The Nitrosating Agent in Mice Exposed to Nitrogen Dioxide: Improved Extraction Method and Localization in the Skin

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ABSTRACT

We reported previously that mice exposed to atmospheric NO₂ contained a nitrosating agent (NSA) that reacted with morpholine in aqueous methanol homogenates of the mice to give N-nitroso- morpholine. We have now found that N-nitrosomorpholine was also produced by reacting morpholine with ether extracts of aqueous homogenates prepared from NO₂-exposed mice. After exposure to NO₂ for 4 hr, mice contained NSA (5.0 nmol/g tissue, corrected to 50 ppm NO₂ and assuming that 1 mol NSA yields 1 mol N-nitrosomorpholine). This is 3.6 times the concentration observed by our previous method. Some NSA (0.6 nmol/g tissue) was also detected in untreated mice. The NSA in ether extracts was nonvolatile and stable on storage at -15° or for short periods in the presence of water at pH 1 to 10, but it was decomposed by a pH 1 solution of nitrite scavengers. It reacted to similar extents with three different secondary amines. Eighty-eight % of the NSA occurred in the skin, one-third of which was in the hair. The high skin concentration occurred when the bodies but not the heads of mice were exposed to NO₂, indicating that the major exposure route was the skin. The NSA might consist of α-nitro or other activated nitrite esters derived from unsaturated lipids.

INTRODUCTION

The air pollutant nitrogen dioxide (NO₂) reacts directly with secondary amines in aqueous solution to produce nitrosamines and nitramines (1). Since most nitrosamines and at least one nitramine are carcinogenic, it seemed likely that human exposure to NO₂ would produce carcinogens in vivo. Iqbal et al. (10) reported that µg amounts of NMOR were produced in mice gavaged with morpholine and then exposed to atmospheric NO₂. The whole mice were frozen in liquid nitrogen and then homogenized in 35% aqueous methanol, and no precautions were adopted to prevent nitrosamine formation during the work-up. The highly sensitive GC-TEA technique was used to determine the NMOR. A similar study was performed on DMN formation from dimethylamine (11).

In 1981, we (15) reported experiments in which mice and rats were exposed to NO₂ and morpholine, as done by Iqbal et al. (10, 11). Before the tissue was homogenized, we added a "stopping solution" that contained ascorbate and sulfamate and was adjusted to pH 1. At this pH, ascorbate and sulfamate react rapidly with nitrite and hence should prevent artifactual nitrosation by any nitrite present. The stopping solution also contained cis,2,6-dimethylmorpholine, and the appearance of DMNM together with NMOR indicated that at least some of the NMOR had been formed during the work-up, i.e., was artifactual. No significant amounts of NMOR were detected when homogenates of the whole mouse or of various rat tissues were worked up by this method, using GC-TEA for detection. However, we obtained NMOR when we repeated the experiment of Iqbal et al. (10), in which mouse homogenate was worked up without adding a stopping solution. We showed that the NMOR was produced in the homogenate by a NSA present in NO₂-exposed mice. (NSA is defined as the agent that is produced in vivo by NO₂ exposure and that reacts in vitro with amines to produce nitrosamines.)

The present paper reports an improved method for determining NSA in tissue, using an ether extract of an aqueous homogenate. The report includes a study of the organ distribution of the NSA, using the new analytical method. Some of these results were recently presented at a symposium (14).

MATERIALS AND METHODS

All solvents were of American Chemical Society reagent grade, except for the ether, which was Nanograde (Mallinckrodt, Inc., St. Louis, Mo.). All procedures were performed without fluorescent lighting.

NO₂ Exposure. We used adult male Swiss mice weighing 30 to 35 g from the Eppley Institute colony. They were maintained on a standard commercial diet (Wayne Lab Blox; Allied Mills, Chicago, Ill.). A NO₂ stream was generated by mixing gas from a cylinder of 105 ppm NO₂ in air (Linde Division, Union Carbide Corp., E. Chicago, Ind.) with air from a compressed air supply, using 2 flowmeters and a mixing chamber connected by Teflon tubing. The mixture was passed at 2 liters/min through a glass cylinder (9 cm diameter x 35 cm) with Plexiglas ends, fitted with inlet and outlet tubes, that could hold 4 mice. These were always exposed to 20 to 50 ppm NO₂ for 4 hr. The apparatus was contained in a chemical hood. The effluent gas was analyzed for NO₂ by the Griess-Salzmann method (12) at hourly intervals, and the NO₂ concentration for each exposure was averaged. The analytical method involved serial passage of the gas through 2 glass bubblers containing Griess reagent. The average S.D. of the 3 to 4 measurements per exposure was 10% of the mean value. Our NO₂ results agreed closely with those derived from the analyses of the NO₂ cylinders by the supplier.

The mice were killed immediately after exposure by immersion in liquid N₂, stored in liquid N₂ for <1 week or in some cases up to 1 month, and then powdered in a Waring blender.

Analysis of Whole-Mouse Homogenates for NSA by Measuring NMOR Formation. The entire powder derived from each NO₂-exposed mouse was homogenized with 200 ml of 0.9% NaCl solution in distilled water for 12 min, using a Waring blender. The homogenate was extracted twice with 200 ml ether, using centrifugation at 5° to separate the 2 phases. In some experiments, 5-g samples of the powder were homogenized in 100 ml of 0.9% NaCl solution and extracted twice with 100 ml ether. The combined ether extract was dried over Na₂SO₄ for 30 min and...
and used immediately or, in some cases, stored at -15° over a new batch of drying agent for up to 1 month. To estimate NSA, 50 ml extract were mixed with a solution of 25 mg morpholine in 1 ml ether, kept for 30 min at room temperature, concentrated in a Kuderna-Danish apparatus to 4 to 6 ml and then with a N2 stream to 1.5 ml, in glass-stoppered tubes at room temperature in the dark for 24 hr, and analyzed for NMOR by GC-TEA. This used a Bendix Model 2200 gas chromatograph, with a 2-μm x 2-mm column of 10% Carbobox 20M/2% KOH on 80 to 100 mesh Chromosorb W AW (Supelco Inc., Bellefonte, Pa.), operated at 145°.

Two samples were injected into the gas chromatograph with a 5-μl Hamilton 85N syringe (Hamilton Co., Reno, Nev.) by drawing up successively a "plug" of solvent, an air bubble, and 1 to 3 μl of sample. For detection, we used a Model 502 Thermal Energy Analyzer (Thermo-Electron Corp., Waltham, Mass.), linked with a Spectraview System 1 integrator. NMOR had a retention time of 450 sec. DMN was determined on the same column, operated at 90°; its retention time was 156 sec. Large solvent front peaks were completely separated from both NMOR and DMN. Stock nitrosamines solutions were analyzed each day as standards. Most results were corrected to an exposure of 50 ppm NO2 by assuming that NMOR formation was proportional to NO2 concentration.

The extraction procedure was efficient, since 2 further extractions, each with 100 ml ether, removed only 6 and 7% (in 2 experiments) of the NSA in the first set of extractions. Most NMOR formation occurred when the ether extracts were boiled for 30 to 45 min in the Kuderna-Danish apparatus, as shown by the analysis of extracts before and after they were concentrated. The NMOR yield increased 13% when the concentrated ether extract was stored for 1 day at room temperature, and the yield decreased when the NMOR was stored for 2 days to 97% of that at Day 0. Hence, the concentrate was kept for 1 day before analysis.

The GC-TEA gave a linear response when 1 to 7 μl of a NMOR solution in ether (0.53 ng/μl) were injected. Ether extracts were prepared from NO2-exposed mice, incubated with morpholine, and concentrated to 1.5 ml as described above. When different volumes of the extract were injected into the GC-TEA, the integrator gave a linear response from 0.5 to 3 μl (derived from 1.7 to 10 mg tissue). However, higher volumes showed higher amounts of NMOR than expected for a linear response, probably because of overlap from the solvent front peak. Hence, we injected extracts of <10 mg tissue into the GC-TEA apparatus.

Duplicate injections into the GC-TEA were made for each sample. In a series of 15 analyses, the difference between individual values and the means was 8 ± 6% (S.D.) of the means. When duplicate 5-g samples of homogenized tissue from 4 NO2-exposed mice were analyzed for NSA, the difference of the individual values from each mean value was 23 ± 7% of the means. In view of this large error, most work on whole mice was done on extracts of the entire mice and not on homogenate samples.

Analysis of a morpholine solution in ether showed that the morpholine contained 1.43 ppm NMOR, i.e., 35.7 ng/25 mg morpholine, corresponding to 7 ng/g tissue in an experiment on 5 g tissue. When 100 ml 0.9% NaCl solution in water were blended and extracted twice with 100 ml ether and the ether was mixed with 25 mg morpholine, concentrated, and analyzed, we obtained 140 ± 40 ng NMOR (3 results), corresponding to a mean of 28 ng (0.24 nmol) of NMOR per g tissue in an experiment on 5 g tissue. This NMOR was derived from NMOR in the morpholine or by the standard procedure, killed by cervical dislocation, and dissected. Corresponding tissues from each mouse were combined and weighed. The carcass comprised all material not used for the other tissues, including the head, limbs, and tail. The entire skin was used, or the hair was shaved and analyzed separately from the skin itself. The tissues were stored in stoppered scintillation vials or (for the carcass) wrapped in aluminum foil in liquid N2 for <1 week, homogenized, and analyzed as described earlier for 5-g tissue samples of whole mice. All the tissue (or, for the carcass, 5 g) was homogenized in 100 ml of 0.9% NaCl solution, except for the skin, where 10 ml/g tissue were used. The entire ether extract was reacted with morpholine, except for the skin and carcass, where one-fourth to one-half of the extract was used.

For the experiments in which only the heads or bodies were exposed to NO2, we constructed a 6- × 2.5- × 2.5-cm box made of galvanized iron wire grid (8 mm mesh), with one side that could be opened, and attached it to the end plate of the NO2 exposure chamber. The end plate had a 1.5-cm-diameter hole, covered with rubber from a surgical rubber glove containing a 1-cm-diameter hole. A mouse was lightly anesthetized with ether and placed in the box with its head protruding through the end plate. The rubber fitted snugly around the neck. By reversing the end plate, either the body or the head could be exposed to NO2. In control experiments, a similar but longer box was used to hold the mouse and was placed completely inside the exposure chamber. Effluent gases were vented via a tube through the end plate to the top of the chemical hood, and NO2 content was estimated from the flowmeter readings. (Since this system was not fully airtight, we could not bubble the gases through Griess solution.) Air was blown lightly over unexposed parts of the mouse to ensure that NO2 was absent. After the exposure, the mice were killed and dissected, and tissues were analyzed as described above.

**RESULTS**

In our previous report (15), we used an aqueous methanol homogenate of NO2-exposed mice to demonstrate the presence of NSA. Preliminary results showed that dichloromethane extracted the NSA from this homogenate. Accordingly, we adopted the more usual procedure for isolating lipids of preparing homogenates in water (without methanol) and extracting them with ether. For 7 mice, we obtained results for NMOR of 580 ± 140 ng/g (5.0 ± 1.2 nmol/g tissue), after correction to an exposure of 50 ppm NO2. (Actual exposure was 27 to 36 ppm.) The mean result corresponded to 5.0 nmol of NSA per g tissue, if we assume that 1 mol NSA produced 1 mol NMOR. Most subsequent results are expressed on this basis as NSA.

The NSA was nonvolatile, since 98.1 and 99.8% (in 2 runs) were recovered when an ether extract containing NSA was evaporated to dryness at 20 torr and room temperature and immediately redissolved in ether. NSA solutions in ether gave similar results when analyzed freshly and after storage over Na2SO4 at -15° for 6 months. When 25-, 50-, and 100-ml portions of a NSA extract in ether were reacted with morpholine under the standard conditions, we obtained 10.9, 19.9, and 37.6 nmol NMOR, respectively, showing that NMOR yield was proportional to the amount of NSA.

NSA stability was measured in the presence of various aqueous solutions. Fifty ml ether extract containing NSA were stirred fast enough to produce a vortex for 60 min at 24° with 50 ml water, adjusted to pH 1 with H2SO4 or buffered to pH 7 (0.05 M phosphate) or 10 (0.05 M borate). The ether phase was dried over Na2SO4 and analyzed for NSA. As the control run, part of the ether extract was analyzed for NSA directly, without stirring with water. Mean NSA recoveries in duplicate experiments were 104% (pH 1), 93% (pH 7), and 79% (pH 10) of that.
for the control run, showing that NSA was stable under these conditions. In a similar experiment, an ether solution of NSA was stirred with 50 ml of the stopping solution used previously (15), containing sodium ascorbate (2.5 mg/ml) and ammonium sulfamate (2.5 mg/ml) and adjusted to pH 1 with H2SO4. NSA recovery relative to the control value was 24% when the mixture was stirred for 60 min at 24° and 34% when it was stirred for 60 min at 0°. Hence, NSA was partly decomposed in the presence of an acidic solution containing nitrite scavengers.

The reactivity of aminopyrine to NSA was examined. An ether extract (100 ml) containing NSA was mixed with 1 ml ether solution containing 25 mg morpholine or 25 mg aminopyrine, kept for 30 min, concentrated to 1.5 ml, and analyzed by GC-TEA. The reactions produced 9.8 nmol NMOR from morpholine (0.0034% yield) and 0.21 nmol DMNM from aminopyrine (0.0002% yield), after subtracting the results of blanks prepared in the absence of NSA. Hence, NSA reacted much more readily with morpholine than with aminopyrine, in contrast to the reaction of aminopyrine with nitrous acid, where the reverse is true (13).

We compared the NSA content of unexposed mice and of mice killed at various times after they were exposed to 34 to 36 ppm NO2 for 4 hr. The extracts gave NSA yields of 6.5 ± 0.8 nmol/g tissue for 3 NO2-exposed mice killed immediately after the exposure, 4.8 ± 0.8 nmol/g for 2 mice killed after 20 hr, and 1.6 nmol/g for a mouse killed after 44 hr. For 5 unexposed mice that were analyzed together with the treated mice, the NSA yield was 0.85 ± 0.27 nmol/g. Hence, most of the NSA persisted for 20 hr after the NO2 exposure, and about 13% of the NSA measured immediately after the NO2 exposure was not produced by this exposure. Of the 0.85-nmol/g yield of NSA measured in the untreated mice, 0.24 nmol/g (corresponding to 28 ng/g NMOR) was attributed to NO2 reaction with absorbed lipids. Hence, NSA reacted much more readily with morpholine than with aminopyrine, in contrast to the reaction of aminopyrine with nitrous acid, where the reverse is true (13).

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Analysis of mice exposed to 35 ppm NO2 for various periods gave results of 1.6 ± 0 (0.5 hr), 4.2 ± 0.4 (1 hr), 4.9 ± 1.1 (1.5 hr), 6.3 ± 1.0 (2 hr), 6.1 ± 0.2 (4 hr), 6.3 ± 0.6 (6 hr), and 6.2 ± 0.1 (8 hr) nmol of NSA per g (2 mice per group). This is a composite of 2 experiments, in each of which 4 pairs of mice were placed in the exposure chamber and separated by wire screens. In another experiment, mice were exposed for 4 hr/day for 1, 2, or 3 days to 26 to 29 ppm NO2 and killed immediately after the last exposure. The homogenates contained 4.3 ± 0.2, 5.6 ± 0.8, and 5.8 ± 1.27 nmol of NSA per g, respectively (2 mice per group). Three unexposed mice analyzed with the exposed mice gave results of 1.0 ± 0.4 nmol of NSA per g. Hence, NSA did not accumulate when the time of a single NO2 exposure was increased above 2 hr and increased only moderately after repeated daily exposures.

We previously compared the nitrosation of morpholine, cis-2,6-dimethylmorpholine, and pyrrolidine by NSA in the 35% methanol homogenate (Ref. 15, Table 3). In that experiment, nitrosamine yield followed the order DMNM > NMOR > N-nitrosopyrrolidine, with an 8-fold difference between the DMNM and N-nitrosopyrrolidine yields. Nitrosation of the same 3 amines was compared using the current technique. Portions of an ether extract containing NSA were reacted with the amines and analyzed by GC-TEA (Table 1). Similar amounts of nitrosamine were produced from each amine and from the run in which all 3 amines were added together, suggesting that all NSA in the ether extract had reacted to form the nitrosamines. Hence, the current method for measuring NSA may be quantitative.

Mice were exposed to NO2, and various tissues were dissected and analyzed separately for NSA (Table 2A). The skin (including the hair) contained 88% of the total NSA. Subsequent analysis showed that the hair contained about one-third of the skin NSA, although it constituted <10% by weight of the skin. NSA concentration in the hair was 6 times higher than that in the skin. This is attributed to the high surface area/weight ratio of the hair and may be due to NO2 reaction with absorbed lipids. Mean NSA concentration was 7 times higher in the skin (including hair) than in the stomach (the tissue with the next highest concentration). The carcass included some skin from the limbs and tail, which was probably sufficient to account for the NSA in this tissue. The other principal tissues with significant amounts of NSA were those of the gastrointestinal tract. In this case, the NSA may well have occurred in the contents, which were analyzed.

Table 1
Nitrosation of 3 amines by a NSA solution in ether

<table>
<thead>
<tr>
<th>Amine added</th>
<th>Amount of amine added (nmol)</th>
<th>Yield of nitrosamine (nmol)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Morpholine</td>
<td>129</td>
<td>36.1</td>
</tr>
<tr>
<td>cis-2,6-Dimethylmorpholine</td>
<td>121</td>
<td>37.3</td>
</tr>
<tr>
<td>Pyrrolidine</td>
<td>103</td>
<td>31.5</td>
</tr>
<tr>
<td>All 3 added together</td>
<td>129</td>
<td>21.1</td>
</tr>
<tr>
<td></td>
<td>121</td>
<td>121.7</td>
</tr>
<tr>
<td></td>
<td>121</td>
<td>6.7</td>
</tr>
<tr>
<td></td>
<td>103</td>
<td>21.3</td>
</tr>
</tbody>
</table>

* The yields were corrected for blanks, in which each amine was worked up in the absence of NSA. These blanks yielded 0.72 nmol NMOR, 0.50 nmol DMNM, and 3.2 nmol N-nitrosopyrrolidine from the corresponding amines.

Table 2
NSA in various tissues after the entire mouse or the head only was exposed to NO2

A. Exposure of entire mouse

<table>
<thead>
<tr>
<th>Tissue</th>
<th>No. of analyses</th>
<th>Mean tissue weight (g/mouse)</th>
<th>NSA concentration (nmol/g tissue)</th>
<th>% of total NSA in tissue</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blood</td>
<td>2</td>
<td>0.48</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td>Stomach</td>
<td>2</td>
<td>0.85</td>
<td>8.1</td>
<td>1.6</td>
</tr>
<tr>
<td>Small intestine</td>
<td>2</td>
<td>1.63</td>
<td>4.5</td>
<td>1.1</td>
</tr>
<tr>
<td>Liver</td>
<td>2</td>
<td>1.55</td>
<td>3.8</td>
<td>1.3</td>
</tr>
<tr>
<td>Kidneys</td>
<td>1</td>
<td>1.91</td>
<td>0.92</td>
<td>0.4</td>
</tr>
<tr>
<td>Lungs</td>
<td>4</td>
<td>0.35</td>
<td>0.55</td>
<td>0.0</td>
</tr>
<tr>
<td>Carcass</td>
<td>2</td>
<td>24.3</td>
<td>0.9</td>
<td>6.6</td>
</tr>
<tr>
<td>Skin with hair</td>
<td>2</td>
<td>7.0</td>
<td>46.7</td>
<td>87.8</td>
</tr>
<tr>
<td>Hair</td>
<td>3</td>
<td>0.40</td>
<td>300 ±150</td>
<td>25.1</td>
</tr>
<tr>
<td>Skin</td>
<td>3</td>
<td>4.64</td>
<td>49 ±28</td>
<td>49.7</td>
</tr>
</tbody>
</table>

* A blank value of 1.21 nmol NSA, corresponding to 140 ng NMOR, was obtained in runs performed in the absence of tissue and subtracted from the results of each analysis. Blanks were run together with each set of analyses. Results are given as individual values or as mean ± S.D.
* The amount of NSA in each tissue (mean NSA concentration x tissue weight) was expressed as a percentage of total NSA in all tissues.
* Results for 2 mice, expressed in the same order for all tissues. The carcass did not include the head and tail.
* Includes the contents.
corresponding organs of the fully exposed mice (Table 2A). The gastrointestinal tract had lower NSA concentrations than did the ingestion of NO2.

indicating that a large proportion of NSA in the skin, carcass, exposed mice. NSA concentrations in unexposed mice (Table 3, contact with the skin. NSA concentration in the carcass and demonstrated that about 95% of the skin NSA arose from N02 exposure. In conclusion, it is unlikely that NSA was produced by NO2 in direct contact with the skin and not by absorption through the skin of the head would also have occurred. NSA concentration in the skin was about 10 times higher in "body-exposed" than in "head-exposed" mice and was similar absorption through the skin of the head would also have occurred. NSA concentration in the skin was about 10 times higher in "body-exposed" than in "head-exposed" mice and was similar absorption through the skin of the head would also have occurred. NSA concentration in the skin was about 10 times higher in "body-exposed" than in "head-exposed" mice and was similar absorption through the skin of the head would also have occurred. NSA concentration in the skin was about 10 times higher in "body-exposed" than in "head-exposed" mice and was similar absorption through the skin of the head would also have occurred. NSA concentration in the skin was about 10 times higher in "body-exposed" than in "head-exposed" mice and was similar absorption through the skin of the head would also have occurred. NSA concentration in the skin was about 10 times higher in "body-exposed" than in "head-exposed" mice and was similar absorption through the skin of the head would also have occurred. NSA concentration in the skin was about 10 times higher in "body-exposed" than in "head-exposed" mice and was similar absorption through the skin of the head would also have occurred. NSA concentration in the skin was about 10 times higher in "body-exposed" than in "head-exposed" mice and was similar absorption through the skin of the head would also have occurred. NSA concentration in the skin was about 10 times higher in "body-exposed" than in "head-exposed" mice and was similar absorption through the skin of the head would also have occurred.
inhaled 15NO2 was rapidly distributed in extrapulmonary sites (8), probably as nitrite and nitrate. When radioactive nitrite (Na15NO2) was injected i.v., it was rapidly distributed in the body and then converted to nitrate (18).

In conclusion, we have shown that NSA is present in the skin of mice exposed to NO2. This NSA could produce N-nitroso compounds in vivo from amines or amides. Since the NSA is fat soluble and most amines occur as hydrophilic salts at neutral pH, the most likely compounds to be nitrosated in vivo by the NSA are lipophilic amines. However, such a nitrosation has not yet been demonstrated to occur in vivo. The NSA is most likely to consist of activated nitrite esters produced from the reaction of NO2 with unsaturated fatty acids in tissue lipids.

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REFERENCES

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