Decreased peroxynitrite inhibitory activity in induced sputum in patients with bronchial asthma

H Kanazawa, S Shiraiishi, K Hirata, J Yoshikawa

Background: The production of peroxynitrite, an extremely potent oxidant, is increased in inflammatory lung disease. It is therefore important to measure antioxidant activity against peroxynitrite in epithelial lining fluid to examine the physiological effects of peroxynitrite in the airways of patients with asthma. This study was designed to determine whether peroxynitrite inhibitory activity in induced sputum is correlated with clinical characteristics and airway inflammatory indices in asthmatic patients.

Methods: Inflammatory indices were measured in induced sputum from 25 patients with asthma and 12 normal control subjects. Peroxynitrite inhibitory activity was also measured by monitoring rhodamine formation in sputum samples.

Results: Peroxynitrite inhibitory activity in induced sputum was significantly lower in asthmatic patients (52.4 ± 24.5%) than in normal control subjects (92.1 ± 3.9%, p<0.0001). Its activity was significantly correlated with forced expiratory volume in 1 second (FEV1) % predicted (r=-0.758, p<0.0001) and bronchial hyperreactivity to methacholine (r=0.464, p=0.023). There was a significant negative correlation between peroxynitrite inhibitory activity and the degree of eosinophilic airway inflammation (% eosinophils, r=-0.758, p=0.0001; eosinophil cationic protein, r=-0.780, p<0.0001).

Conclusions: Decreased peroxynitrite inhibitory activity occurs in induced sputum of asthmatic patients. Since even in patients with stable asthma the airway lining fluid lacks peroxynitrite inhibitory activity, large amounts of peroxynitrite, which are further increased during an acute asthma attack, would not be completely inactivated and asthmatic airways might have markedly increased susceptibility to peroxynitrite induced airway injury.
returned to baseline value. The sputum sample diluted with phosphate buffer solution (PBS) containing dithiothreitol (DTT, final concentration 1 mmol/l) was then centrifuged at 400g for 10 minutes and the cell pellet was resuspended. Total cell counts were performed with a haemocytometer and slides were made using a cytospin (Cytospin 3; Shandon, Tokyo, Japan) and stained with May-Grunwald-Giemsa stain for differential cell counts. The supernatant was stored at –70°C for subsequent assay for eosinophil cationic protein (ECP). The ECP concentration was measured using a radioimmunoassay kit (Pharmacia Diagnostics, Uppsala, Sweden). The sol phase was obtained by ultracentrifuging the supernatant for 60 minutes at 4°C; this was stored at –70°C for subsequent assay for peroxynitrite inhibitory activity. All subjects produced an adequate specimen of sputum of at least 2 ml which, on differential cell counting, contained <10% squamous cells.

Measurement of peroxynitrite inhibitory activity

Working solutions of peroxynitrite (Wako Pure Chemical Industries Ltd, Osaka Japan) were prepared by dilution in phosphate buffer solution (PBS) containing dithiothreitol (DTT, 0.1 N NaOH just before use as 10⁻² mol/l solutions, and further dilutions were made in PBS. The peroxynitrite concentration was determined spectrophotometrically by measuring the absorption at 302 nm (εM=1670 M⁻¹ cm⁻¹). Peroxynitrite readily oxidises dihydrorhodamine-123 whereas superoxide anion, H₂O₂, and NO alone do not. A standard curve of oxidising activity of dihydrorhodamine-123 to rhodamine was constructed with peroxynitrite. Peroxynitrite inhibitory activity was assayed by monitoring rhodamine formation at 500 nm in reaction mixtures containing 200 µl sputum, 1.3 ml dihydrorhodamine-123 diluted with PBS (pH 7.4), and 500 µl peroxynitrite for 30 minutes at 37°C. Peroxynitrite inhibitory activity was assayed in at least triplicate and reproducibility of the assay was confirmed by repeat measurements in the same subjects on separate days. A recent study supports the specificity of this assay system for peroxynitrite.

**Statistical analysis**

All values are presented as mean (SD). The Mann-Whitney U test was used for intergroup comparisons and the significance of correlations was evaluated by determining Spearman rank correlation coefficients. A p value of less than 0.05 was considered significant.

**RESULTS**

Baseline lung function and bronchial hyperreactivity to methacholine in the 25 asthmatic patients are shown in table 1. The percentage of eosinophils and concentration of ECP in induced sputum were significantly higher in asthmatic patients (% eosinophils 15.6 (9.1)%, p<0.0001; ECP 699.6 (408.9) ng/ml, p<0.0001) than in control subjects (% eosinophils 0.78 (0.50)%; ECP 118.3 (40) ng/ml).

Peroxynitrite inhibitory activity in induced sputum was significantly lower in asthmatic patients than in control subjects (asthmatics 52.4 (24.5)%, normal controls 92.1 (3.9)%, p<0.0001; fig 1) and was significantly correlated with FEV₁ % predicted (r=–0.774, p<0.0001; fig 2) and bronchial hyperreactivity to methacholine (r=–0.464, p=0.023; fig 3). Moreover, there was a significant negative correlation between peroxynitrite inhibitory activity and the degree of eosinophilic airway inflammation (% eosinophils: r=–0.758, p<0.0001; ECP: r=–0.780, p<0.0001; fig 4).

**Table 1 Clinical characteristics of study subjects**

<table>
<thead>
<tr>
<th></th>
<th>Asthmatic patients (n=25)</th>
<th>Normal controls (n=12)</th>
</tr>
</thead>
<tbody>
<tr>
<td>M:F</td>
<td>14:11</td>
<td>7:5</td>
</tr>
<tr>
<td>Age (years)</td>
<td>39.8 (20–59)*</td>
<td>31.8 (24–40)</td>
</tr>
<tr>
<td>FEV₁ [%]</td>
<td>90.7 (75–115)**</td>
<td>115.7 (105–127)</td>
</tr>
<tr>
<td>P&lt;sub&gt;C&lt;/sub&gt;₉₀ methacholine (µg/ml)†</td>
<td>3.28 (2.80–3.96)</td>
<td>ND</td>
</tr>
<tr>
<td>Sputum Eosinophils [%]</td>
<td>15.6 (1.0–32.0)**</td>
<td>0.78 (0.1–1.5)</td>
</tr>
<tr>
<td>ECP (ng/ml)</td>
<td>699.6 (90–1400)**</td>
<td>118.3 (50–180)</td>
</tr>
</tbody>
</table>

Values are mean (range). FEV₁=forced expiratory volume in 1 second; P<sub>C</sub>₉₀=concentration of methacholine causing a 20% fall in FEV₁; ND=not determined. *p<0.05, **p<0.01 asthmatic patients vs normal controls. †Geometric mean.
The airways of asthmatic patients are often inflamed and it has been shown that the production of superoxide anion by airway inflammatory cells is increased. The concentration of NO in exhaled air of asthmatic patients is also increased, and we have previously found a higher concentration of NO derivatives in induced sputum of patients with asthma. From the existing evidence it is likely that peroxynitrite is formed in the respiratory tract. However, the cellular source of peroxynitrite in asthmatic airways is unclear. Previous studies have reported that the production of peroxynitrite is increased in inflammatory cells and eosinophils in asthmatic patients compared with normal control subjects. It is possible that decreased peroxynitrite inhibitory activity is likely to reflect the increases in reactive oxygen and nitrogen intermediates in asthmatic airway fluid. However, we did not measure peroxynitrite or nitric oxide levels in the airways of our asthmatic patients, nor did we determine peroxynitrite inhibitory factors in induced sputum as we did not directly measure the levels of airway thiols such as glutathione, cysteine, and albumin. In future studies we plan to measure the levels of peroxynitrite and airway thiols directly.

Barnes has suggested that peroxynitrite may indirectly exacerbate the airway inflammatory response by inducing the shedding of airway epithelial cells—which may occur even in patients with mild asthma—thereby exposing afferent nerve endings. This might induce the release of sensory neuropeptides through axon reflexes and result in bronchoconstriction, mucus hypersecretion, and microvascular leakage leading to oedema of the airway wall and extravasation of plasma into the airway lumen. We have also previously reported that peroxynitrite altered β2 adrenoceptor function and inactivated neutral endopeptidase in the airways. An imbalance of peroxynitrite stress/antioxidant defence may therefore contribute to the pathogenesis of bronchial asthma. The reaction of peroxynitrite with airway thiols is associated with their oxidation to the corresponding disulfide. Our method would evaluate the reduced form antioxidant activity against peroxynitrite. A previous study found that asthmatic patients have increased levels of total glutathione (oxidised plus reduced forms) in bronchoalveolar lavage fluid. However, it is important to emphasise that local antioxidant defences in asthmatic airway fluid are not completely inactivated, even in acute asthma exacerbation. In patients with stable asthma, large amounts of peroxynitrite inhibitory activity in the airway lining fluid of asthmatic airway fluid. However, it is important to emphasise that local antioxidant defences in asthmatic airways are not completely inactivated, even in acute asthma exacerbation, the reduced antioxidants are diminished and therefore the asthmatic airways have markedly increased susceptibility to peroxynitrite.

In conclusion, we have found decreased peroxynitrite inhibitory activity in the airway lining fluid of asthmatic patients. Since ELF lacks peroxynitrite inhibitory activity, even in patients with stable asthma, large amounts of peroxynitrite would not be completely inactivated, even in acute asthma attack, and the asthmatic airway might markedly increase the susceptibility to peroxynitrite induced airway injury.

ACKNOWLEDGEMENT
This work was supported by grant-in-aid for Scientific Research (13670611) from the Ministry of Education, Science and Culture, Japan.

Authors’ affiliations
H Kanazawa, S Shiraishi, K Hirata, J Yoshikawa, Department of Respiratory Medicine, Graduate School of Medicine, Osaka City University, 1-4-3 Asahi-machi, Abenoku, Osaka, 545-8585, Japan

REFERENCES
1 Beckman JS, Beckman TW, Chen J, et al. Apparent hydroxyl radical production by peroxynitrite: implication for endothelial injury from nitric oxide and superoxide. Proc Natl Acad Sci USA 1990;87:1620–4.

Figure 3 Correlation between peroxynitrite inhibitory activity in induced sputum and bronchial hyperreactivity to methacholine.

Figure 4 Correlation between peroxynitrite inhibitory activity in induced sputum and (A) percentage eosinophils and (B) ECP concentration in induced sputum. O normal controls; • asthmatic patients.

DISCUSSION
In this study peroxynitrite inhibitory activity was assayed by monitoring rhodamine formation since the oxidation of dihydrorhodamine-123 to rhodamine is mediated by peroxynitrite and not by either NO or superoxide anion alone. Using this method, we found that peroxynitrite inhibitory activity in induced sputum was significantly lower in asthmatic patients than in normal controls, and that its activity was correlated with the degree of airway obstruction and airway hyperreactivity to methacholine. We also found a significant negative correlation between peroxynitrite inhibitory activity in induced sputum and the degree of eosinophilic airway inflammation.

The airways of asthmatic patients are often inflamed and it has been shown that the production of superoxide anion by airway inflammatory cells is increased. The concentration of NO in exhaled air of asthmatic patients is also increased, and we have previously found a higher concentration of NO derivatives in induced sputum of patients with asthma. From the existing evidence it is likely that peroxynitrite is formed in the respiratory tract. However, the cellular source of peroxynitrite in asthmatic airways is unclear. Previous studies have reported that the production of peroxynitrite is increased in inflammatory cells and eosinophils in asthmatic patients compared with normal control subjects. It is possible that decreased peroxynitrite inhibitory activity is likely to reflect the increases in reactive oxygen and nitrogen intermediates in asthmatic airway fluid. However, we did not measure peroxynitrite or nitric oxide levels in the airways of our asthmatic patients, nor did we determine peroxynitrite inhibitory factors in induced sputum as we did not directly measure the levels of airway thiols such as glutathione, cysteine, and albumin. In future studies we plan to measure the levels of peroxynitrite and airway thiols directly.

Barnes has suggested that peroxynitrite may indirectly exacerbate the airway inflammatory response by inducing the shedding of airway epithelial cells—which may occur even in patients with mild asthma—thereby exposing afferent nerve endings. This might induce the release of sensory neuropeptides through axon reflexes and result in bronchoconstriction, mucus hypersecretion, and microvascular leakage leading to oedema of the airway wall and extravasation of plasma into the airway lumen. We have also previously reported that peroxynitrite altered β2 adrenoceptor function and inactivated neutral endopeptidase in the airways. An imbalance of peroxynitrite stress/antioxidant defence may therefore contribute to the pathogenesis of bronchial asthma. The reaction of peroxynitrite with airway thiols is associated with their oxidation to the corresponding disulfide. Our method would evaluate the reduced form antioxidant activity against peroxynitrite. A previous study found that asthmatic patients have increased levels of total glutathione (oxidised plus reduced forms) in bronchoalveolar lavage fluid. However, it is important to emphasise that local antioxidant defences in asthmatic airway fluid are not completely inactivated, even in acute asthma exacerbation. In patients with stable asthma, large amounts of peroxynitrite inhibitory activity in the airway lining fluid of asthmatic patients. Since ELF lacks peroxynitrite inhibitory activity, even in patients with stable asthma, large amounts of peroxynitrite would not be completely inactivated, even in acute asthma attack, and the asthmatic airway might markedly increase the susceptibility to peroxynitrite induced airway injury.

ACKNOWLEDGEMENT
This work was supported by grant-in-aid for Scientific Research (13670611) from the Ministry of Education, Science and Culture, Japan.

Authors’ affiliations
H Kanazawa, S Shiraishi, K Hirata, J Yoshikawa, Department of Respiratory Medicine, Graduate School of Medicine, Osaka City University, 1-4-3 Asahi-machi, Abenoku, Osaka, 545-8585, Japan

REFERENCES
1 Beckman JS, Beckman TW, Chen J, et al. Apparent hydroxyl radical production by peroxynitrite: implication for endothelial injury from nitric oxide and superoxide. Proc Natl Acad Sci USA 1990;87:1620–4.


Clinical Evidence—Call for contributors

Clinical Evidence is a regularly updated evidence based journal available world wide both as a paper version and on the internet. Clinical Evidence urgently needs to recruit a number of new contributors. Contributors are health care professionals or epidemiologists with experience in evidence based medicine and the ability to write in a concise and structured way.

We are presently interested in finding contributors with an interest in the following clinical areas:

- Acute bronchitis
- Acute sinusitis
- Cataract
- Genital warts
- Hepatitis B
- Hepatitis C
- HIV

Being a contributor involves:

- Appraising the results of literature searches (performed by our Information Specialists) to identify high quality evidence for inclusion in the journal.
- Writing to a highly structured template (about 1500–3000 words), using evidence from selected studies, within 6–8 weeks of receiving the literature search results.
- Working with Clinical Evidence Editors to ensure that the text meets rigorous epidemiological and style standards.
- Updating the text every eight months to incorporate new evidence.
- Expanding the topic to include new questions once every 12–18 months.

If you would like to become a contributor for Clinical Evidence or require more information about what this involves please send your contact details and a copy of your CV, clearly stating the clinical area you are interested in, to Polly Brown (pbrown@bmjgroup.com).
Decreased peroxynitrite inhibitory activity in induced sputum in patients with bronchial asthma
H Kanazawa, S Shiraishi, K Hirata and J Yoshikawa

Thorax 2002 57: 509-512
doi: 10.1136/thorax.57.6.509

Updated information and services can be found at: http://thorax.bmj.com/content/57/6/509

These include:

References
This article cites 16 articles, 3 of which you can access for free at:
http://thorax.bmj.com/content/57/6/509#BIBL

Email alerting service
Receive free email alerts when new articles cite this article. Sign up in the box at the top right corner of the online article.

Topic Collections
Articles on similar topics can be found in the following collections

- Asthma (1782)
- Airway biology (1100)
- Inflammation (1020)
- Lung function (773)

Notes

To request permissions go to:
http://group.bmj.com/group/rights-licensing/permissions

To order reprints go to:
http://journals.bmj.com/cgi/reprintform

To subscribe to BMJ go to:
http://group.bmj.com/subscribe/