THE EFFECTS OF MULTIPLE DAY EXPOSURE TO NITROGEN DIOXIDE ON HUMAN CELLULAR IMMUNITY: HUMAN MACROPHAGE RESPONSES

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Abstract

Fifteen healthy volunteers were recruited, provided informed consent and were characterized with respect to pulmonary function, airway resistance and non-specific airway reactivity. The subjects were exposed to filtered air (FA) or 2.0 ppm nitrogen dioxide (NO₂) for 4-hr per day, alternating 30-min. periods of rest and exercise, for 3 consecutive days. Bronchoscopy and bronchoalveolar lavage were performed 18-hr after the 3rd day of either FA or NO₂ exposure. Macrophages were recovered from the bronchoalveolar lavage fluid and selected macrophage functions were assayed. Assays were performed for superoxide free radical production by the macrophages before and after stimulation with phorbol myristate acetate (PMA) and before and after stimulation with serum-incubated yeast particles (opsonized zymosan). This function, known as respiratory burst activity, is an important part of lung defenses against infectious agents. However, excessive free radical production can overwhelm tissue defenses and cause injury. Other assays that were performed (when there were sufficient numbers of macrophages) included phagocytic activity of the macrophages and production of beta glucuronidase (a lysosomal enzyme important in lung defense against pathogens). These latter assays did not show any clear exposure-related effects, however NO₂ exposure increased superoxide release by macrophages incubated with either of the two stimulants; the increase was significant (p ≤ 0.05) for PMA stimulation but not for zymosan. Analysis of the data also revealed a significant, but hitherto unreported, relationship between baseline (i.e. unstimulated) free radical release of macrophages and specific airway resistance. We found, in fact, that those subjects with relatively high airway resistance had macrophages with suppressed baseline superoxide production. The findings of this study suggest that macrophages may play a role in mediating normal airway resistance. They also demonstrate that NO₂ exposure can interfere with mechanisms that control macrophage responses to stimulation, such that they produce greater amounts of toxic free radicals, compared to macrophages from the same individuals after FA exposure.
Executive Summary

Background
The Air Resources Board funded a study at the Lung Biology Center (LBC), University of California, San Francisco to test the hypothesis that serial-day exposure to 2.0 ppm NO₂ would change the distribution of leukocytes and lymphocyte subsets and activation of lymphocytes. Since macrophage responses might well be involved in NO₂-induced health effects, this project was performed, in conjunction with Dr. John Balmes of the LBC, to test the hypothesis that serial-day exposure to 2.0 ppm NO₂ would change macrophage functions associated with defenses against respiratory infections.

Methods
Fifteen healthy volunteers were recruited, provided informed consent and were characterized with respect to pulmonary function, airway resistance and non-specific airway reactivity. The subjects were exposed to filtered air (FA) or 2.0 ppm nitrogen dioxide (NO₂) for 4-hr per day, alternating 30-min. periods of rest and exercise, for 3 consecutive days. Bronchoscopy and bronchoalveolar lavage were performed 18-hr after the 3rd day of either FA or NO₂ exposure. Macrophages were recovered from the bronchoalveolar lavage fluid and macrophage phagocytic activity, production of superoxide radicals, and production of lysosomal enzymes and cytokines were assayed.

Results
There were no changes in baseline (unstimulated) superoxide production. Stimulation of macrophages by opsonized zymosan and phorbol myristate acetate (PMA) both elicited increased superoxide production from the macrophages, however differences between FA and NO₂ exposures were significant only for macrophages stimulated with PMA. β-glucuronidase, interleukin-1 and tumor necrosis factor were analyzed in samples of macrophages obtained after FA and NO₂ exposures. There were no significant exposure-related differences in concentrations released by unstimulated macrophages after 24-hr incubations. Phagocytic activity was measured in macrophages after both FA and NO₂ exposures. There were no differences in macrophage phagocytic activity.

Conclusions
Exposure to NO₂ at the concentration used in this study (2 ppm) resulted in significant increases in the release of free radicals by alveolar macrophages. These free radicals have been implicated in the damage of lung tissues following pollutant exposures. However, macrophage phagocytic activity and release of lysosomal enzymes and cytokines were not significantly altered. The results of this study suggest that NO₂ exposure can interfere with mechanisms that control macrophage responses to stimulation, such that they produce greater amounts of toxic free radicals, compared to macrophages from the same individuals after FA exposure. We have also observed a correlation between specific airway resistance and release of free radicals by macrophages. This correlation does not necessarily imply causality, but it suggests that macrophage responses may provide a useful biomarker for evaluating the potential susceptibility of individuals to the toxic effects of air pollution.
The Effects of Multiple-Day Exposure to Nitrogen Dioxide on Human Cellular Immunity: Human Macrophage Responses

Introduction

Epidemiological data suggest that exposure to ambient NO\textsubscript{2} is associated with an increased incidence of respiratory symptoms and illness (Detels et al., 1981, Helsing et al., 1982, Koo et al., 1990; Speizer and Ferris, 1973; Yokoyama et al., 1985). However, other studies have indicated no association between NO\textsubscript{2} exposure and respiratory symptoms or illness (Cohen et al., 1972; Euler et al., 1988; Keller et al., 1979). A meta-analysis of the data from studies, involving children (age = 5 - 12 yr), indicated that a long-term increase in NO\textsubscript{2} exposure of 0.015 ppm was associated with a 20% increase in the odds of respiratory illness (Hasselbad, Eddy, and Kotehmar. 1992).

Due to its cytotoxicity, inhalation of NO\textsubscript{2} could produce airway inflammation and change the leukocyte distribution in the airway lining fluid. Exposure to 4.0 ppm NO\textsubscript{2} for 20 min increased the number of lymphocytes and decreased the number of macrophages at 4, 8, and 24 h post-exposure, and increased the number of mast cells at 4 and 24 h post-exposure, compared to pre-exposure (Sandstrom et al., 1990). Similarly, exposure for 20 min to 4.0, and 5.5 ppm NO\textsubscript{2} increased the number of lymphocytes and decreased the number of macrophages, and exposure to 2.25, 4.0, and 5.5 ppm increased the number of mast cells measured at 24 post-exposure compared to pre-exposure (Sandstrom et al., 1991). The effect of exposure to NO\textsubscript{2} on cellular immunity has been assessed via determination of host defense to induced respiratory tract infection.

Following exposures to 0.6 ppm NO\textsubscript{2} for 3 h, or 1.03.0 ppm NO\textsubscript{2} for 2 h per day for 3 days, there was no significant change in the in vitro inactivation of influenza A virus by macrophages collected from BAL (Frampton et al., 1989). Nor was the rate of infection from influenza A virus increased in the subjects exposed to NO\textsubscript{2} (Giongs et al., 1989).

Cumulatively, the reviewed literature indicate that there is some degree of re-distribution of lymphocyte subsets following NO\textsubscript{2} exposure, although it appears that there may be a decreased inflammatory response following multiple-day, compared to single-day NO\textsubscript{2} exposure. Whether lymphocyte activation is changed following NO\textsubscript{2} exposure is unknown. The Air Resources Board funded a study at the Lung Biology Center (LBC), University of California, San Francisco to test the hypothesis that serial-day exposure to 2.0 ppm NO\textsubscript{2} would change the distribution of leukocytes and lymphocyte subsets and activation of lymphocytes. Since macrophage responses might well be involved in NO\textsubscript{2}-induced health effects, we developed this project, in conjunction with Dr. John Balmes of the LBC to test the hypothesis that serial-day exposure to 2.0 ppm NO\textsubscript{2} would change macrophage functions associated with defenses against respiratory infections.
Materials and Methods

A group consisting of 15 healthy volunteers were characterized by physical characteristics, spirometric pulmonary function, specific airway resistance and non-specific airway responsiveness. All subjects completed a medical history questionnaire, denied a history of pulmonary dysfunction, were non-smokers, and had no respiratory-tract illness in the three weeks preceding, or during, the experiment. Subjects abstained from strenuous exercise for 12 h and caffeine for 4 h prior to each session. Each subject was informed of the risks of the experiment and provided informed consent prior to participation.

Each subject was exposed, during separate exposure periods, to a control condition of filtered air (FA) and to 2.0 ppm NO₂ in filtered air. Subjects were unaware of the exposure condition. The exposure periods were for 4 h per day for three consecutive days. A minimum of three weeks separated the two exposure condition periods. Thus each subject acted as his/her own control.

The subjects were bronchoscopyied and bronchoalveolar lavage was performed 18-hr following the last exposure of each 3-day series. Leukocytes, including macrophages, were recovered from the lavage fluid. Total and differential cell counts, lymphocyte subset counts and activation, and total protein and lactate dehydrogenase (LDH) were measured in BAL post-exposure. Lymphocyte subset counts and activation were also measured in peripheral blood pre- and post-exposure. The staff at the LBC performed these assays. The macrophages were centrifuged and resuspended in RPMI-1640 medium with 10% heat inactivated fetal calf serum and supplemented with penicillin and streptomycin. The macrophage samples were shipped, refrigerated, to UCI by overnight courier for functional assays.

Details of the methods are described below.

Spirometric Pulmonary Function, Specific Airway Resistance, and Non-Specific Airway Responsiveness. Forced expired spirometry for the determination of forced vital capacity (FVC) and forced expired volume in one second (FEVI) was performed using a dry-rolling-seal spirometer (Anderson Instruments; Spirotech Division, Model No. S400) using standardized procedures (ATS, 1995). Specific airway resistance (SRaw) was calculated as the product of total airway resistance and thoracic gas volume, which were both measured using a constant-volume body plethysmograph (Warren E. Collins). Airway responsiveness was determined by the FEV₁, response to inhalation of nebulized (Devilbiss, Model No. 646) phosphate-buffered saline (PBS) followed by increasing doses (0.26, 1.53, 4.09, 10.48 umol) of methacholine in PBS delivered via a dosimeter (Rosenthal) at the rate of 0.01 ml breath⁻¹ (Kanner et al., 1994). The airway responsiveness value was taken as either the cumulative dose of methacholine that produced a 20% decrease in FEV₁ from baseline (log-linear interpolation) or, when FEV₁ did not decrease by 20%, as the maximum dose of 10.48 μmol.
**Exposure Chamber and Atmospheric Monitoring:** The exposure sessions were performed at the LBC in a custom-built steel and glass exposure chamber (Nor-Lake Inc., Model No. W00327-3R), which is 2.5 m x 2.5 m x 2.4 m in size, and has an average airflow rate of 300 m³ min⁻¹. The chamber air was supplied from ambient air which was filtered by passing through purifying (Purafil, Model No. 6239) and high efficiency particle filters (Aeropac. Model No 53 HEPA 95). The filtered air was dehumidified by passing through a drier (Cargocaire Engineering Corp., Model No. HC-575). Subsequently, the air temperature was decreased with a chilled-water coil, and the humidity increased with steam (Nortec. Model No. NHMC-050), to obtain the pre-set temperature (20.0 °C) and relative humidity (50%) conditions in the chamber. The temperature and relative humidity inside the chamber were monitored (3-min intervals) and controlled throughout the exposures (Johnson Controls, Model No. DSC 8500). For the NO₂ exposures, NO₂ was supplied from gas cylinders containing 250-500 ppm NO₂ in air (Liquid Carbonic Corp.) which was piped through Teflon® tubing directly into the intake duct of the chamber. The NO₂ concentration in the chamber was monitored continuously (30-s intervals) throughout the exposures using a chemiluminescent NOX analyzer (Monitor Labs Inc., Model No. 8840). The NOX analyzer was calibrated using a permeation tube calibrator (VICI Metronics, Model No. 340) using ultrapure air (NOX < 0.001 ppm) and a certified NO₂ permeation source that released NO₂ at a known, constant rate.

**Exercise and Pulmonary Ventilation:** During each exposure, subjects exercised by either walking/running on a treadmill (Precor Model No. M9.1), or pedaling a cycle-ergometer (Monark, Model No. 908 1 8e). The exercise intensity was adjusted for each subject to a target expired minute ventilation (VE) of 25 L min⁻¹ m⁻² body surface area. During exercise, VE was calculated from tidal volume and breathing frequency measured using a pneumotachograph (Fleisch, Model No. 3) at the 1- and 20-min interval of each 30-min exercise period. There was no significant difference in the mean VE between the FA and NO₃ conditions (x ± SD; 47.8±5.6 L min⁻¹ vs. 47.5±4.8 L min⁻¹; P=0.80).

**Bronchoalveolar Lavage:** Bronchoscopies were performed in a dedicated room at San Francisco General Hospital. Vital signs were measured pre- and post-bronchoscopy. Throughout the procedure, intravenous access was maintained, and arterial hemoglobin oxygen percent saturation (Ohmeda, Model No. Biox 3700) and the electrocardiograph were monitored. Atropine, to decrease airway secretions, and if required, midazolam, to maintain subject comfort, were administered intravenously. The posterior pharynx was anesthetized using a 4% lidocaine gargle, a 1% lidocaine spray, and 4% lidocaine-soaked, cotton-tipped pledgets applied to the mucosa over the ninth cranial nerve. Supplemental 0, was delivered via a nasal canula at 2 l min⁻¹. The bronchoscope (Pentax, Model No. FB 1 8x), tipped with lidocaine jelly, was introduced through the mouth, and the larynx and airways were anesthetized using 1% lidocaine solution as required. The bronchoscope was initially directed and wedged into the right middle lobe orifice (3 x 50 ml lavage), and subsequently the lingula (1 x 50 ml lavage). The lingual lavage provided the alveolar macrophages for functional assays that were then shipped to UCI for this study. The lavages were performed using 0.9% saline heated to 37°C. The first 15 ml of lavage fluid returned was designated the bronchial fraction (BFx). The PB
collections were performed immediately before each exposure period and pre-bronchoscopy.

**Cell Enumeration:** All lavage samples were immediately placed on ice following collection. A 1 ml aliquot was removed for the total and differential cell counts. For the BAL and BFe fluids, total cell counts were performed using a hemocytometer (Fisher Scientific, Cat. No. 0267110), and differential cell counts were performed from cytopsins subsequently stained (Diff-Quik, Baxter, Cat. No. B4132-1). Total and differential (400 cells) cell counts were performed in duplicate by two counters. A certified commercial laboratory (Metwest) performed total and differential cell counts.

**Macrophage Function Assays:** Superoxide production by macrophages during respiratory burst activity was measured before and after stimulation with opsonized zymosan (Sigma Chemical, St. Louis, MO), and before and after stimulation with phorbol myristate acetate (PMA) were measured in the presence and absence of superoxide dismutase (SOD) as follows. Macrophages were centrifuged and resuspended in HEPES-buffered Hanks Balanced Salt Solution (HHBS). The total number of cells was determined by hemocytometry and cell viability was assessed by Trypan Blue exclusion. The samples were diluted such that suspensions contained 5 x 10⁵ viable cells per ml. Viable macrophages (5 x 10⁵) were added to luminometer cuvettes (LKB Pharmacia) in 1 ml HHBS. Samples were incubated at 37°C in 5% CO₂ for 90 min and non-adherent cells were removed by gentle washing. The medium was replaced with RPMI 1640 with 10% fetal calf serum (heat inactivated) supplemented with penicillin and streptomycin and the samples were incubated overnight. SOD-inhibitable superoxide production was determined by lucigenin-amplified chemiluminescence using an LKB-Pharmacia Model 1251 Luminometer. Chemiluminescence measurements were made beginning immediately after the addition of 200 mM bis N Methylacridinium Nitrate (Lucigenin, Sigma), with or without stimulating agent. Measurements were continued until readings returned to near baseline levels (typically 30 min). Duplicate cuvettes with SOD-treated samples were used to correct readings to yield SOD-inhibitable chemiluminescence readings. Phagocytic activity was measured by incubating 5 x 10⁵ macrophages with 1 μm diameter fluorescently-labeled polystyrene latex microspheres (10⁶ microspheres in 2 ml RPMI 1640 medium) for 1-hr in a shaking water bath at 37°C. The samples were cytocentrifuged onto glass slides, fixed with methanol and air dried. Samples were treated with xylene to quench fluorescence of uningested microspheres, counted using a fluorescence microscope and macrophages were scored as positive if they ingested 2 or more microspheres. A total of 200 cells were counted per sample. β-glucuronidase was measured in macrophage conditioned medium and in lysed macrophages using a commercial kit (Sigma). Measurements for the cytokines interleukin-1 (IL-1) and tumor necrosis factor (TNF) were made using an enzyme linked immunoassay (ELISA) method.

**Statistical Analyses:** Data transforms or nonparametric statistical methods were used to analyze variables that were not normally distributed and for comparing groups that had unequal variances. Two-tailed tests with α ≤ 0.05 were used to establish statistical significance. Regression analysis was used to determine the strengths of intervariable relationships.
Results

The subject group consisted of 15 healthy volunteers who were characterized by physical characteristics, spirometric pulmonary function, specific airway resistance and non-specific airway responsiveness (Table 1.). Each subject completed nine laboratory sessions. Session one was for screening and characterization, subjects performing all pulmonary and airway function tests. Sessions two through four were the first exposure condition (FA or NO2) period, and session five was for bronchoscopy, which was performed 18-hr following the end of session four. Following the minimum three week inter-condition period, sessions six-eight were performed for the other exposure condition (FA or NO2), and session nine was for the bronchoscopy. Each exposure condition period consisted of 4-hr exposures performed at the same time on three consecutive days.

For each of the exposure sessions, pulmonary function tests were performed immediately pre- and post-exposure, and peak flow was monitored during the exposure at the end of each exercise period. During the exposure sessions, subjects alternated 30-min periods of rest and exercise. Following the bronchoscopy sessions, subjects were transferred to the General Clinical Research Center, where they were observed and released when recovered. The values for pulmonary function and airway resistance are in the range expected for these individuals based on their heights, weights and genders. The airway responsiveness (PD20) results do not indicate airway hyperreactivity.

The concentrations (Mean ± SD) of NO2 in the chamber during the exposures are summarized in Table 2. The NO2 in the chamber during the FA exposures was not detectable (N.D.); detection limit for the chemiluminescent detector is estimated to be about 30 ppb.

Bronchoalveolar lavage samples were shipped, by overnight courier, to UCI for analyses of macrophage-related endpoints. There were no exposure-related differences in numbers of cells yielded, reported as millions of viable cells (FA – 5.9 ± 1.1; NO2 – 6.1 ± 0.8). The viability was assessed using the Trypan Blue exclusion method. The samples were allowed to adhere to plastic cuvettes, non-adherent cells were removed by washing, and the cells were incubated in culture medium (RPMI 1640, supplemented with 10% fetal calf serum and penicillin/streptomycin) overnight to allow the macrophages to recover from any activation due to the adherence process. Baseline rates of superoxide production were measured by lucigenin-amplified chemiluminescence. PMA and opsonized zymosan were then added and counts were continued for 200 min (sufficient time for the samples to exhibit a respiratory burst and to return to near-baseline levels. The mean values for superoxide production after FA and NO2 exposures are shown in Figure 1. There were no changes in baseline (unstimulated) superoxide production. Opsonized zymosan and phorbol myristate acetate (PMA) both elicited increased superoxide production from the macrophages, however differences between FA and NO2 exposures were significant only for macrophages stimulated with PMA.

β-glucuronidase, interleukin-1 and tumor necrosis factor were analyzed in samples of macrophages obtained after FA and NO2 exposures. There were no significant exposure-
related differences in concentrations released by unstimulated macrophages after 24-hr incubations.

Phagocytic activity was measured in macrophages after both FA and NO₂ exposures. There were no differences in macrophage phagocytic activity.

Table 1. Individual subjects' physical, spirometric lung function and airway responsiveness characteristics.

<table>
<thead>
<tr>
<th>Subj</th>
<th>Gender</th>
<th>Age (yr)</th>
<th>Height (cm)</th>
<th>Mass (kg)</th>
<th>FVC (L)</th>
<th>FEV1 (L)</th>
<th>FEV1/FVC</th>
<th>SRaw (L x cmH2O l s)</th>
<th>PD20 (1l mol)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>M</td>
<td>25</td>
<td>176</td>
<td>63.6</td>
<td>6.20</td>
<td>4.97</td>
<td>80</td>
<td>3.7</td>
<td>10.48</td>
</tr>
<tr>
<td>2</td>
<td>M</td>
<td>40</td>
<td>177</td>
<td>78.2</td>
<td>5.02</td>
<td>4.23</td>
<td>84</td>
<td>3.0</td>
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<td>3</td>
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<td>26</td>
<td>185</td>
<td>80.9</td>
<td>5.89</td>
<td>5.04</td>
<td>86</td>
<td>2.5</td>
<td>10.48</td>
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Mean: 29.3  175.4  75.4  5.40  4.38  81.4  3.3  8.90
± SD: 4.8  6.8  8.6  1.04  0.78  5.3  1.3  2.99

FVC, FEV₁, FEV₁/FVC (%), SRaw, and PD20 data are individual means of pre-exposure data for the FA and NO₂ conditions. PD20 = methacholine dose (cumulative), at which FEV₁ decreased 20%.

Table 2 Exposure Atmospheric Characteristics.

<table>
<thead>
<tr>
<th>Exposure Condition</th>
<th>FA</th>
<th>NO₂</th>
</tr>
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<tr>
<td>Nitrogen Dioxide (ppm)</td>
<td>N.D.</td>
<td>1.95 ± 0.14</td>
</tr>
<tr>
<td>Temperature (°C)</td>
<td>19.9 ± 0.1</td>
<td>20.2 ± 0.4</td>
</tr>
<tr>
<td>Relative Humidity (%)</td>
<td>50.0 ± 7.3</td>
<td>56.4 ± 66</td>
</tr>
</tbody>
</table>

Values are means ± SD. Abbreviations: FA = filtered air; NO₂ = nitrogen dioxide.
Figure 1 Exposure to NO₂ Increases Respiratory Burst Activity in Macrophages From Healthy Subjects.
Although it was not included as an hypothesis for this experiment, there is some rationale for expecting that the background level of free radical production in the lung might be correlated with airway resistance. As shown in Figure 2, there is a statistically significant negative correlation ($r = 0.40; p \leq 0.05$) between specific airway resistance ($S_{raw}$) and baseline superoxide release by macrophages from BAL. It is not necessary that this correlation represent a causal relationship. It may be, in the case of healthy individuals, that some factor that moderates macrophages’ ability to produce mediators can also affect airway dilation.
Figure 2. Specific Airway Resistance is Negatively Correlated with Baseline Free Radical Production by Macrophages.
Discussion

Exposure to low concentrations of NO₂ can cause functional alterations such as airflow limitation and airway hyperresponsiveness to bronchoconstrictor stimuli which are generally associated with epithelial injury, edema and airway and parenchymal infiltration by inflammatory cells. Biochemical effects can include lipid peroxidation, increased antioxidant metabolism, and alteration of enzyme activity. Nitrogen dioxide may also alter the immunological response and reduce the defense against infections, increasing susceptibility of exposed individuals to infections (Chitano et al., 1995). This study, in conjunction with the Lung Biology Center at UCSF examined several of these possibilities in a group of healthy human volunteers. Pulmonary functions and lymphocyte populations were examined at UCSF. Macrophage functions were examined at UCI.

Exposure to NO₂ under the conditions used in the current experiment resulted in a decrease in T-helper cells in bronchoalveolar fluid (BAL), as measured by collaborators at the UC San Francisco Lung Biology Center. This was not accompanied by an increase in the numbers of inflammatory cells (neutrophils and macrophages) in the BAL. However, there was an increase in the number of neutrophils recovered from the lavage of just the bronchial airways, which might suggest that there could be a regional difference in pulmonary response to NO₂.

It has been shown that NO₂ exposure can alter macrophage functions. For example, the migration rate of macrophages (compared to air exposure) was reduced by 33% with 1.0 ppm NO₂ and by 61% with 5.0 ppm (Kienast et al., 1994a). Migration rate is one of the important factors in phagocytosis. There was no significant cytotoxic effect of NO₂ exposure at 1.0 and 3.0 ppm in the Kienast (1994a) study. Their results indicate that NO₂ concentrations relevant to indoor conditions affect the chemotaxis of macrophages without relevant cytotoxicity. Nonsmokers exposed to NO₂ reacted with an increase of neutrophils in BP, an increase of lymphocytes in BAP, and a tendency to reduced AM phagocytosis (Helleday et al., 1994). Although NO₂ exposures in the range used in the present study can alter chemotaxis and macrophage migration, we showed no significant changes in phagocytic activity. However, the suspension assay that we used is not highly influenced by changes in migration, thus our data suggest that although NO₂ can inhibit macrophage migration, phagocytosis of particles or bacteria in contact with the plasma membrane may not be inhibited.

In vitro tests have demonstrated that NO₂ exposure can alter macrophage production of free radicals. Exposure of alveolar macrophages to NO₂ for 30 to 120 min yielded a dose-dependent increase in the production of reactive oxygen intermediates of 1.7- to 2.9-fold of control (Kienast et al., 1994b). This degree of activation is very similar to that observed in the present study. Macrophage-related production of free radicals stimulated by both phorbol myristate acetate (PMA) and opsonized zymosan were both increased after NO₂ exposure as compared to FA exposure, however only PMA produced a statistically significant increase. PMA activation is mediated through protein kinase C (PKC) and is not calcium-dependent, while opsonized zymosan activates respiratory
burst activity via other mechanisms. Thus the results of this study suggest that NO₂ may alter signal transduction pathways mediated by PKC and that other PKC-mediated responses could be affected by NO₂, as well.

In our study we did not see changes in cytokine production in macrophages from NO₂ exposed subjects. This confirms earlier studies by Kienast et al (1996). In the Kienast study, NO₂ exposure of nonstimulated macrophages did not result in changes in IL-1 beta, IL-6, TNF-alpha, and TGF-beta release, compared to the situation with control experiments. Exposure for 30 min to NO₂ induced a significant decrease of LPS-stimulated IL-1 Beta, IL-6, IL-8, and TNF-alpha (p < .05). The release of TGF-beta was not significantly affected by NO₂ exposure. Dandria et al. (1998) also found that NO₂ diminished the capability of lipopolysaccaride (LPS)-stimulated macrophages to secrete cytokines; this effect was minimal in non-smokers but was more pronounced in smokers. We did not perform assays with and without LPS stimulation, however we do confirm that NO₂ exposure did not alter baseline releases of a lysosomal enzyme (B-glucuronidase) or the cytokines (IL-1 and TNF). LPS is a material derived from bacterial cell walls. Hence it is possible that NO₂ exposure, concurrent with a respiratory infection, could result in aberrant production of cytokines and enzymes.

This study in healthy normal subjects did not demonstrate an effect of NO₂ exposure on specific airway resistance (Sraw) or on bronchial reactivity after provocation with methylcholine. Other investigators (Strand et al., 1996) have, however, shown increased histamine-induced bronchial reactivity in NO₂-exposed asthmatic subjects; there was no effect on Sraw in these asthmatics. Our finding that macrophage superoxide production was negatively correlated with Sraw could be indirectly linked to nitric oxide. Nitric oxide (NO) is an important mediator of bronchial smooth muscle relaxation and hence could be a mediator of Sraw. We found that NO₂ exposure resulted in increased macrophage production of superoxide after stimulation with PMA. A recent finding from our studies with animals suggests that NO production is lower in macrophages that have high baseline levels of superoxide production. Thus, if NO₂ exposure increases the release of superoxide by airway and alveolar macrophages, the production of NO might be reduced, and this could contribute to increased airway resistance. There have been no prior reports suggesting that mediators released by macrophages are important contributors to airway resistance, however macrophages are an important source of inducible nitric oxide synthase. Thus, moderation of airway tone may be another type of macrophage-mediated physiological response.

**Summary and Conclusions**

Exposure to NO₂ at the concentration used in this study (2 ppm) resulted in significant increases in the release of free radicals by alveolar macrophages. These free radicals have been implicated in the damage of lung tissues following pollutant exposures. However, macrophage phagocytic activity and release of lysosomal enzymes and cytokines were not significantly altered. The increase in release of free radicals achieved significance in macrophages stimulated with phorbol myristate acetate, a compound that stimulates the protein kinase C signaling pathway, but not in macrophages stimulated by opsonized zymosan, which is a Ca-dependent pathway. Macrophage free radical and NO
production are important in a number of physiological responses and defensive systems. Thus, this study suggests that NO₂ exposure can interfere with mechanisms that control macrophage responses to stimulation, such that they produce greater amounts of toxic free radicals, compared to macrophages from the same individuals after FA exposure. We have also observed a correlation between specific airway resistance and release of free radicals by macrophages. This correlation does not necessarily imply causality, but it suggests that macrophage responses may provide a useful biomarker for evaluating the potential susceptibility of individuals to the toxic effects of air pollution.

Table 3. Summary of Effects of NO₂ on Human Macrophage Responses

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Direction of Change</th>
<th>Significance (ANOVA: NO₂ vs. FA)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Respiratory Burst:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Baseline</td>
<td>+</td>
<td>N.S.</td>
</tr>
<tr>
<td>PMA</td>
<td>+</td>
<td>P ≤ 0.05</td>
</tr>
<tr>
<td>Opsonized Zymosan</td>
<td>+</td>
<td>N.S.</td>
</tr>
<tr>
<td>Phagocytosis</td>
<td>-</td>
<td>N.S.</td>
</tr>
<tr>
<td>β-Glucuronidase</td>
<td>-</td>
<td>N.S.</td>
</tr>
<tr>
<td>IL-1</td>
<td>+</td>
<td>N.S.</td>
</tr>
<tr>
<td>TNF</td>
<td>+</td>
<td>N.S.</td>
</tr>
</tbody>
</table>

Note: N.S. - not significant (p ≥ 0.05)

Recommendations
This study tested the responses of normal, healthy individuals to repeated exposures to NO₂. The studies were of relatively short duration (3 days) and the number of subjects were relatively small (15). However, the study did demonstrate significant findings related possible immunological compromise of exposed individuals. We therefore recommend that studies are needed in populations of subjects with lung diseases such as asthma. In addition, we have observed a hitherto unreported correlation between a measure of lung mechanics (airway resistance) and release of mediators by macrophages. This correlation needs to be explored more fully to determine whether it can provide a marker for evaluating potential susceptibility to the toxic effects of air pollution.
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