Nitrosamine Formation from Amines Applied to the Skin of Mice after and before Exposure to Nitrogen Dioxide

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ABSTRACT

Skin lipids of mice exposed to NO2 contain lipid-soluble nitrosating agent(s) (NSA) that react in vitro with amines to produce nitrosamines. To test whether this reaction occurs in skin, we exposed mice to 50 ppm NO2 for 4 h and, 20 h later, applied 25 mg morpholine or N-methylamine to the skin, which was then analyzed for the corresponding nitrosamine. When morpholine was applied, mean N-nitrosomorpholine yield was only 0.3 nmol/mouse (not significant). When N-methylamine was applied and mice were killed after 10—40 min, N-nitroso-N-methylamine yield in the skin was 13—21 nmol/mouse of which 87% occurred in the hair. NSA formation when mice were exposed to 6.5 ppm NO2 was only 0.15% of that for exposure to 50 ppm NO2. NSA occurred mostly in surface lipids of the skin and its in vitro reaction to give nitrosamines was not inhibited by a-tocopherol. When morpholine was painted on the skin and mice were then exposed to 55 ppm NO2 for 30 min, the skins contained 19 nmol N-nitrosomorpholine/mouse, attributed to a direct reaction between NO2 and the amine. We concluded that nitrosamine formation in skin by this direct reaction may be more important than the reaction of amines with NO2-derived NSA.

INTRODUCTION

Exposure of mice to 50 ppm NO2 in air produced lipid-soluble NSA, which occurred only in the skin and was defined and determined by its in vitro reaction with morpholine to produce the nitrosamine, NMOR (4, 5). Most NSA was derived from cholesterol and was almost certainly cholesterol-3-β-nitrite (6). Some NSA originated from triglycerides (6) and was probably derived from a peroxidized product (1, 7). NSA also occurs in the lipids of fried bacon, where it probably contributes to nitrosamine formation (8). Triglyceride-derived NSA could be simple nitrite esters derived from alcohols produced by peroxidation, or could be α-nitronitroso or related compounds derived by addition of N2O3 or N2O4 to ethylene groups (9, 10). Since nitrosamines are produced from nitrite esters at <60°C (4, 5) and from α-nitronitroso compounds only at >80°C (8), nitrite esters may be more relevant to potential nitrosamine formation in vivo.

NSA derived from NO2 in the skin could be environmentally relevant if it reacted with amines or amides in or on the skin to produce N-nitroso compounds. However, we failed to detect significant NMOR in vivo when rats were gavaged with morpholine prior to NO2 exposure (4). Up to 4 nmol NMOR/mouse was detected in similar experiments performed elsewhere (11, 12), but it was not shown which tissues contained the nitrosamine. This formation was inhibited by the administration of α-tocopherol (13). NMOR was detected when morpholine was added to perfusates of isolated lungs exposed to NO2 (14).

To examine whether the reaction between NO2-derived skin NSA and amines could occur in or on the skin, we tested whether 2 secondary amines, morpholine and MANL, are nitrosated when they are applied to the skin 20 h after mice are exposed to NO2. The NO2 is expected to diffuse away during this period and any nitrosamine formation would be attributed to a reaction between the amines and NSA in the skin lipids. The amines were applied to the skin because NSA is formed in this tissue (5). Morpholine was used because it was the standard amine in our previous research (4, 5, 7). MANL was examined mainly because it has a pKo of 4.85 and is almost completely nonionized at physiological pH. In contrast, morpholine (pKo 8.4) is about 90% protonated at pH 7.4 (15). Only nonionized species are likely to be lipid soluble and hence able to react with the lipophilic NSA.

To help evaluate the hazard of nitrosation in the skin by NO2, we also measured NMOR formation when morpholine was painted on the skin and the mice were then exposed to NO2. We expected positive results because NO2 (as its dimer, N2O4) was known to react with amines in nonpolar solvents to produce nitrosamines (as well as nitramines) (16). These reactions also occur when NO2 is bubbled into aqueous solutions of amines (17—19). When morpholine was gavaged or injected i.p. before NO2 exposure (4, 11—14), NMOR could have been produced directly from NO2 and morpholine present in the stomach, mouth, or lungs. Our results demonstrated that a nitrosamine was produced when MANL, but not morpholine, was applied to the skin of mice previously exposed to NO2 and when morpholine was applied to the skin of mice which were then exposed to NO2. Further information is also supplied about skin NSA.

MATERIALS AND METHODS

1. Chemicals, Animals, and Apparatus. Cylinders of 13 and 105 ppm NO2 in air (Linde Division, Union Carbide Corp., New York, NY) were used directly or diluted <3-fold with air using flow meters. Morpholine was obtained from Fisher Chemical Company (Fairlawn, NJ), MANL from Aldrich Chemical Company (Milwaukee, WI), and N-methyl-p-toluidine from Kodak Chemicals (Rochester, NY). The last 2 amines were redistilled before use. cis-2,6-Dimethylmorpholine was prepared as before (5). DCM, n-hexane, and acetone were of analytical grade. 2-Methylpentane (98%) was obtained from Sigma Chemical Company (St. Louis, MO). We used adult male Swiss mice that were 6—8 wk old and were maintained on a standard commercial diet. Nitrosamines are volatile carcinogens and were handled with due precautions.

Gas chromatography of amines was performed on a 2 mm x 2 m column of 4% Carbowax-0.8% KOH on Carbopak B (Supelco Inc., Bellefonte, PA) at 137°C (flow rate, 20 ml He/min) with a TSD N- and P-specific detector (Varian Associates, Palo Alto, CA). Column temperature and retention times were 137°C, 306 sec (MANL), and 130°C, 55 sec (morpholine). Nitrosamines were determined by gas chromatography-thermal energy analysis of 2—5 μl samples in DCM with a 2 mm x 2 mm column of 10% Carbowax 20 M-TPA on 80—100 mesh Chromosorb WAW (Supelco Inc.) flow rate, 20 ml He/min. The detector was a thermal energy analyzer (Thermedics Corp., Woburn, MA). Column temperatures and retention times were 180°C, 250 sec
(NMOR); 180°C, 200 sec (DMNM); 150°C, 320 sec (NMA); and 150°C, 510 sec (NMT). Thermal energy analysis response/mol was 50–60% (for NMA) and 30–45% (for NMT) of that for NMOR.

2. Studies on NSA. To determine skin NSA, mice were exposed to NO2 by methods used before (5), killed by cervical dislocation, and skinned on waxed paper. The skins were cut up and homogenized in an Osterizer Galaxie blender (Oster Corp., Milwaukee, WI) with 100 ml DCM and 10 g Na2SO4. The extracts were filtered, dried over another 10 g Na2SO4, and evaporated under vacuum. To samples of the residues (which were weighed) in 25 ml DCM were added 25 mg 2,6-dimethylmorpholine/1 ml DCM. The solutions were concentrated to 4–6 ml by boiling for 30 min in a Kuderna-Danish apparatus, concentrated to 1.5 ml with a N2 stream, kept overnight at 23–25°C, and analyzed for NMOR.

To obtain NSA for in vitro studies, mice were exposed to 50 ppm NO2 for 4 h, left 20 h, and killed. Each skin (weighing 3–5 g) was homogenized in 200 ml 0.9% aqueous NaCl. The homogenate was extracted with 2 x 200 ml DCM using centrifugation to separate the phases. The extract (with 400 mg lipid/skin) was dried over Na2SO4 and stored at −15°C. Samples (25 ml) were mixed with 25 mg morpholine or 17 mg MANL (and, in some experiments, 50 mg α-tocopherol), concentrated, kept overnight, and analyzed as described above. In the dipping experiment, 5 mice were exposed to 50 ppm NO2 for 4 h, kept 20 h, and killed. The bodies were dipped successively into 4 beakers, each containing 100 ml DCM, while the furs were ruffled with a spatula. The skins were homogenized and extracted with DCM. The washings and extract were analyzed for NSA.

3. Analysis for NMOR and NMA in Tissues after Application of the Amines or Nitrosamines to Skin of NO2-Exposed Mice. Mice were exposed for 4 h to 50 ppm NO2 and left for 20 h in a cage with a wire mesh floor supplied with chow and water. In experiments on nitrosamine formation, a solution of 25 mg morpholine or MANL/100 μl acetone was pipetted onto each unshaved dorsal skin (“painted”); this wetted a 4 x 2 cm area of skin. In experiments on nitrosamine recovery, NMOR or NMA solutions in 100 μl acetone were applied similarly. Each mouse was placed in a restraining cage to prevent skin licking. After various times, the mice were killed and skinned, which took 10 min. In some experiments, the hair was separated with an electric clipper, which took another 10 min. In other experiments, the combined noncutaneous tissues were frozen in liquid N2, and pulverized, and 5-g samples were analyzed.

For NMOR analysis, each skin was homogenized as in section 2 in 25 ml of a 9:1 mixture of methanol and “stopping solution” (2.5 g ascorbic acid, 2.5 g ammonium sulfamate, 1.0 g monitor amine (cis,2,6-dimethylmorpholine for NMOR analyses and N-methyltoluidine for NMA analyses) in 1 liter water, adjusted to pH 1 with H2SO4. Each homogenate was extracted with 25 ml hexane. The methanol phase was mixed with 100 ml water and extracted with 3 x 50 ml DCM. The DCM extract was dried (Na2SO4), concentrated, and analyzed. For NMA analysis, each skin was homogenized in 25 ml stopping solution and 50 ml N-pentane containing 50 mg α-tocopherol. The 2 phases were separated and the aqueous phase was extracted with 3 x 50 ml pentane. The combined pentane phase was extracted with 2 x 50 ml stopping solution which lacked monitor amine, dried, concentrated, and analyzed on the same day as the mice were painted.

Three μg NMOR, 0.6 μg DMNM, 1.5 μg NMA, or 2.9 μg NMT were added to homogenates of single skins and recovery was determined by the appropriate methods. The recoveries were, respectively, 88 ± 20% (9), 74 ± 5% (4), 85 ± 24% (10), and 64 ± 14% (7). [A ± B (C) always indicates mean ± SD (number of analyses).] NMA recovery from the combined noncutaneous tissues was 46 ± 16% (10). Results were not corrected for these losses.

RESULTS

Most of section 1 describes the determination of skin NSA, formed in vitro by exposure to NO2. The NSA was determined by its in vitro reaction with morpholine to yield NMOR, on the assumption that 1 mol nitrosamine arose from 1 mol NSA (2).

Sections 2 and 3 describe nitrosamine formation in mouse skin when amines were applied, respectively, before and after mice were exposed to NO2. Section 4 describes the recovery of nitrosamines applied to skin. All experiments were performed on mice.

1. Preliminary Experiments. Mice were exposed for 4 h to 50 ppm NO2 at 1 liter/min, kept 20 h to allow NO2 to diffuse away, and killed. The bodies were dipped successively in beakers of DCM for 5 sec (beaker 1) or 5 min (beakers 2–4). The DCM solutions in beakers 1–4 and the washed skin contained, respectively, 38, 29, 10, 5, and 18% of the recovered NSA, which totaled a mean of 200 nmol/mouse. These results suggest that most, but not all, NSA occurred in surface lipids of the skin and hair. NSA was not detected in the s.c. fat.

Skin lipid NSA was determined after 8 mice were exposed for 24 h to 6.5 ppm NO2 at 250 ml/min. We obtained 0.93 ± 0.22 nmol NSA/mouse (2.7 ± 0.8 nmol NSA/g lipid). Analysis of 8 mice not exposed to NO2 yielded 0.24 ± 0.16 nmol NSA/mouse (0.9 ± 0.7 nmol/g lipid). The results, expressed either way, were not significantly different (0.10 > P > 0.05).

The partition ratios of the test amines for ether to pH 7.4 buffer (0.01 M Na phosphate) were 0.005 for morpholine and >50 for MANL. The ratios for ether to pH 12 solution were 0.02 for morpholine and >50 for MANL.

2. NMOR Formation in Mice Exposed to NO2 and Then Painted with Morpholine or MANL. We exposed mice to 50 ppm NO2 for 4 h, kept them for 20 h, and applied 25 mg morpholine to each skin. The skins were analyzed for NMOR immediately after the painting ("zero time") or after 0.5–2 h. To prevent artifactual nitrosation during the analysis, we used a stopping solution that contained nitrite scavengers and cis,2,6-dimethylmorpholine as a monitor amine, the nitrosation of which would indicate that nitrosation of the test amine was artifactual (4, 5). NMOR yield was 0.3 ± 0.1 (7 mice) at zero time, 0.3 ± 0.3 (6 mice) at 0.5 h, 0.3 ± 0.2 (5 mice) at 1 h, and 0.3 ± 0.3 (18 mice) nmol/mouse for all times combined. NMOR was not detected in the skin of 6 of these mice or in the combined noncutaneous tissues of 5 mice killed after 0.5 h. The skin of 3 mice killed 0.5–2 h after painting with morpholine, but not exposed to NO2, yielded 0.1 ± 0.2 nmol NMOR/mouse. Five morpholine samples that were worked up similarly without tissue yielded 0.2 ± 0.3 nmol NMOR. No DMNM was detected in these analyses. We concluded that NMOR formation in the experimental animals was not significant and that most of the detected NMOR was an impurity in the morpholine.

When 3 mice were exposed to 55 ppm NO2 for 4 h and the skins were analyzed by the method used for NMOR, but with MANL added to the stopping solution, 1000 ± 300 ng NMA were observed, indicating that artifactual formation of NMA had occurred. When pentane solutions of NSA (skin lipids of mice exposed to 55 ppm NO2 for 4 h) were reacted with an equimolar mixture of MANL and N-methyltoluidine, we obtained 3.3 nmol NMA and 11 nmol NMT, indicating that NMA reacted readily with both of these amines.

We then developed a method for determining NMA without artifactual nitrosation. This method involved homogenizing the skins in a mixture of aqueous stopping solution and n-pentane followed by analysis of the aqueous phase. The method was tested by incubating NSA solutions in pentane with MANL under different conditions until a minimum NMA yield was achieved. Incubation of NSA with MANL in pentane yielded 18 ± 9 nmol NMA (2 runs). When the same incubation mixture was shaken with stopping solution immediately after MANL was added, NMA yield was 3.0 ± 0.6 nmol NMA (4 results).
When, in addition, the pentane phase contained $\alpha$-tocopherol (1 mg/ml), NMA yield was reduced to 0.1 ± 0.2 nmol (4 results). Hence, both stopping solution and $\alpha$-tocopherol were used in the final method of analysis.

We also tested whether $\alpha$-tocopherol could inhibit nitrosamine formation from NSA. Samples of skin-lipid NSA were reacted in DCM with morpholine (3 experiments) or MANL (2 experiments). Each experiment included 2–3 samples with and 2–3 without $\alpha$-tocopherol. Mean percentage of inhibition by the vitamin was 13, −22, and −28% for NMOR formation, and 22 and 37% for NMA formation. $\alpha$-Tocopherol clearly did not significantly inhibit nitrosamine formation from NSA.

Table 1 shows experiments in which MANL was applied to skin of mice exposed previously to NO2 and the skin was analyzed for NMA. The maximum NMA yield of 18 nmol/mouse amounted to 0.008% conversion of the MANL. NMA yield was > 7 times that of NMT derived from the monitor amine, except for 3 results which were excluded. The observed NMA was chiefly derived from in vivo reaction of MANL with substances (NSA) derived from NO2, since mice not exposed to NO2 but painted with MANL yielded only 2.1 nmol NMA/mouse (Table 1, line 3), and 4 MANL samples worked up without tissue yielded 0.3 ± 0.6 nmol NMA/sample. NMA was not detected in the combined noncutaneous tissues. MANL was applied as before to unshaved mice, but the hair and shaved skin were separated after death and analyzed separately (Table 2, lines 1 and 2). About 90% of NMA occurred in the hair, but NMA seemed to disappear from hair more rapidly than from skin, probably by evaporation (see next section). Table 2 also shows the amount of lipid in the hair and shaved skin and its content of NSA and cholestrol (the main NSA precursor (6)).

3. NMOR Formation in Mice Painted with Morpholine before NO2 Exposure. Mice were painted with morpholine and immediately exposed to NO2 for 30 min, and the skin was analyzed for NMOR (Table 3). NMOR formation was 5 times that of DMNM formed from the monitor amine, showing that NMOR was produced in vivo. The mice were exposed to NO2 in a cylindrical glass chamber, usually with 2 mice/chamber. The gas inlet and outlet were at opposite ends and each mouse was placed closer to the inlet ("upstream") or the outlet ("downstream"). Skin NMOR in the upstream mice exposed to 55 or 10 ppm NO2 (Table 3, experiments 1 and 2) was significantly ($P < 0.001$) greater than in mice treated with morpholine but not exposed to NO2 (experiment 3). Exposure to 55 ppm NO2 yielded 17 times more NMOR than exposure to 10 ppm NO2. The downstream mice showed higher NMOR yields than those placed upstream (experiments 1 and 2). This difference was significant for both NO2 levels ($P < 0.01$), is attributed to trapping by the downstream mice of NMOR that evaporated from the upstream mice, and indicates that nitrosamines can readily evaporate from skin. The hair and shaved skin contained similar amounts of NMOR (experiment 4).

4. Recovery of Nitrosamines Applied to the Skin. We measured the recovery from skin of NMOR (1.2 µg/mouse) applied in acetone solution to the skin. The mice were killed 1 min later. NMOR recovery was 21 ± 3% (6 mice). Recovery was also measured of 3–150 µg NMA applied similarly, with the mice killed after 10–40 min. NMOR recovery did not seem to be affected by the dose or time interval and was 23 ± 13% (9 results). These low recoveries are in contrast to the 85–88% recoveries when the same nitrosamines were added to skin homogenate ("Materials and Methods," Section 3) and are attributed mainly to evaporation.

DISCUSSION

Our previous studies (4, 5) used mice exposed to about 50 ppm NO2, whereas humans are generally exposed to <1 ppm NO2, i.e., <1850 µg/m3 (20). Cigarette smoke contains up to 420 µg NO/cigarette (21) and this is slowly oxidized to NO2. The mean skin NSA level in mice exposed to 50 ppm NO2 for 4 h was 420 nmol/mouse (5) and that in mice exposed to 6.5 ppm NO2 for 24 h was 0.1 nmol/mouse, not significantly greater than the level in unexposed mice. This large difference may be due to 2 factors: (a) NSA, which probably consists mainly of the nitrite ester cholesteryl nitrite (6), is likely to be produced from the dimer, $N_2O_4$ [by analogy to nitrosamine production from NO2 (22–24)], and hence its formation rate may be proportional to NO2 concentration squared, (b) NSA degradation could be more significant at low NSA levels.

\[ 2N_2O_4 \rightarrow RONO + HNO_3 \]

Table 1. NMA yield in unshaved skin after mice were exposed to 50 ppm NO2 for 4 h, kept 20 h, painted with 25 mg MANL/mouse, and killed after various times

<table>
<thead>
<tr>
<th>NO2 exposure (min)</th>
<th>Time (min)*</th>
<th>No. of mice</th>
<th>NMA yield (nmol/mouse, mean ± SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>+</td>
<td>10</td>
<td>8</td>
<td>11 ± 5.1′</td>
</tr>
<tr>
<td>+</td>
<td>25</td>
<td>14</td>
<td>13 ± 7′</td>
</tr>
<tr>
<td>−</td>
<td>25</td>
<td>4</td>
<td>2.1 ± 2.5</td>
</tr>
<tr>
<td>+</td>
<td>40</td>
<td>8</td>
<td>18 ± 7′</td>
</tr>
<tr>
<td>+</td>
<td>70</td>
<td>11</td>
<td>5.4 ± 4.9</td>
</tr>
<tr>
<td>+</td>
<td>130</td>
<td>6</td>
<td>0.15 ± 0.29</td>
</tr>
</tbody>
</table>

* Time from painting MANL until skin was homogenized.

Table 2. Results for hair and shaved skin

<table>
<thead>
<tr>
<th>Parameter*</th>
<th>No. of data</th>
<th>Results/mouse (mean ± SD)</th>
<th>Results/mg lipid (mean ± SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Shaved skin</td>
<td>Hair</td>
</tr>
<tr>
<td>NMA yield after 50 min</td>
<td>4</td>
<td>0.9 ± 0.3</td>
<td>6.2 ± 0.8</td>
</tr>
<tr>
<td>(nmol)b</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NMA yield after 70 min</td>
<td>4</td>
<td>1.0 ± 0.8</td>
<td>1.8 ± 1.6</td>
</tr>
<tr>
<td>(nmol)b</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NSA (nmol)</td>
<td>4</td>
<td>7.0 ± 2.8</td>
<td>6.1 ± 4.0</td>
</tr>
<tr>
<td>Cholesterol (nmol)</td>
<td>4</td>
<td>8900 ± 5500</td>
<td>5300 ± 1500</td>
</tr>
<tr>
<td>Lipid weight (mg)</td>
<td>4</td>
<td>205 ± 170</td>
<td>15 ± 7</td>
</tr>
<tr>
<td>Tissue weight (mg)</td>
<td>4</td>
<td>3100 ± 500</td>
<td>480 ± 60</td>
</tr>
</tbody>
</table>

* For parameters in lines 3–5, DCM extracts of the hair and shaved skin were evaporated, weighed, and analyzed for NSA and colorimetrically for cholesterol (6).

b NMA was determined 50 or 70 min after MANL was applied. NMT was not detected.

For parameters in lines 3–5, DCM extracts of the hair and shaved skin were evaporated, weighed, and analyzed for NSA and colorimetrically for cholesterol (6).
The effect of α-tocopherol in reducing artifactual NMA formation is of interest. In the absence of α-tocopherol, NO would be produced from NSA reduction by ascorbate in the aqueous phase, pass into the organic phase, and be oxidized there to NO₂, which would nitrosate MANL (13, 25, 26). Presumably, α-tocopherol reduced this NO₂ (a known reaction (27)) and hence prevented NMA formation in the pentane phase. Use of the stopping solution of sulfamate (which yields N₂, not NO) without ascorbate may have obviated the need for α-tocopherol. α-Tocopherol did not inhibit nitrosamine formation from NSA in lipids, even though it inhibited N-nitroso-N-butyric chloride formation in hexane solutions of NO₂ (27), its emulsions inhibited nitrosamine formation from nitrite in aqueous solution (26), and it inhibited NMAO formation when fed to mice treated with morpholine and then exposed to NO₂ (13). Hence, these results indicate that NMA reacted directly with the amine and not via liberation of NO₂ or nitrite. This is consistent with the literature, since nitrite esters react directly with amines to produce nitrosamines (22–24).

\[
\text{RONO} + R'\text{NH} \rightarrow \text{ROH} + R'R''\text{NNO}
\]

Nanomole amounts of NMA were produced when MANL was applied to the skin 20 h after mice were exposed to NO₂ (Table 1) and this is attributed to a reaction between MANL and skin NSA. NMAO yield was insignificant when morpholine was applied under similar conditions. This difference was not due to differences in nitrosamine recovery (“Results,” section 4). Unlike morpholine, MANL showed high ether:water partition ratios at both pH 7.4, and 12. This indicates that MANL is extracted by lipid more readily than morpholine from neutral solutions because more MANL is nonionized at neutral pH and because nonionized MANL is more lipophilic than nonionized morpholine. Therefore, the difference between the NMA and NMAO yields is attributed mainly to the greater solubility of the amine MANL in the lipid phase, where nitrosation is believed to occur. The facile nitrosation of MANL (15) may be a contributory factor.

The high NMA yield in hair is attributed to the high NSA level in hair lipids (Table 2) and the likelihood that MANL concentration was also high in these lipids. NMA yield in the hair was similar to NSA content, indicating that nearly all NSA in the hair reacted to form NMA. (This did not apply to the shaved skin.) This result fits in with the “dipping experiment” (“Results,” section 1), showing that most NSA occurred in surface lipids of the skin.

Nitrosamine formation by NO₂-derived NSA in the skin may not present much hazard to humans because (a) only an amine (MANL) that is unusually lipophilic at neutral pH reacted in vivo with skin NSA; (b) more NSA occurred in hair, where nitrosamines are less likely to be absorbed, than in unshaved skin; and (c) NSA was not produced by exposure to 6.5 ppm NO₂, indicating that little NSA would result from exposure to ambient NO₂ levels of < 1 ppm. However, N-substituted amides, which can produce nitrosamines, might react with NO₂-derived NSA more readily than most amines, because amides are nonionized and hence may be lipid soluble at neutral pH.

Maximal NMAO yield in mice painted with morpholine and then exposed to 55 ppm NO₂ was 19 nmol/mouse (Table 3), similar to the NMAO yield in mice painted with MANL after NO₂ exposure (Table 1) and in contrast to the absence of NMO in mice exposed to NO₂ exposure. This NMAO formation is attributed to a direct reaction of the amine with NO₂ (17–19, 28), whereas NMAO production after NO₂ exposure is attributed to an indirect reaction via NSA formation. The direct reaction is probably more hazardous to humans than the indirect reaction, because (a) it is expected to occur with a wide range of amines and, perhaps, amides, and was observed with the rather slowly nitrosated and poorly lipid-soluble morpholine, whereas the indirect reaction occurred only with MANL; and (b) equal amounts of NO₂-derived NMAO were detected in shaved skin (where it could readily be absorbed) and hair (Table 3), whereas NSA-derived NMAO was detected mostly in the hair (Table 1). The direct reaction probably accounts for NMAO detected by other workers in mice exposed to morpholine and then to NO₂ (11–13). Both the direct and indirect reactions could occur with exogenous amines (or perhaps amides) in contact with the skin as components of cosmetics and pesticides and as solutions, such as metal-working fluids, used in industry (28). Reaction with endogenous amides in contrast with skin lipids might also occur. Such amides include lipoproteins (peptide bonds are amide groups) and ceramides.

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### REFERENCES


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