Formation of Strong Airway Irritants in Mixtures of Isoprene/Ozone and Isoprene/Ozone/Nitrogen Dioxide

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We evaluated the airway irritation of isoprene, isoprene/ozone, and isoprene/ozone/nitrogen dioxide mixtures using a mouse bioassay, from which we calculated sensory irritation, bronchial constriction, and pulmonary irritation. We observed significant sensory irritation (approximately 50% reduction of mean respiratory rate) by dynamically exposing the mice, over 30 min, to mixtures of isoprene and O₃ or isoprene, O₃, and NO₂. The starting concentrations were approximately 4 ppm O₃ and 500 ppm isoprene (+ approximately 4 ppm NO₂). The reaction mixtures after approximately 30 sec contained < 0.2 ppm O₃. Addition of the effects of the residual reactants and the identified stable irritant products (formaldehyde, formic acid, acetic acid, methacrolein, and methylvinyl ketone) could explain only partially the observed sensory irritation. This suggests that one or more strong airway irritants were formed. It is thus possible that oxidation reactions of common unsaturated compounds may be relevant for indoor air quality.

Key words: airway irritation, indoor air chemistry, isoprene, mouse irritation bioassay, nitrogen dioxide, ozone.

It has been proposed that reactions between unsaturated volatile organic compounds (VOCs) and oxidants (e.g., terpenes and ozone) may produce chemically reactive products that irritate the eye and airway (1). Some of the known reaction products are aldehydes, carboxylic acids, and hydroperoxides, which may cause irritation at concentrations relevant for indoor air (1,2). Some epidemiologic studies of airway irritation symptoms are consistent with the hypothesis that ozone in combination with unsaturated VOCs (e.g., from human activities or building furnishings) contribute to nasal resistance and eye irritation (3). However, until the recent report of the formation of irritants from the reactions of O₃ and (+)-α-pinene (4) and O₃ and limonene (5), the only experimental evidence that supports this hypothesis was the observations of indoor air oxidation reactions reported by Weschler and Shields (6,7).

Our objective was, using a mouse bioassay, to provide experimental evidence for the formation of irritating substances in mixtures of O₃ and isoprene, a common plant and microbial metabolite (8,9) and one of the major organic constituents of air exhaled by humans (10,11). This assay analyzes the respiratory pattern of mice exposed to airborne chemicals (e.g., VOCs). When the upper airway is exposed to irritants, the respiratory rate is reduced because stimulation of the nasal trigeminal nerves reflexively induces a break in breathing after inhalation. When pulmonary irritants are present, the vagal nerves are stimulated, which often creates a pause in breathing before inhalation and thus also a reduction of the respiratory rate. These effects are concentration-dependent over a wide range of concentrations and they are distinguished by analysis of the respiratory parameters (12). They are usually expressed as percent of baseline or percent decrease from baseline. Thus the threshold concentration for reduction of the respiratory rate (RD₅₀), which can be estimated from the dose-response relationship, corresponds to the no-effect level (NOEL). The RD₅₀ used here is the concentration of a substance required to cause 50% decrease in respiratory rate.

The atmospheric chemistry of isoprene with ozone and nitrate radicals has been investigated extensively (13–18). The ozone reaction is reported to give methacrolein, methyl vinyl ketone, hydroxy hydroperoxides, the two isomeric monoxides, 3-methylfur-furan, propene, and many secondary oxidation products of these, depending on the reaction conditions (13–15). The reported reaction products of isoprene with NO₂ consist primarily of nitro or hydroxy aldehydes formed by 1,4 addition processes (16–18).

Experimental Details

Chemicals. Isoprene (>98%, cat. no. 59250) was obtained from Fluka (Fluka Chemie AG, Copenhagen, Denmark) and contained approximately 1% C₁₀ impurities determined by gas chromatography—mass spectrometry (GC-MS) analysis. Methyl vinyl ketone, methacrolein, 2-methyl-2-vinylxirane, and 3-methyl-2(5H)-furane were supplied by Aldrich Chemicals (www.sigma-aldrich.com). We used O₃ (99.999%, N₂ < 5 ppm (product no. 500158, H ydrogas Denmark, Glostrup, Denmark)) to generate O₃ to avoid contamination with nitrogen oxides. Nitrogen containing 150 ppm nitrogen dioxide was diluted to approximately 4 ppm in the O₃/NO₂ reaction.

Methods. We generated O₃ photochemically (19) with a mercury lamp in a thermostated lamp housing controlled by a high-performance variable power supply, as described earlier (4). We transferred O₃ in pure O₂ at 0.5 L/min through a steel tube (internal diameter (i.d.) = 2 mm) into an approximately 13 m Teflon reaction flow tube (i.d. = 2.2 cm) and diluted to an airflow of approximately 18 L/min from the VOC generator or directly with similar dilution without the VOC generator (O₃ exposure) to the mouse exposure chamber, a cylindrical glass vessel (vol 2.3 L), mounted vertically with coplanar mouse ports at 90° angles. The Teflon reaction flow tube was connected directly to the isoprene vapor generator. Pitt N. O. 1 (20), which was fed by an ice-cooled syringe pump. The isoprene concentration was monitored by infrared spectroscopy (M i ran 1A; Foxboro Co., Foxboro, MA, USA). The steel tube from the O₃ generator protruded through the wall of the Teflon reaction flow tube so that the outlet was directed downstream. O₃ concentrations were monitored by a Photometric O₃ Analyzer (M odel 400; A PI, Inc., San Diego, CA, USA), interfaced to a personal computer. The sampling cycle was 8 sec. The analyzer was calibrated with an internal O₃ source at six concentrations. We measured the O₃ concentration loss through the Teflon reaction flow tube at less than 1%. Variation of O₃ concentrations in the mouse chamber was ± 2% over 1 hr. The age of the reaction mixture, determined by the transport time through the flow tube and exposure chamber, was approximately 30 sec, and 96% of the O₃ was consumed.

We analyzed the aldehydes (C₁₋C₄) by sampling on DNH-coated silica gel and then by DAD/HPLC (21) while we collected acids (C₁₋C₂) on Nₐ₂CₐO₃-coated...
Chromosorb PAW (Dansk Miljøcenter A/S, Galtén, Denmark) and analyzed them by gas chromatography-flame ionization detection (GC-FID) of their methyl esters (22). VOCs (methylvinyl ketone, methacrolein, and unidentified products) were concentrated on Tenax TA (Supelco International, Bellefonte, PA, USA) with a syringe (100 mL) and analyzed immediately by GC-M S after thermal desorption (4). We took all air samples in duplicate, in the same plane as the mouse ports and as close as possible to the breathing zones of the mice. Variation in the monitored reactant concentrations were < 5%.

Experimental protocol. The biologic test method followed the American Society for Testing and Materials method (23), further developed and computerized by Boylstein et al. (24,25) and Vijayaraghavan et al. (26,27). We recorded plethysmograph data by a datalogger and analyzed each curve with a computer to calculate the parameters. Experiments were performed between 0900 and 1700 hr.

Effects

Sensory irritation. When a substance stimulates the trigeminal nerve endings, it may cause a painful sensation in humans (12). In mice, it causes a reflexively induced decrease in respiratory rate (fr), which is caused by an elongation of the period from the end of the inspiration until the start of the expiration, called the time of break (TB).

Airflow limitation. This effect is caused by bronchoconstriction, edema, or accumulation of mucus in the conducting airways. This increases the time of expiration (TE) and decreases expiratory flow rate. The parameter used to characterize airflow limitation is the expiratory flow rate at half of the tidal volume (VT), which is abbreviated VD. When VT changes, VD is expected to change as well. Thus, one adjusts for changes in VT by plotting the VD/VT ratio versus the exposure concentration.

Pulmonary irritation. Stimulation of the vagal nerves at the alveolar level may produce two types of respiratory effects. One is a rapid shallow breathing, in which f is increased and VT is decreased. The other is an increase in time from the end of the expiration to the initiation of the following inspiration, called the time of pause (TP). This effect can be recognized and quantified by either the effect on TP or the decrease in fr. The concentration that causes a 50% decrease in respiratory rate is called RD₀.5, and the concentration found by extrapolating the dose–response curve to 0 response is the RD₀.

Because we observed only sensory irritation, we report only changes in respiratory rate.

Experiments. Each experiment consisted of a 15-min preexposure period during which we recorded breathing parameters for the unexposed mice. The exposure period was 30 min, followed by a 15-min recovery period. These parameters were uniformly applied to dose–response experiments for mixtures, pure substances, and air blanks. The chamber conditions were 23 ± 2°C and 10 ± 5% relative humidity. All experiments with O₂ were performed at 22% O₂ content. The total airflow through the exposure chamber was approximately 17–18 L min⁻¹ for all experiments, and the air was introduced in a uniform manner. In each experiment, a naive group of 4 male BALB/c mice (M & B A/S, Rý, Denmark), maintained under standard conditions, was exposed, head only, in separate body plethysmographs. We compared the mean effect for 12 mice for the period between the 11th and 20th minute to values obtained during the pre-exposure period, to determine the exposure effects. Data for the pre-exposure period were not significantly different for different groups of mice.

To facilitate comparison, differences in effects were expressed as percent of baseline or relative decrease from baseline. Time dependence was studied by two-way analysis of variance and regression analysis, using Minitab statistical software (Minitab 13 for Windows; Minitab Inc., State College, PA, USA). p-Values < 0.05 were considered statistically significant.

Dose–response curves were established for isoprene, methacrolein (28), O₃ and formaldehyde (29): we used data for methylvinyl ketone, allylglycidyl ether, NO₂, formic acid, acetic acid, acetone, saturated aldehydes, and 3-methylfuran to calculate/estimate the response for these substances in the reaction mixtures. We estimated the total irritation attributable to identified components. Detailed kinetic studies have shown that mixtures of irritants exhibit competitive agonism, and that in 10–60% reduction of breathing frequency, effects are hypoadaptive (30,31). We performed control experiments using laboratory air. The starting concentrations of the mixture of isoprene and O₃ (and isoprene, O₃, and NO₂) were approximately 500 ppm and 3.7 ppm (~ 500 ppm, 3.7 ppm, and 3.9 ppm) respectively. The high concentration of isoprene was necessary to ensure the reaction of > 90% of the ozone so the concentration was close to the NOEL. The irritation effect of methylvinyl ketone was calculated from literature data (32).

Results and Discussion

Breathing parameter response during exposure to isoprene. We observed no significant effects on breathing parameters during exposure to isoprene at concentrations ≤ 15 × 10³ ppm. No animals died during the exposure or recovery periods.

Reaction of isoprene with O₃. About 0.2 ppm ozone (~ 4% of the original concentration) and approximately 500 ppm isoprene were unreacted and approximately 0.3 ppm formaldehyde, 1.4 ppm methacrolein, 0.6 ppm methylvinyl ketone, 0.3 ppm acetone, 0.7 ppm formic acid, and 0.4 ppm acetic acid were formed in the approximately 30-s reaction mixture (Figure 1). The concentrations of some of the irritants (especially formaldehyde, methylvinyl ketone, and methacrolein) may be slightly underestimated because of reactions with O₃ during sampling on Tenax TA. The linear saturated aldehydes with more than two carbon atoms, identified in the DNPH analysis, were probably artifacts from contaminants in the flow system. In addition to the identified

Figure 1. Average reduction in respiratory rate caused by exposure to isoprene/oxidant mixtures and controls with 95% confidence limits (11th to 20th min of exposure).
products, there were two major products of unknown structure in the carbonyl analyses and several small unidentified peaks in the GC-MS analysis. We observed only traces of T enax T artifacts (< 0.1 ppm), undoubtedly because of the small sample volume. 

Small amounts (~ 50 ppb) of C10 oxidation products were detected from the approximately 1% C10 oxidation impurities in both reactions. We disregarded these because of their low levels.

Assuming that isoprene reacted with one mole of ozone (thus neglecting secondary reactions, e.g., hydroxyl radicals), approximately 2.3 ppm of products (C3 or larger) could be accounted for from 3.7 ppm ozone.

The bioassay results include only effects on the respiratory rate (Figure 1), because sensory irritation was the dominating effect of the individual substances and the mixture described by analysis of the respiratory parameters. The concentrations of all substances identified in the reaction mixture, except for methacrolein, were below or equal to established NOEL or estimated irritation threshold levels (RD50). Thus we expected their contributions to be minor, as reflected in both NOEL and RD50.

The amounts of methacrolein and methyl vinyl ketone reported here agree essentially with those reported earlier, in our experiments (in our experiments < 10%).

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The Nd 0 values for formaldehyde, methyl vinyl ketone, and methacrolein to contribute significantly to irritation of the reaction mixture, reflected by their calculated effect levels and the sum of these (Table 1). The sum of the effects should be an overestimation of the actual contributions because these effects can be hypoadditive. We observed a mean reduction in the respiratory rate of 58% for the mixture isoprene/O3/N2O2 (Figure 1).

For convenience, we used the RD50 values to evaluate the biologic effects, instead of the actual results (52 and 58%, respectively).

Table 1. Calculated reduction in the respiratory rate caused by reactants and products from reaction 1 (isoprene/ozone) and reaction 2 (isoprene/ozone/N2O2) including sensory irritation parameters (NOEL or RD0 and RD50).

| Substance                  | Concentration (ppm) | NOEL (ppm) | RD50 (ppm) | Calculated effect (% reduction) |
|---------------------------|---------------------|------------|------------|--------------------------------|
| Isoprene                  | ~ 500               | ~ 11,000   | ~ 57,200   | 2%                             |
| Formaldehyde              | 0.3                 | 0.3 (29)   | 3.1        |                                |
| Formaldehyde              | 0.7                 | 10 (RD0)   | 480        |                                |
| Acetic acid               | 0.4                 | 13 (RD0)   | 310        |                                |
| Acetone                   | 0.1                 | 0.1 (< 0.001) | 450 |                                |
| Methyl vinyl ketone       | 1.4                 | 1.3 (RD0)  | 10.4       | 2                              |
| Methacrolein              | ~ 0.3               | 100 (RD0)  | 2%         |                                |
| C2–C6 linear saturated    |                     |            |            |                                |
| aldehydes                 | ozone               | 0.14       | 1 (29)     |                                |
| Sum of calculated effects | 500                 | ~ 11,000   | ~ 57,200   | 2%                             |
| Isoprene                  | ~ 500               | ~ 11,000   | ~ 57,200   | 2%                             |
| Formaldehyde              | 0.5                 | 0.3 (29)   | 3.1        |                                |
| Formaldehyde              | 0.9                 | 10 (RD0)   | 480        |                                |
| Acetic acid               | 0.5                 | 13 (RD0)   | 310        |                                |
| Acetone                   | ~ 0.1               | 0.1 (< 0.001) | 450 |                                |
| 3-Methyl furan            | 0.2                 | ~ 0.8 (29) | 5.2        |                                |
| Methyl vinyl ketone       | ~ 1.4               | 1.3 (RD0)  | 10.4       | 2                              |
| Methacrolein              | ~ 0.5               | 1.4 (RD0)  | 7.50       |                                |
| Isoprene epoxides         | ~ 0.3               | 100 (RD0)  | 2%         |                                |
| C2–C6 linear saturated    | aldehydes            | ozone      | 0.17       | 1 (29)                         |
| Sum of calculated effects |                     |           | ~ 0.02     | 9                               |

*Calculated from dose-response data. **The NOEL of the sensory irritation effect in mice, if not available, is set equal to the threshold concentration (RD50), obtained from the curvilinear relationship of the percent decrease in respiratory rate versus the logarithm of the exposure concentration (ppm), taken from the cited literature. The similarity between NOEL and RD50 for formaldehyde may justify the use of RD50 as NOEL. The NOEL of the sensory irritation effect in mice, if not available, is set equal to the threshold concentration (RD50), obtained from the curvilinear relationship of the percent decrease in respiratory rate versus the logarithm of the exposure concentration (ppm), taken from the cited literature. The similarity between NOEL and RD50 for formaldehyde may justify the use of RD50 as NOEL. The NOEL of the sensory irritation effect in mice, if not available, is set equal to the threshold concentration (RD50), obtained from the curvilinear relationship of the percent decrease in respiratory rate versus the logarithm of the exposure concentration (ppm), taken from the cited literature. The similarity between NOEL and RD50 for formaldehyde may justify the use of RD50 as NOEL. The NOEL of the sensory irritation effect in mice, if not available, is set equal to the threshold concentration (RD50), obtained from the curvilinear relationship of the percent decrease in respiratory rate versus the logarithm of the exposure concentration (ppm), taken from the cited literature. The similarity between NOEL and RD50 for formaldehyde may justify the use of RD50 as NOEL.
has been reported from the reaction of furan unsubstituted furanone [2(3H)-furanone] reactions have been described previously. All of the products identified in the two reactions of O3 with isoprene have been reported in model mixtures of R(α)-limonene/ozone (formation of peroxyacetyl nitrate), limonene/ozone, and methyl vinyl ketone. Because the rate of the reaction of O3 with N2O5 is somewhat larger than that of O3 with isoprene (3.2 compared to 1.43 cm3 molecule−1 sec−1), we anticipated that the O3 reaction will be the major contributor to the formation of peroxyacetyl nitrate. Ozone concentrations of 0.03 parts per million (ppm) or 0.1 ppm will lead to the formation of 0.1–10–12 molecule/sec). It is highly likely that unstable products such as hydroperoxy-nitrates or nitrate-aldehydes decompose during thermal desorption (38). All of the products identified in the two reactions have been described previously except the two furanone derivatives. The unsubstituted furanone [2(3H)-furanone] has been reported from the reaction of furan with N2O5 (39). It is possible that the furanones identified in this work are the result of oxidation of 3-methylfuran or that they are formed by rearrangements of products, in which the terminal carbon atoms of isoprene are oxidized, followed by ring closure. Alternatively, they may be artifacts caused by thermal degradation of labile open chain derivatives during thermal desorption. Formation of epoxides was reported in reactions of O3 with α-pinene (40) and limonene (5) under similar conditions, so identification of the three epoxides here, isoprene monoxideepoxides (two isomers) and methylvinyl ketone epoxide was consistent with earlier work.

Further examination of reaction intermediates and their decomposition, involving cold isolation procedures and kinetic ultraviolet-visible spectroscopy and Fourier transform infrared spectroscopy will probably provide more detailed product identification and mechanistic characterization of these reactions under the special flow reaction conditions used.

The identified VOs expected to contribute most to the sensory irritation effects of the mixtures are formaldehyde, methacrolein, and methylvinyl ketone. Because the effects of airway irritants are assumed to be additive or hypoadaptive (1), the substances measured cannot account for the observed effect (Table 1). That the calculated effects for the two reaction mixtures were considerably less than those observed suggests that (potent) unidentified irritant(s) were formed in the reaction mixtures. Possibly, unstable reaction product(s) such as hydroperoxides or, in the O3/N2O5 reaction, peroxyketyl nitrate, may be responsible for the unexplained upper airway irritation. The formation of peroxycetyl nitrate has been reported in model mixtures of α-pinene, O3, and N2O5 (41).

An intriguing question, which is partly the basis for the investigation, is whether these oxidative processes contribute significantly to human airway irritation during periods of elevated indoor O3 (and/or NO2) concentrations and/or in crowded buildings with low air exchange rates (elevated isoprene and other reactive olefin concentrations). The concentration of isoprene in the lungs is low (25 ppm) and somewhat lower in an occupied room (20 ppm) (42). At these low concentrations, the oxidation processes are much slower, but it is possible that during extended exposure they could contribute to the reported airway irritation. Aldehydes have been identified in bronchoalveolar lavage of rats exposed to 0.10–10 ppm ozone undoubtedly from the oxidation of unsaturated fatty acids (43). These authors suggested that oxidation intermediates (epoxides, hydroxy-hydroperoxides) might be involved in the inflammatory process.

It is also interesting that blood levels of isoprene in humans have been reported to be 1–5 mg/g rum (44), whereas the other mammals investigated had blood levels of < 70 μg/mL (44), suggesting that some aspects of terpene biosynthesis in humans are unique.

The upper airway irritation observed in mice exposed to isoprene/oxygen mixtures could not be explained by the concentrations of residual reactants and reaction products identified by some conventional sampling/analytic procedures. It is likely that strongly irritating, unstable products are responsible for the unexplained airway irritation. The practical and rather ironic implication of these observations may be that humans themselves produce one of the compounds, which may contribute to upper airway irritation. References and Notes

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