has also been suggested to correspond to the lowest-observed-adverse-effect level (LOAEL) in humans (Nielsen et al. 2007). Considering the RD0 in mice to be the LOEAL in humans and that an assess factor of 2 is the median of the ratio of LOAEL/NOAEL in humans for mild respiratory effects (Alexeeff et al. 2002), this suggests 92 ppm as another estimate of the human threshold for sensory irritation. Overall, the range of no adverse effects is from 30 to 100 ppm. The metabolism in humans and mice has to be sufficiently similar for the estimate to be reliable. Mice have a slightly higher nasal carboxylesterase activity than rats (Stott and McKenna 1985), and rats have a considerably higher activity than humans (Mattes and Mattes 1992), which for the olfactory epithelium may cause an 18-fold higher tissue concentration of acid (Frederick et al. 2002). Depending on the role of the metabolism, the sensory irritation effect in humans may be overestimated from the mouse data. The potential bias cannot be addressed quantitatively, but accepting a certain bias in the estimate and accepting the vague data from human studies, it seems justified to conclude that no relevant sensory irritation will appear in humans if the occupational exposure level does not exceed 100 ppm.

Conflict of interest The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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