

# Nitration of Proteins in Bronchoalveolar Lavage Fluid from Patients with Acute Respiratory Distress Syndrome Receiving Inhaled Nitric Oxide

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Inhaled nitric oxide ( $\cdot\text{NO}$ ) is used to improve gas exchange and reduce pulmonary vascular resistance (PVR) in patients with the acute respiratory distress syndrome (ARDS). Although controlled studies have shown no survival benefit, some investigators have suggested that inhaled  $\cdot\text{NO}$  may have anti-inflammatory properties under these circumstances. In contrast, others have speculated that  $\cdot\text{NO}$  given by inhalation could be cytotoxic, as it combines with superoxide at near diffusion-limited rates to produce the highly reactive oxidant peroxynitrite ( $\text{ONOO}^-$ ). We therefore quantified levels of 3-nitrotyrosine, a marker for  $\text{ONOO}^-$  formation, in bronchoalveolar lavage fluid (BAL) from patients with ARDS receiving inhaled  $\cdot\text{NO}$ , and from patients with comparable lung injury who were not so treated. We also measured levels of 3-chlorotyrosine as an index of neutrophil activation to assess indirectly the effects of inhaled  $\cdot\text{NO}$  on lung inflammation. Patients receiving  $\cdot\text{NO}$  had increased levels of 3-nitrotyrosine ( $6.76 \pm 2.79$  versus  $0.4 \pm 0.15$  nmol/mg of protein,  $p < 0.05$ ) and 3-chlorotyrosine ( $7.97 \pm 2.74$  versus  $1.53 \pm 1.09$  nmol/mg of protein,  $p < 0.05$ ) in BAL protein compared with controls. In patients with ARDS, inhaled  $\cdot\text{NO}$  increases the formation of 3-nitrotyrosine and is accompanied by an increase in levels of 3-chlorotyrosine (a marker of neutrophil activation). The possible long-term consequences of these observations remain to be evaluated. Lamb NJ, Quinlan GJ, Westerman ST, Gutteridge JMC, Evans TW. Nitration of proteins in bronchoalveolar lavage fluid from patients with acute respiratory distress syndrome receiving inhaled nitric oxide.

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Inhaled nitric oxide ( $\cdot\text{NO}$ ) improves oxygenation by diminishing shunt fraction and reduces pulmonary vascular resistance (PVR) in a proportion of patients with the acute respiratory distress syndrome (ARDS). Although it does not appear to confer a survival benefit (1),  $\cdot\text{NO}$  given by inhalation has been shown to influence favorably indices of inflammation detectable in bronchoalveolar lavage (BAL) fluid from such patients (2); and theoretically has antioxidant capacity, by terminating lipid peroxidation (3). In contrast, inhaled  $\cdot\text{NO}$  potentially reacts with superoxide ( $\text{O}_2^-$ ), at near diffusion-limited rates to form the toxic and reactive oxidant peroxynitrite ( $\text{ONOO}^-$ ) (4).  $\text{ONOO}^-$  is capable of inflicting damage on biological molecules similar to that caused by the hydroxyl radical ( $\cdot\text{OH}$ ), and also nitrates tyrosine and tryptophan amino acids and residues and nitrosates thiol groups (reviewed in Reference 5). Both oxidation and nitration reactions represent potentially deleterious events by inflicting damage on biological molecules and loss of function. Moreover, tyrosine nitration indi-

cated by 3-nitrotyrosine formation, a marker for  $\text{ONOO}^-$ , has been found in the lungs and in BAL samples taken from patients with ARDS (6, 7).

Inhaled  $\cdot\text{NO}$  may therefore lead to oxidative tissue damage and protein nitration, but might also have beneficial anti-inflammatory effects in patients with ARDS. To explore further these possibilities, we measured levels of 3-nitrotyrosine in BAL protein from patients with ARDS receiving inhaled  $\cdot\text{NO}$ , and compared them with those of patients not receiving this intervention. Second, we measured neutrophil counts, and levels of 3-chlorotyrosine in the same BAL samples as an index of neutrophil activation, to assess indirectly the possible effects of inhaled  $\cdot\text{NO}$  on lung inflammation.

## METHODS

### Patients

Twenty sequentially admitted patients with established ARDS as defined by the American European Consensus Guidelines were investigated. A dose response to inhaled  $\cdot\text{NO}$  (0-20 ppm) was performed and responders ( $n = 10$ , defined as a  $> 20\%$  change in  $\text{PaO}_2:\text{FiO}_2$ ) continued to receive nitric oxide at the minimum effective dose according to published guidelines (8). Nonresponders ( $n = 10$ ) received identical mechanical ventilatory support without inhaled  $\cdot\text{NO}$ . Ethics committee approval was gained for patients to undergo BAL, at clinically relevant times not less than 24 h after the commencement of the study. No control patient underwent BAL less than 24 h after the  $\cdot\text{NO}$  dose-response test. It was not possible to attain informed consent

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TABLE 1  
CLINICAL DETAILS OF PATIENTS IN STUDY

| Patient | Age/Sex | Days* | Primary Insult             | Fl <sub>O</sub> <sub>2</sub> | All | LIS | 3-CT  | 3-NT  | ·NO | Cells <sup>†</sup> | %N    | Prot <sup>‡</sup> |
|---------|---------|-------|----------------------------|------------------------------|-----|-----|-------|-------|-----|--------------------|-------|-------------------|
| A (S)   | 28/F    | 1     | Pneumonia                  | 0.5                          | 15  | 3.3 | 0     | 0.49  | —   | 0.45               | 98.4  | 1.1               |
| B (NS)  | 44/F    | 1     | Pneumonia                  | 0.99                         | 18  | 3   | 1     | 0.77  | —   | 3.43               | 59.28 | 4.1               |
| C (NS)  | 64/M    | 7     | CPB                        | 0.75                         | 13  | 3   | 0.006 | 0.002 | —   | 0.15               | 16.82 | 30.3              |
| D (S)   | 18/M    | 6     | Trauma                     | 0.99                         | 20  | 2.5 | 0.57  | 0.068 | —   | 0.27               | 78.49 | 6.0               |
| E (S)   | 33/M    | 1     | Trauma                     | 1                            | 18  | 4   | 11.19 | 0.084 | —   | 0.63               | 0     | 1.4               |
| F (NS)  | 54/M    | 1     | Pneumonia                  | 0.64                         | 16  | 3   | 0     | 0.045 | —   | 0.39               | 87.46 | 1.1               |
| G (S)   | 75/M    | 1     | CPB                        | 0.55                         | 12  | 3   | 0     | 1.13  | —   | 0.51               | 11.04 | 0.7               |
| H (S)   | 38/M    | 2     | Lung resection             | 0.99                         | 6   | 3.3 | 0.063 | 1.33  | —   | 0.16               | 44.34 | 15.4              |
| I (NS)  | 30/F    | 6     | Pneumonia                  | 0.63                         | 9   | 3   | 0.31  | 0.28  | —   | 0.21               | 31.65 | 40                |
| J (S)   | 20/M    | 1     | Trauma                     | 0.45                         | 7   | 2.5 | 2.17  | 0.53  | —   | 0.60               | 48.0  | 1.8               |
| K (S)   | 28/M    | 2     | Pneumonia                  | 1                            | 11  | 3.5 | 20.81 | 26.77 | 20  | 3.48               | 21.18 | 0.4               |
| L (NS)  | 48/M    | 1     | Pneumonectomy              | 0.89                         | 20  | 3   | 1.64  | 0.46  | 20  | 0.96               | 90.36 | 35.2              |
| M (NS)  | 43/M    | 2     | Trauma                     | 0.87                         | 20  | 3.3 | 24.55 | 16.36 | 10  | 2.79               | 83.83 | 0.3               |
| N (S)   | 24/M    | 2     | Trauma                     | 0.82                         | 20  | 4   | 7.85  | 8.33  | 10  | 1.09               | 95.73 | 11.4              |
| O (S)   | 19/M    | 2     | Trauma                     | 0.6                          | 11  | 2.8 | 5.79  | 0.79  | 14  | 0.68               | 94.34 | 5.2               |
| P (NS)  | 33/F    | 2     | Pneumonia                  | 0.99                         | 9   | 2.8 | 6.19  | 2.02  | 5   | 0.33               | 93.21 | 1.1               |
| Q (S)   | 22/M    | 1     | Trauma                     | 0.99                         | 9   | 3   | 0.043 | 0.72  | 7   | 0.45               | 90.83 | 2.4               |
| R (S)   | 24/M    | 1     | Pneumonia                  | 0.45                         | 5   | 3.5 | 0.11  | 9.62  | 10  | 1.55               | 91.16 | 2.5               |
| S (S)   | 24/F    | 2     | Cesarian section           | 1                            | 14  | 3.5 | 11.6  | 2     | 12  | 0.04               | 18.09 | 2                 |
| T (S)   | 29/M    | 2     | Pancreatitis<br>laparotomy | 0.99                         | 10  | 3   | 1.07  | 0.52  | 10  | 3.0                | 86.71 | 6                 |

Definition of abbreviations: All = APACHE II score; CPB = cardiopulmonary bypass; LIS = lung injury score; N = neutrophils; NS = nonsurvivor; S = survivor; 3-CT = 3-chlorotyrosine; 3-NT = 3-nitrotyrosine.

\* Days in ICU before lavage.

<sup>†</sup> BAL cell count ( $\times 10^6/\text{ml}$ ).

<sup>‡</sup> BAL fluid protein (mg/ml after concentration).

from patients, and United Kingdom law does not permit families and relatives to provide consent in lieu.

#### Bronchoalveolar Lavage

BAL was performed by standard techniques described in detail elsewhere (7).

#### Analysis of Samples

Neutrophil counts were performed as described previously (9). BAL was concentrated by centrifugation through Centrifree (Millipore, Herts, UK) micropartition devices. Protein concentrations were determined before and after ultrafiltration, using the Lowry method. The retentate was collected, and hydrolyzed by adding 5  $\mu\text{l}$  of mercaptoethanol and 0.5 ml of HCl (6 M) under vacuum in hydrolysis tubes for 18 h at 110° C. When cooled, the free amino acids were purified by cation exchange, as described elsewhere (10). The resultant amino acid samples were lyophilized for 18 h, resuspended in 1 ml of high-performance liquid chromatography running buffer, and ion-exchange and lyophilization procedures repeated. The samples were resuspended in 200  $\mu\text{l}$  of running buffer ready for analysis by high-performance liquid chromatography (HPLC), using a method adapted from Reference 11. Peaks were identified and quantified as previously described (7). Results were corrected to total protein content and expressed as nanomoles per milligram of protein.

#### RESULTS

Patient demographics, BAL 3-nitrotyrosine and 3-chlorotyrosine levels, and neutrophil counts are shown in Table 1. Patients receiving inhaled  $\cdot\text{NO}$  ( $11.8 \pm 1.57$  ppm) showed significantly increased levels of 3-nitrotyrosine in their BAL proteins compared with those who did not receive inhaled  $\cdot\text{NO}$  ( $6.76 \pm 2.79$  versus  $0.4 \pm 0.15$  nmol/mg of protein,  $p < 0.05$ , Figure 1). Furthermore, in patients receiving inhaled  $\cdot\text{NO}$  significantly higher levels of 3-chlorotyrosine were detected than in control patients ( $7.97 \pm 2.74$  versus  $1.53 \pm 1.09$  nmol/mg of protein, respectively;  $p < 0.05$ ).

A positive relationship between the level of  $\cdot\text{NO}$  administered in ppm and the amount of 3-nitrotyrosine measured in

BAL protein was observed ( $r = 0.4$ ), although this did not reach significance, suggesting that inhaled  $\cdot\text{NO}$  is not the only factor involved in determining the amount of protein nitration seen in these patients. However, BAL samples obtained from one patient with ARDS treated with varying levels of  $\cdot\text{NO}$  at three different time points did reveal a possible relationship

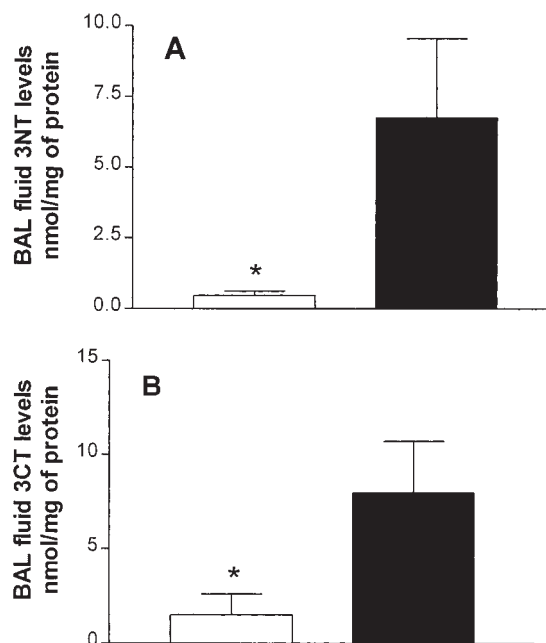
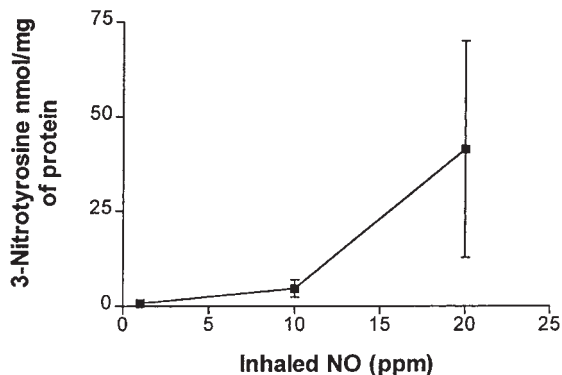


Figure 1. Levels of (A) 3-nitrotyrosine (3NT) and (B) 3-chlorotyrosine (3CT) in patients with ARDS who received inhaled  $\cdot\text{NO}$  (filled bars,  $n = 10$ ) compared with patients who did not receive  $\cdot\text{NO}$  (open bars,  $n = 10$ ). \* $p < 0.05$  relative to filled bars.



**Figure 2.** Levels of 3-nitrotyrosine in patient K subsequent to administration of three different concentrations of inhaled  $\cdot\text{NO}$ . Patient K received 20 ppm for 9 d, then 10 ppm for 12 d, followed by 1 ppm for 2 d. Standard deviation represents the average of triplicate measurements of each sample; the large SD at 20 ppm is due to one replicate skewing the results.

between protein nitration and the level of  $\cdot\text{NO}$  administered (Figure 2).

Patients receiving inhaled  $\cdot\text{NO}$  had higher percentages of neutrophils in their BAL ( $69.68 \pm 11.08$  versus  $47.55 \pm 10.58\%$  for patients receiving  $\cdot\text{NO}$  and control patients, respectively), although this was not significant. BAL cell counts correlated with 3-chlorotyrosine levels ( $r = 0.46$ ,  $p = 0.039$ ), but this was not so for neutrophil levels ( $r = 0.17$ ,  $p = 0.46$ ), a finding that may be related to the degree of neutrophil activation in these patients.

## DISCUSSION

Inhaled  $\cdot\text{NO}$  has been used to improve arterial oxygenation and lower PVR in critically ill patients suffering from ARDS. However, controversy exists regarding possible adverse effects associated with this form of therapy, particularly the formation of the reactive oxidant and nitrating agent  $\text{ONOO}^-$ . Normally,  $\text{ONOO}^-$  formation is limited because intracellular levels of  $\text{O}_2^-$  are kept to a minimum by endogenous antioxidants, particularly superoxide dismutase (SOD). However, during the inflammatory response,  $\text{O}_2^-$  and  $\cdot\text{NO}$  production is greatly enhanced to levels that favor  $\text{ONOO}^-$  production (12). In its protonated form as peroxyxynitrous acid,  $\text{ONOO}^-$  can decompose to form an intermediate with reactivity similar to that of the hydroxyl radical, together with nitrogen dioxide and nitrate (13), although the mechanism involved is subject to speculation (14).  $\text{ONOO}^-$  is also a powerful nitrating agent of tryptophan and tyrosine residues, reactions that are greatly enhanced in the presence of bicarbonate/carbon dioxide (15). Indeed, levels of bicarbonate may be a factor determining tyrosine nitration in these patients.

There are data suggesting that inhaled  $\cdot\text{NO}$  has adverse effects on the lungs of newborn animals exposed to high concentrations, both in terms of surfactant dysfunction and pulmonary inflammation (16, 17); but as far as we are aware, ours is the first report to show increased 3-nitrotyrosine and 3-chlorotyrosine levels in patients receiving inhaled nitric oxide compared with patients not receiving this intervention. Thus, we clearly demonstrate increased protein nitration (tyrosine) in patients with ARDS receiving inhaled  $\cdot\text{NO}$  compared with those patients who did not (Figure 1). Interestingly, in one of our previous studies (7), 3-NT levels measured in BAL fluid from patients with ARDS exceeded those of the control pa-

tients investigated in the current study ( $2.21 \pm 0.65$  versus  $0.4 \pm 0.15$  nmol/mg of protein, respectively), although they were still significantly less than those seen in the  $\cdot\text{NO}$  group ( $6.76 \pm 2.79$  nmol/mg of protein). In fact, a number of patients in the original study were receiving inhaled  $\cdot\text{NO}$ , which might account for this discrepancy, thereby confirming the results we report here.

An absolute relationship between the level of  $\cdot\text{NO}$  administered and the concentration of 3-nitrotyrosine formed could not be demonstrated conclusively, but a positive relationship was identified ( $r = 0.4$ ). This result is not entirely unexpected, given the heterogeneous nature of the patient population, in which many factors may also influence  $\text{ONOO}^-$  synthesis. However, in a single patient lavaged for clinical reasons on three occasions while receiving different concentrations of  $\cdot\text{NO}$ , a clear relationship between levels of administered nitric oxide and BAL protein tyrosine nitration was seen (Figure 2).

We have previously demonstrated a relationship between 3-chlorotyrosine formation and myeloperoxidase release (an indicator of neutrophil activation) in BAL fluid from patients with ARDS. Here, increased levels of 3-chlorotyrosine were detected, providing indirect evidence of neutrophil activation in the inhaled  $\cdot\text{NO}$  group, and again suggesting a proinflammatory effect of the therapy. Also, neutrophil counts were elevated in the patients receiving  $\cdot\text{NO}$  compared with control patients, although this did not achieve significance. Others (2) have shown diminished neutrophil production of hydrogen peroxide ( $\text{H}_2\text{O}_2$ ) after 4 d of  $\cdot\text{NO}$  administered at generally higher concentrations (18 ppm) with an associated fall in BAL cytokine concentrations.

Differences between these data and our own may be attributable to the nature and severity of the lung injury, the days of mechanical ventilation before the commencement of  $\cdot\text{NO}$ , and the duration and dose of therapy administered before BAL. Moreover, we used a different index of neutrophil activation, tyrosine chlorination, a marker of hypochlorous acid formation *in vivo*. An alternative mechanism for tyrosine nitration independent of  $\text{ONOO}^-$  formation has been proposed, involving the interaction of hypochlorous acid with nitrite, a breakdown product of  $\cdot\text{NO}$ . This mechanism also leads in the formation of intermediates capable of chlorinating tyrosine, although such reactions probably make only a small contribution to overall chlorination, which mainly occurs owing to the action of hypochlorous acid (18). The nitration and chlorination observed here may therefore be mediated by more than one mechanism. Tyrosine nitration not only serves as a marker for  $\text{ONOO}^-$  formation, but may also have biological consequences. Thus, nitration induces a loss of function of surfactant protein A (SP-A) and the  $\alpha_1$ -antitrypsin inhibitor, resulting in a loss of its ability to inhibit the action of elastase; both consequences of clinical significance in ARDS (19, 20). The dosage administered is also important in determining adverse consequences of inhaled NO therapy as shown in Reference 17, where 6 h of inhaled nitric oxide at 200 and 80 ppm reduced surfactant function, while 20 ppm did not. Duration of  $\cdot\text{NO}$  treatment may also influence levels of 3-nitrotyrosine detected, because the *in vivo* half-life of endogenously formed 3-NT is 6 d in plasma (21). We found no significant correlation between duration of inhaled  $\cdot\text{NO}$  therapy and BAL nitrotyrosine levels ( $r = 0.25$ ,  $p > 0.5$ ). But it is difficult to draw firm conclusions from these results, as patients were receiving different amounts of inhaled  $\cdot\text{NO}$  over time. In our study 7 of 20 patients died, 3 of whom were receiving inhaled  $\cdot\text{NO}$ . On the basis of these results, it would seem inappropriate to speculate as to the role of inhaled  $\cdot\text{NO}$  in contributing to clinical outcome.

In summary, we have demonstrated an association between

the use of inhaled ·NO in patients with ARDS and increased 3-nitrotyrosine formation. The mechanism by which nitration occurs is probably mediated via ONOO<sup>-</sup> but may also involve interaction between nitrite and hypochlorous acid. The consequences of protein nitration in these patients remain to be decided.

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