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Original Paper

Type 2 nitric oxide synthase and protein nitration in chronic lung infection

Natalie Hopkins, Elaine Cadogan, Shay Giles, John Bannigan and Paul McLoughlin*
Department of Anatomy and Physiology, University College Dublin, Dublin, Ireland

**Correspondence to:*
Paul McLoughlin, Department of Human Anatomy and Physiology, Conway Institute of Biomolecular and Biomedical Research, University College Dublin, Earlsfort Terrace, Dublin 2, Ireland.
E-mail: paul.mcloughlin@ucd.ie

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Abstract

Inflammation in the lung can lead to increased expression of inducible nitric oxide synthase (iNOS) and enhanced NO production. It has been postulated that the resultant highly reactive NO metabolites may have an important role in host defence, although they might also contribute to tissue damage. However, in a number of inflammatory lung diseases, including bronchiectasis, iNOS expression is increased but no elevation of airway NO can be detected. A potential explanation for this finding is that NO is rapidly scavenged by reaction with superoxide radicals, forming peroxynitrite, which is preferentially metabolized via nitration and nitrosation reactions. To test this hypothesis, anaesthetized, specific pathogen-free rats were inoculated with *Pseudomonas aeruginosa* incorporated into agar beads (chronically infected group) or sterile agar beads (control group). Ten to 15 days later, the lungs were isolated and fixed. *Pseudomonas* organisms were isolated from the lungs of the chronically infected group. These lungs showed extensive inflammatory cell infiltration and tissue damage, which were not observed in control lungs. Expression of iNOS was increased in the chronically infected group when compared with the control group. However, the mean number of cells staining for nitrotyrosine in the chronically infected group was not significantly different from that in the controls, nor was there an excess of nitrotyrosine, nitrate, nitrite or nitrosothiol concentrations in the infected lungs. Thus, no evidence was found of increased NO metabolites in chronically infected lungs, including products of the peroxynitrite pathway. These findings suggest that chronic infection does not cause increased iNOS activity in the lung, despite increased expression of iNOS.

Keywords: **pulmonary vascular resistance; nitrate; nitrite; nitrosothiols**

Introduction

Infection, lipopolysaccharide (LPS), and pro-inflammatory cytokines lead to increased expression of the high output, calcium-independent nitric oxide synthase (NOS)-2 in a variety of cells in the lung. Through this mechanism, inflammatory diseases of the lung can cause increased NO production, which is thought to have an important anti-microbial role [1–3]. However, it also has the potential to cause damage to the host cells and tissues. This dual potential for both beneficial and harmful actions is clearly illustrated by the observations that NOS-2 knock-out mice are more susceptible to some infectious agents than wild-type controls, yet seem to suffer less during infection by other agents [3]. Thus, increased expression of this transcriptionally regulated enzyme plays a key role in the pulmonary response to inflammatory stimuli.

NO produced in the lungs can be detected in exhaled gas from normal lungs and altered NO excretion from the airways has been reported in respiratory disease conditions [4,5]. In particular, expression of the high output isoform of nitric oxide synthase (iNOS or type 2 NOS) is increased in asthma and is associated with abnormally elevated nitric oxide concentrations in exhaled gases [1,6]. Exhaled NO may be reduced to normal values by inhalation of selective inhibitors of iNOS or glucocorticoids, which inhibit the expression of this isoform [7–9]. Increased concentrations of NO have also been reported in exhaled gases in several other lung diseases [10]. Thus, it has been suggested that production of NO in the lungs is increased by pulmonary inflammatory diseases in general and that measurement of exhaled NO may be a useful index of airway inflammation.

More recently, it has been reported that in inflammatory lung diseases caused by chronic infection, exhaled NO is not elevated [11–13]. This finding is unexpected given that both in bronchiectasis in human subjects and in animal models of chronic airway infection, iNOS expression is increased above normal [11,14,15]. Interestingly, in other inflammatory lung diseases, including stable chronic obstructive pulmonary disease, adult respiratory distress syndrome and sarcoidosis, in which there is no known defect of iNOS expression, exhaled NO is not elevated [16–20]. A potential explanation is that in chronic infection NO may be scavenged by superoxide radical, in a reaction whose rate is so rapid that it is nearly

diffusion-limited [21,22], and lead to the formation of peroxynitrite at, or near to, the sites of NO production. This peroxynitrite could cause nitrosation at multiple nearby sites by reacting with nucleophilic centres and cause nitration of amino acid residues in proteins. Peroxynitrite, amongst other oxides of nitrogen, supports nitrosation reactions that occur preferentially at thiol groups, but also at multiple other sites [23,24]. Such a series of reactions could account for the failure to detect abnormal elevation of exhaled NO in these conditions.

The aim of this study was to test the hypothesis that in chronic lung infection, excess NO derived from NOS-2 activity is rapidly scavenged by reaction with superoxide radical, producing peroxynitrite, which causes abnormal nitration and nitrosation reactions. We used a previously described model to establish chronic *Pseudomonas aeruginosa* airway infection in rats [25,26] and compared nitrotyrosine, nitrate/nitrite, and nitrosothiol formation in these lungs with that in control lungs.

Materials and methods

A mucoid *Pseudomonas aeruginosa* strain, isolated from a patient with cystic fibrosis, was used to prepare the inoculum for all experiments. Chronic infection was produced by incorporating the organism into agarose beads as previously described [25,26]. Adult male (300–400 g) specific pathogen-free Sprague–Dawley rats (Harlan, Bicester, UK) were anaesthetized and inoculated with either *Pseudomonas aeruginosa* in agar beads (*Pseudomonas*-inoculated group) or agar beads alone (placebo-inoculated group) as previously described [15]. Isolation of lungs was carried out under anaesthesia 10–15 days post-inoculation as previously described [15]. In brief, isolated lungs were ventilated and artificially perfused by continuous recirculation of a mixture of blood and physiological saline solution. Samples of perfusate were removed after 30 and 90 min and stored at −80 °C with light excluded for later analysis of NO metabolites (NOx). Following the haemodynamic studies, bronchoalveolar lavage (BAL) was carried out and differential cell counts were performed [15,27].

After BAL, the lungs were inflated by intra-tracheal instillation of optimal cutting temperature embedding compound (OCT, Lennox Lab Supplies, Ireland). Random blocks of tissue were removed from the lungs, frozen in OCT, and cryostat sections from each block were prepared on gelatine-coated slides. After BAL, immunoperoxidase localization of nitrotyrosine was carried out with an anti-nitrotyrosine antibody (dilution 1/100; TCS Biologicals, UK). Negative control slides included (a) the standard staining procedure with omission of the anti-nitrotyrosine antibody; (b) substitution of pre-immune rabbit serum; and (c) the standard procedure with substitution of affinity-purified polyclonal rabbit anti-neuronal pentraxin antibody for the anti-nitrotyrosine antibody. No immunostaining was observed in the negative controls. Specificity was confirmed by demonstrating complete absence of staining when the antibody (dilution 1/100) was pre-incubated with 10 mM nitrotyrosine. Some sections were incubated prior to immunostaining, with 1 mM sodium nitrite, 1 mM hydrogen peroxide in acetate buffer (Sigma), pH 5.0, at room temperature. This solution artificially nitrates tyrosine residues in the tissue that subsequently can be stained with the anti-nitrotyrosine antibody (TCS Biologicals, UK). Immunostaining of iNOS (TCS Biologicals, UK) was carried out using a previously described method [15]. Intensity of immunoperoxidase staining was assessed independently by three blinded reviewers, who used a semi-quantitative scale (0–4) to score each lung. The final score assigned to an individual lung was the mean of the three independent determinations. Reviewers were given reference slides representative of scores of 0, 2, and 4, to which they could refer at any stage during the scoring procedure.

The number of cells intensely stained for nitrotyrosine was quantified using the double dissector [28–30] as previously described [27]. For the dissector analysis, random pairs of serial sections separated by 7 μm were obtained by optical sectioning (water immersion objective × 40, numerical aperture 1.2) using confocal microscopy (Biorad, MRC 1024). Calibration of images in the X- and Y-axes was carried out using calibration graticules, while in the Z-direction calibration was confirmed using fluorescent micro-spheres of known diameters (Cambridge Bioscience, Cambridge, UK). The concentrations of total NOx (NO, nitrate, nitrite, and nitrosothiols) in lung perfusate and BAL fluid were determined by reduction of NO metabolites in a vanadium chloride solution and assay of the evolved NO by chemiluminescence [31]. Nitrotyrosine concentrations in BAL fluid were measured by enzyme-linked immunosorbent assay (ELISA; Cayman Chemical Co, Ann Arbor, MI, USA). Due to technical difficulties with the assays of NOx and nitrotyrosine, some samples were lost, so that for these determinations $n = 6$ or 7.

Data are presented as mean values (± SEM) and statistical comparisons of means were made using unpaired t-tests [32].

Results

Mucoid colonies of *Pseudomonas aeruginosa* were grown on blood agar plates from the BAL fluid obtained from each chronically infected lung. Organisms were not isolated from the placebo-inoculated lungs. The mean total cell count in the BAL fluid from *Pseudomonas*-infected lungs was significantly greater than that in the placebo-inoculated group (Table 1). Differential cell counts showed that the mean percentages of neutrophils and lymphocytes

Table 1. Mean (± SEM) total and differential cell counts in BAL fluid from *Pseudomonas*-inoculated and placebo-inoculated lungs

	***Pseudomonas*-inoculated ($n = 8$)**	**Placebo-inoculated ($n = 8$)**
Total cell count ($\times 10^7$ cells/ml)	21.3 (7.2)*	3.5 (0.4)
Neutrophils (% of total)	26.2 (5.0)*	5.1 (0.8)
Macrophages (% of total)	69.0 (5.2)*	92.9 (1.0)
Lymphocytes (% of total)	4.9 (1.5)*	2.0 (0.9)

* Significantly different from the placebo-inoculated group ($p < 0.05$, *t*-test).

in the *Pseudomonas*-infected lungs were significantly elevated above those in the placebo-inoculated group (Table 1).

On routine histological examination, lungs from placebo-inoculated animals showed a normal alveolar structure with no evidence of inflammatory cell infiltration (Figure 1A). In contrast, in the *Pseudomonas*-inoculated group, there was extensive thickening of the alveolar walls due to infiltration of inflammatory cells and loss of the normal alveolar structure in many areas (Figure 1B).

Nitrotyrosine staining was observed in both chronically infected and placebo-inoculated lungs (Figures 1C and 1D). In chronically infected lungs, a population of intensely staining cells was observed within the alveolar walls. Lighter staining of nitrotyrosine was detected in both the airway and the vascular smooth muscle cells, and in the epithelium of the larger airways (Figure 1D). In placebo-inoculated lungs, the pattern and intensity of nitrotyrosine staining were similar to those observed in the *Pseudomonas*-inoculated group, despite the absence of any evidence of chronic inflammation (Figure 1C). The mean score for nitrotyrosine staining in the chronically infected lungs, determined by blinded review, was not significantly different from the mean score for the placebo-inoculated group (Table 2).

Immunostaining for NOS-2 demonstrated that *Pseudomonas*-inoculated lungs showed increased expression of NOS-2 when compared with the placebo-inoculated group (Figure 2). The mean score for iNOS staining in the chronically infected lungs, determined by blinded review, was significantly different from the mean score for the placebo-inoculated group (Table 2).

Table 2. Mean (± SEM) intensity of immunostaining for nitrotyrosine and NOS-2 in *Pseudomonas*-inoculated and placebo-inoculated lungs

	***Pseudomonas*-inoculated ($n = 8$)**	**Placebo-inoculated ($n = 8$)**
Nitrotyrosine score	3.2 (0.3)	3.2 (0.5)
NOS-2 score	2.7 (0.2)*	1.3 (0.3)

No significant difference in the intensity of nitrotyrosine immunostaining in the *Pseudomonas*-inoculated and placebo-inoculated groups.
* Significantly different from the placebo-inoculated group ($p < 0.05$, *t*-test).

To quantify the total number of cells intensely staining for nitrotyrosine, immunofluorescence labelling of the anti-nitrotyrosine antibody was undertaken and visualized using confocal microscopy (Figure 3). The mean number ($\times 10^7$) of intensely labelled cells per left lung in the chronically infected group ($n = 8$) was 18.0 (±2.0), which was not significantly different from that (17.0 ± 3.0) in the placebo-inoculated lungs ($n = 8$). Based on our observations in the immunoperoxidase-stained control tissue, we suspected that the intensely stained cells scattered throughout the lung parenchyma were macrophages. Double immunofluorescence staining with the anti-nitrotyrosine antibody and an antibody against a cell surface marker of rat macrophages (anti-ED 1) showed that most cells that stained intensely for nitrotyrosine were also labelled by the anti-ED 1 antibody (data not shown).

The mean nitrotyrosine concentration in BAL fluid from chronically infected ($n = 6$) lungs (28.8 ± 0.7 ng/ml) was not significantly different from that in placebo-inoculated (29.7 ± 1.1 ng/ml) lungs ($n = 6$). Similarly, the mean NO metabolite concentration in BAL fluid from chronically infected rats ($n = 6$) was 4.9 (±1.6) μM, not significantly different from that in placebo-inoculated (2.7 ± 0.3 μM) animals ($n = 6$). The mean concentrations of NO metabolites in the perfusate of isolated lungs following 30 min and 90 min of recirculation were not significantly different in the chronically infected and placebo-inoculated groups (Table 3).

Discussion

To establish chronic pulmonary *Pseudomonas* infection, we used a previously described model that leads to pathological changes characteristic of bronchiectasis [25,26]. We confirmed that chronic infection was successfully established by demonstrating that *Pseudomonas* organisms could be isolated from BAL fluid of the infected lungs. The elevated total cell counts in this BAL fluid, together with an increased proportion of neutrophils and lymphocytes, are compatible with an airway inflammatory response to chronic infection. Furthermore, the histopathological changes that we observed in chronically infected lungs were similar to those previously reported in this model and also to those observed in bronchiectasis in humans [25,26]. In placebo-inoculated lungs, there was no elevation in the total cell counts in BAL fluid and no histological evidence of lung damage, and *Pseudomonas aeruginosa* organisms were not found. iNOS expression was markedly increased in the chronically infected lungs when compared with the agar-inoculated control lungs, a finding in agreement with previous reports of increased iNOS expression in chronically infected rat

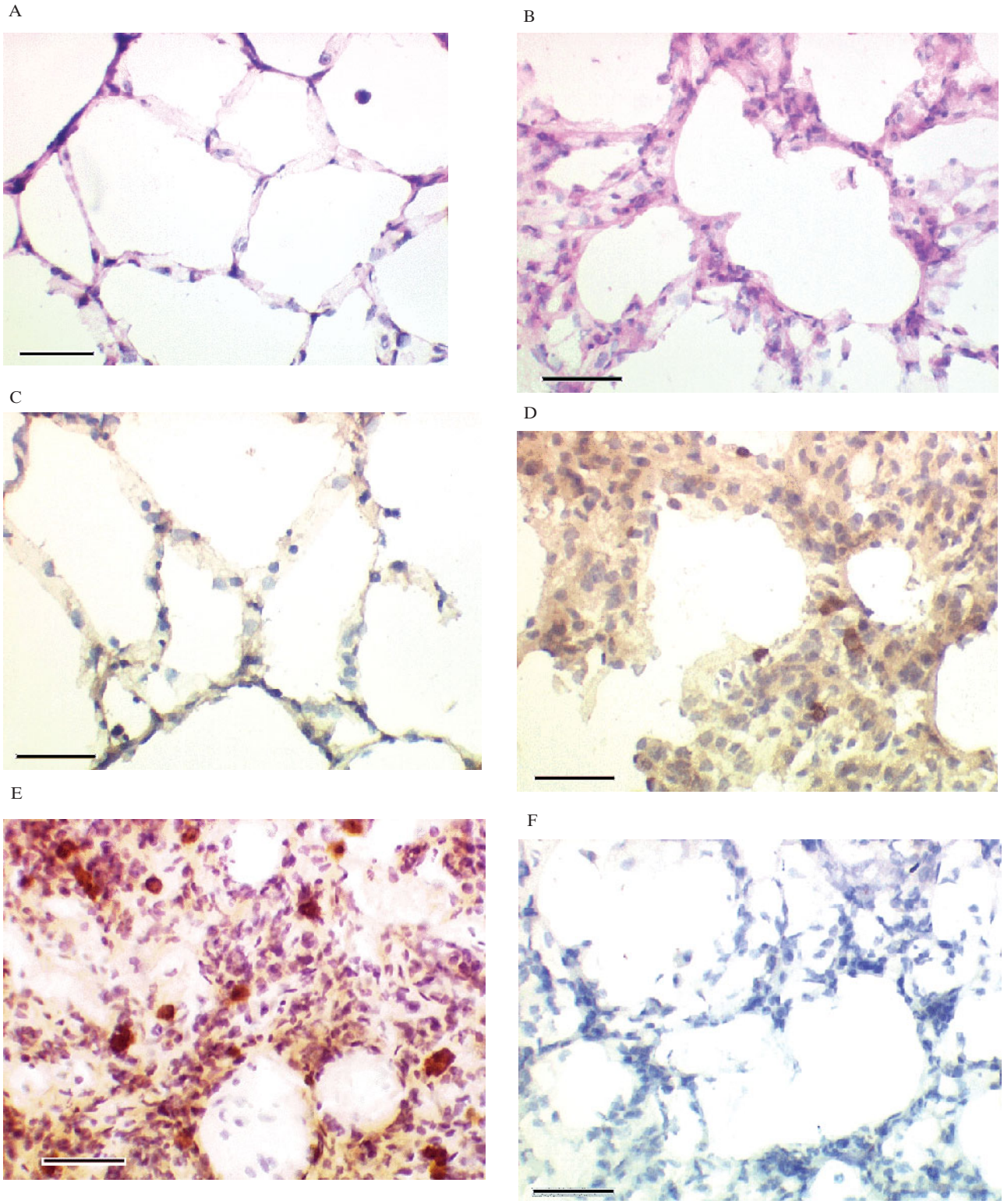

Figure 1. Photomicrographs of placebo- and *Pseudomonas*-inoculated lung tissue. (A) Placebo-inoculated lungs showing a normal alveolar structure with no evidence of inflammatory cell infiltration. (B) Chronically infected lung tissue showing extensive thickening of the alveolar walls due to infiltration of inflammatory cells and loss of the normal alveolar structure in many areas. (C) Photomicrograph of immunostaining for nitrotyrosine in placebo-inoculated lung tissue showing intense staining of nitrotyrosine in alveolar walls. (D) *Pseudomonas*-inoculated lung tissue showing intense immunoperoxidase staining of nitrotyrosine in alveolar walls, similar to that observed in placebo-inoculated lungs. (E) Chronically infected lung tissue pre-incubated with a solution that artificially nitrates tyrosine residues, which will subsequently stain with the nitrotyrosine antibody. (F) Absence of staining in *Pseudomonas*-inoculated lung tissue when polyclonal rabbit anti-nitrotyrosine antibody was pre-incubated with nitrotyrosine. Scale bars = 40 μm

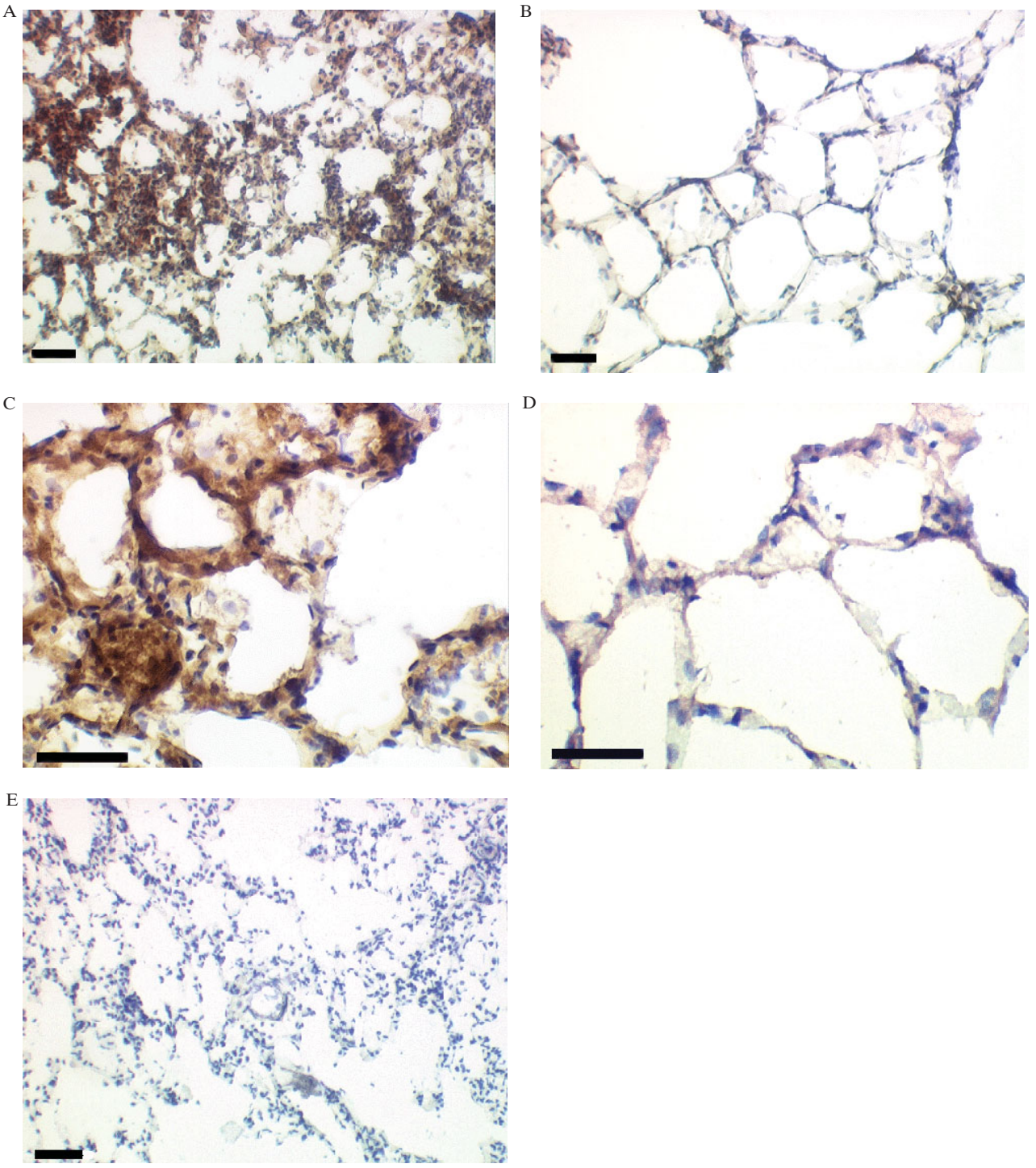

Figure 2. Photomicrographs of placebo- and *Pseudomonas*-inoculated lung tissue (14 μm cryosections) immunostained (brown) for inducible nitric oxide synthase (iNOS). (A) Low-power photomicrograph of *Pseudomonas*-inoculated lung showing extensive intense immunoperoxidase staining of iNOS in the gas exchange region of lungs (×10 objective). (B) Low-power photomicrograph of placebo-inoculated lung tissue showing minimal immunostaining for iNOS in the gas exchange region (×10 objective). (C) Higher-power photomicrograph of *Pseudomonas*-inoculated lung tissue showing intense staining of iNOS within the alveolar walls (×20 objective). (D) Higher-power photomicrograph of placebo-inoculated lung tissue showing minimal iNOS staining within the alveolar walls, compared with *Pseudomonas*-inoculated lung (×20 objective). (E) Absence of staining in *Pseudomonas*-inoculated lung tissue when the polyclonal rabbit anti-iNOS antibody was pre-incubated with iNOS peptide (× 10 objective). This section was obtained from the same lung as that in panel A. Scale bars = 40 μm

lungs [14,15]. Similar elevation of iNOS expression occurs in human bronchiectasis [33]. Taken together, these findings demonstrate that the technique of *Pseudomonas* inoculation leads to chronic infective lung damage and increased iNOS expression. Furthermore, the damage observed was specifically due to chronic infection and was not secondary to the inoculation of agar beads.

Our experiments were undertaken to test the hypothesis that in chronic lung infection, excess NO derived from NOS-2 activity is rapidly scavenged by reaction with superoxide radical, producing peroxynitrite, which causes abnormal nitration and nitrosation reactions. To examine NO metabolism through nitration pathways, we assessed nitrotyrosine formation in the lung tissue and in airway fluid. Our finding that nitrotyrosine residues were present in control lungs (Table 3) is compatible with previous reports that nitrotyrosine is present in normal lungs [34–37]. However, there was no detectable difference between *Pseudomonas*-infected and control lungs, either in the number of nitrotyrosine-stained cells or in the intensity of nitrotyrosine staining (Table 2), both of which argue against the hypothesis that there was increased peroxynitrite production in the chronically infected lungs. The finding that nitrotyrosine concentrations in the BAL fluid of chronically infected lungs were not elevated above control values also indicates that there was no excess of peroxynitrite production in these circumstances. This also argues against the hypothesis that there was excess peroxynitrite formation in the chronically infected lungs. These data support recent evidence that in iNOS knockout mice, nitrotyrosine formation in the presence of lung infection is not reduced compared with wild-type controls, which implies that the iNOS expressed in these conditions in wild types does not contribute to nitrotyrosine formation in the lung [38]. However, the role of iNOS in this regard may be disease-specific, as others have reported that in other models of lung inflammation, inhibition of iNOS activity markedly reduced nitrotyrosine formation [39,40].

The observation that in isolated lungs, the rate of accumulation of NOx was not different in *Pseudomonas*-inoculated and placebo-inoculated lungs (Table 3) strongly supports the finding that nitrotyrosine formation was not different in the two

Table 3. Mean concentrations of the stable end products of NO metabolism (NOx) in the perfusate of the isolated lungs over 30-min and 90-min periods

	n	NOx concentration (μM/30 min)	NOx concentration (μM/90 min)
Pseudomonas-inoculated	7	3.0 (0.6)	3.2 (0.8)
Placebo-inoculated	6	3.2 (0.4)	4.3 (0.5)

Values are means (±SEM). NOx concentration indicates the concentration of NO metabolites in isolated lung perfusate over 30-min and 90-min periods.

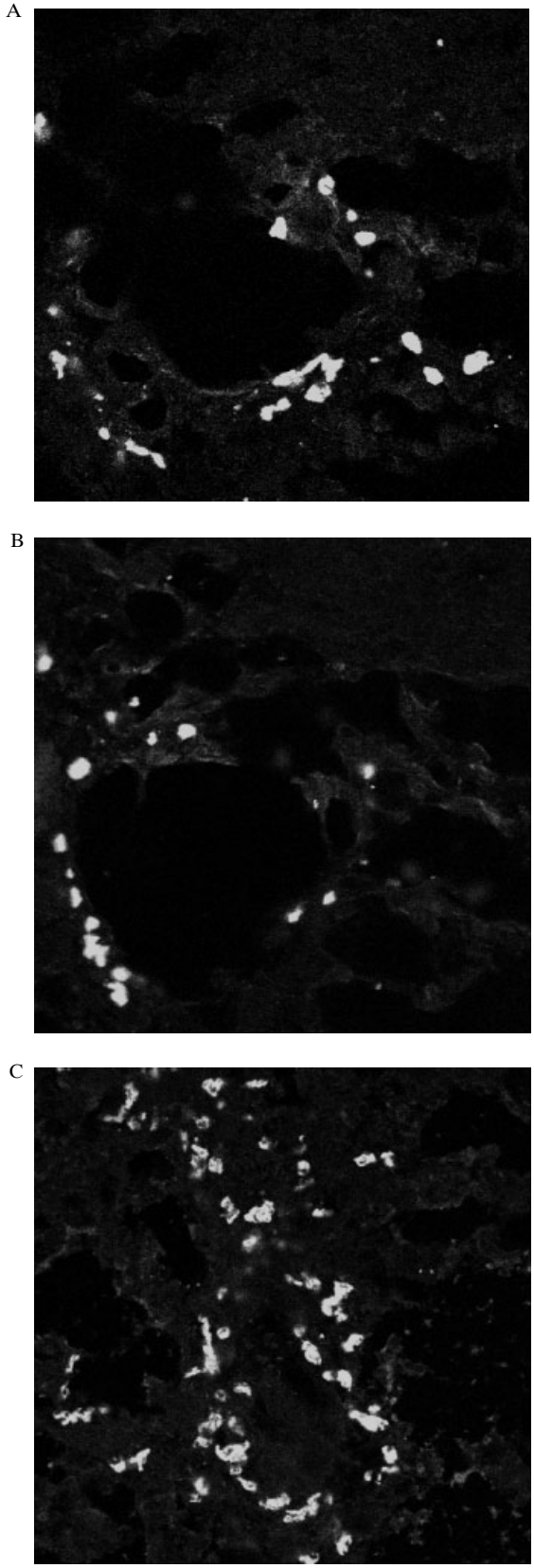

Figure 3. (A) Photomicrograph of chronically infected lung tissue showing cells immunofluorescently staining for nitrotyrosine labelled with FITC. (B) Image of a serial section separated from that in panel A by 7 μm in the *Z*-direction. Cells were immunofluorescently stained for nitrotyrosine using FITC. (C) Photomicrograph of placebo-inoculated lung tissue showing cells immunofluorescently staining for nitrotyrosine labelled with FITC

groups. In addition to nitration reactions, peroxynitrate can undergo intramolecular rearrangement to form nitrate anion, which can be detected by the vanadium chloride-based reduction assay used in the present series of experiments [31]. We found no evidence of the production of large quantities of nitrate anion, behaviour considered to be typical of iNOS [1–3], despite the increased expression of this isoform that was observed (Table 3). These findings support previous reports of increased iNOS expression in chronic lung infection without evidence of high output NO production [14,15]. It is interesting to note that increased iNOS expression without increased activity has been previously reported in chronically hypoxic lungs [41,42]. Taken together, these data call into question the paradigm that iNOS, once expressed, leads to sustained high output of NO.

While our findings support previous reports that iNOS can be expressed in the lung without causing elevated NO production, we have extended those previous findings in an important way. Peroxynitrite can support nitrosation reactions, leading to the formation of nitrosothiols. Indeed, S-nitrosylation can be the preferred nitrosation reaction under physiological conditions [23,43]. Such nitrosation pathways can exert biological effects distinct from those of NO and mediated by signalling mechanisms that are independent of NO [44–46]. In previous experiments on chronically infected lungs, NO metabolites were measured by enzymatic reduction and the Griess reagent, a method that detects only nitrate and nitrite. Thus, there remained the possibility that in the tissue environment of chronic infection, excess NO derived from NOS-2 activity was metabolized to alternative end products such as polynitrosylated and nitrated proteins, which are not detected by the Griess reaction [31]. In the present series of experiments, NO metabolites were measured by reduction in the presence of vanadium chloride and subsequent chemiluminescent detection of the evolved gaseous NO, a technique that, in addition to measuring NO_2 and NO_3, also detects NO bound to thiol groups [31]. Thus, the initial hypothesis predicted that excess nitrosothiol would have been observed in the chronically infected group, either alone or together with excess nitrate formation. However, this was not the case, either in BAL fluid or in lung perfusate (Table 3). Thus, we could find no evidence of preferential metabolism of NO via nitrosation reactions.

What are the potential mechanisms that might account for our findings of increased expression without increased activity? One possible explanation is that reduced substrate or co-factor availability might have impaired NOS activity, as has been previously reported in both pulmonary and cardiovascular diseases [47–50]. A second possible explanation is suggested by our previous observation that eNOS was down-regulated following chronic lung infection [15]. Relatively normal NO metabolite production could have been achieved if decreased eNOS activity was counterbalanced by increased iNOS activity. Finally, it is possible that NO produced in the airway epithelium was immediately excreted into the airway lumen without being further metabolized to higher oxides or via nitrosylation pathways. Such a circumstance might occur if superoxide radical production was not elevated for some unsuspected reason. Elucidation of the precise mechanisms involved will require further specifically designed experiments.

In summary, we have demonstrated that in chronically infected lungs, NOS-2 expression was increased but that there was no corresponding increase in total NO metabolites, including NO_2, NO_3, nitrosothiols or nitrotyrosine residues, when compared with placebo-inoculated lungs. These data imply that increased iNOS expression does not contribute to peroxynitrite production and nitrotyrosine formation in chronically infected lungs.

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