

ORIGINAL ARTICLE

The effect of N-acetylcysteine on Clara cells and Clara cell 16 kDa protein in a murine model of allergen-induced airway inflammation

XIAOMENG NIE, QIANG LI, GANG CAI, YIMIN DAI AND JUN ZHANG

Department of Respiratory Medicine, Changhai Hospital, Shanghai, China

The effect of N-acetylcysteine on Clara cells and Clara cell 16 kDa protein in a murine model of allergen-induced airway inflammation

NIE X, LI Q, CAI G, DAI Y, ZHANG J. *Respirology* 2005; 10: 157–163

Objective: The aim of this study was to investigate the number of Clara cells and the production and secretion of Clara cell 16 kDa protein (CC16) in a murine model of allergen-induced airway inflammation, as well as the effects of N-acetylcysteine (NAC) on CC16 and Clara cell numbers, in order to determine the mechanism of the anti-inflammatory effect of NAC.

Methodology: BALB/c mice were divided into control, ovalbumin (OVA) and NAC groups. An allergen-induced airway inflammation model (OVA group) was established by sensitizing and challenging mice with OVA. NAC was administered as an oral treatment. The number of Clara cells and the production of CC16 were determined by immunohistochemistry. The CC16 levels in bronchoalveolar lavage fluid (BALF) were determined by Western blotting.

Results: The proportion of Clara cells in terminal and respiratory bronchioles significantly decreased in the OVA group compared to the control group ($P < 0.01$). NAC treatment did not change the proportion of Clara cells in the OVA group ($P > 0.05$). CC16 production by Clara cells in the OVA groups was significantly lower than that of the control group ($P < 0.01$), but was elevated following NAC treatment ($P < 0.05$). The CC16 level in BALF of the OVA group was lower than that of the control group ($P < 0.01$), but was elevated by NAC treatment ($P < 0.05$). NAC reduced the total number of white cells and the percentage of eosinophils in BALF. Moreover, it inhibited airway inflammation.

Conclusions: The number of Clara cells and the production and secretion of CC16 were reduced in a murine model of allergen-induced airway inflammation. Antioxidants can enhance the expression of CC16, which might be a mechanism by which they suppress airway inflammation.

Key words: allergen-induced airway inflammation, Clara cell 16 kDa protein, Clara cell, N-acetylcysteine.

INTRODUCTION

Clara cells are non-ciliated cells that are mainly present in the epithelium of mammalian peripheral conducting airways. Clara cell 16 kDa protein (CC16) is the predominant product of these cells. The protein has a molecular weight of 16 908 Da in the rat,¹ and 15 840 Da in humans.² There is growing evidence that CC16 may inhibit the activity of phospholipase (PL)A₂,³ a key enzyme in the production of prostag-

landins and leukotrienes, as well as the production and activity of interferon (IFN)- γ . Therefore, CC16 may act as an important endogenous anti-inflammatory molecule. Some studies have suggested that airway inflammation causes a reduction in the number of Clara cells, as well as in the concentration of CC16 in bronchoalveolar lavage fluid (BALF).^{4,5} The objective of the present study was to investigate the potential role of Clara cells and CC16 in allergen-induced airway inflammation. We assessed the number of Clara cells and CC16 production by immunohistochemistry and determined CC16 levels in BALF by Western blotting. Increasingly, clinical, epidemiological and experimental evidence indicates that there may be excessive production of reactive oxygen and nitrogen species as well as defective endogenous antioxidant defence mechanisms in asthma.^{6,7} Therefore, antioxidant therapy might be of clinical benefit in the treatment of asthma. As such, we investigated the

Correspondence: Xiaomeng Nie, Department of Respiratory Diseases, Changhai Hospital, 174 Changhai road, Shanghai 200433, China.
Email: xmnjie@hotmail.com

Received 31 December 2003; revised 2 November 2004; accepted for publication 7 December 2004.

effects of the antioxidant drug, N-acetylcysteine (NAC), on Clara cells and CC16, and also its effect on allergen-induced airway inflammation.

MATERIALS AND METHODS

Reagents

Ovalbumin (OVA; grade V), poly L-lysine, 3,3'-diaminobenzidine and bovine serum albumin (BSA) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Prestained protein marker, nitrocellulose membrane (0.45 μ m) and 5-bromo-4-chloro-3-indolyl phosphate/nitroblue tetrazolium (BCIP/NBT) were obtained from Watson Co. Ltd. (Shanghai, China). Goat polyclonal antibody against murine CC16 was purchased from Santa Cruz (Santa Cruz, California, USA). Biotinylated pig antigoat IgG, alkaline phosphatase-conjugated rabbit antigoat IgG and streptavidin-horseradish peroxidase (HRP) conjugate were supplied from Zhongshan Biotechnology Co. Ltd. (Beijing, China). The bicinchoninic acid (BCA) kit for protein determination was from Shenergy Biocolor Biological Science & Technology Co. (Shanghai, China). The Centrивap concentrator was from Labconco Co. (Kansas City, MO, USA). The results of Western blotting were analyzed on a Fluro-S multiimager (Bio-Rad Laboratories, Hercules, CA, USA).

Animals

Male and female BALB/c mice (6 weeks of age) of clean grade were obtained from Sippr/DK Co. Ltd. (Shanghai, China), and weighed approximately 15–20 g. The animals were housed in an air-conditioned room (25°C, 50% relative humidity) with a 12-h light : dark cycle.

Allergen sensitization and challenge of the mice

The mice were divided into three groups (control, OVA and NAC), with 10 mice in each group. As pulmonary inflammation with eosinophil (EO) infiltration is a prominent feature of allergic respiratory diseases, repeated sensitization and challenge of the mice with OVA was the method used to induce severe pulmonary eosinophilia in the mice.⁸ After preliminary experiments, we sensitized the OVA and NAC groups by intraperitoneal injection of a 0.2 mL suspension of 20 μ g OVA and 2.6 mg aluminium hydroxide on days 0, 7 and 14. Mice in the control group received an injection of normal saline instead of the OVA/aluminium hydroxide suspension. On day 21, allergen challenge was performed by dropping 0.05 mL of a solution of 2 mg/mL OVA diluted in normal saline into the nose of the mice in both the OVA and NAC groups. OVA challenge was performed for 6 consecutive days until day 26. Control mice were subjected to normal saline inhalation under the same conditions. As Kung *et al.*⁹ had observed, there were

few EO in the lung tissue and BALF 4–8 h after challenge, but by 24 h the number of EO had peaked in the lung and we sacrificed the mice 24 h after challenge. On days 15–26, a solution of 230 mg/kg NAC diluted in pure water was administered orally by gavage, as a single dose per day, to the NAC group. On days 21–26, NAC administrations were carried out 1 h before OVA inhalation. Oral NAC was selected, as this is the usual method of administration in the clinical setting. We used a relatively low dose of NAC, which has been reported to be effective against asthmatic inflammation in rats.¹⁰

Bronchoalveolar lavage

All mice were sacrificed 24 h after the last OVA challenge. Lungs were lavaged with 1 mL of normal saline via a tracheal cannula on three occasions.¹¹ BALF was centrifuged (1000 r.p.m., 10 min, 4°C) and the resultant cell-free supernatant was stored at –80°C until analysis. The cell pellet was resuspended in 200 μ L of normal saline. Aliquots of the cell suspension were used to determine the cell numbers using a haemocytometer. Cytospin samples were used for differential cell counts after staining with May–Grünwald–Giemsa. A total of 200 cells were counted in each cytospin sample.

Histological examination and immunohistochemistry

Lungs were fixed in 10% neutralized buffered formalin and embedded in paraffin. Serial 4- μ m sections were obtained from blocks. Some sections were mounted on common glass slides for haematoxylin and eosin (HE) staining and the others were mounted on poly L-lysine – coated glass slides, deparaffinized in xylene, rehydrated in graded alcohol, and rinsed in distilled water. Cells containing CC16 were localized in lung sections with a goat polyclonal antibody against murine CC16 using a slightly modified streptavidin-biotin immunoperoxidase method.¹² Before immunostaining, rehydrated sections were immersed in 0.3% hydrogen peroxide for 30 min, then placed in plastic jars containing 10 mM citrate buffer in distilled water, pH 6. The jars were microwaved at 98°C for 10 min to unmask antigens and increase the overall reaction sensitivity. After being cooled for about 20 min, the pretreated sections were washed in phosphate-buffered saline (PBS) and incubated in 10% normal pig serum in PBS for 20 min. Slides were then incubated sequentially at 37°C in the following solutions: (i) goat polyclonal antibody against murine CC16 (diluted 1:4000) for 1 h; (ii) biotinylated pig antigoat IgG (diluted 1:200) for 30 min; and (iii) streptavidin-HRP conjugate (diluted 1:200) for 30 min. Bound HRP activity was visualized by incubation with 0.02% 3,3'-diaminobenzidine-0.01% H₂O₂ in PBS, producing a dark brown plasma immunostaining. After each step of the immunostaining procedure, the sections were rinsed in PBS three times. The sections were finally counterstained with haematox-

mlin, dehydrated, and mounted with a permanent mounting medium.

Controls for the specificity of immunolabelling included the omission of the primary antibody or the substitution of the primary antibody with non-immune serum. In each case, these assays confirmed the specificity of the observed immunolabelling. In addition, the antiserum used in this study was checked to ensure it gave clear-cut immunostaining patterns on control lung sections, namely labelling restricted to Clara cells. The analysis of CC16-immunoreactive cells was performed on one lung section per experimental animal by scanning at a magnification of 400. Five terminal and five respiratory bronchioles were selected at random in the section. Only nucleated epithelial cells were counted manually. The total number of epithelial cells was assessed by counting all nuclei located above the basement membrane. A cell was identified as a Clara cell when a typical dome-shaped cytoplasm was distinctly immunostained for CC16. The proportion of Clara cells was calculated. A total of 50 immunoreactive cells (Clara cells) were selected randomly from the terminal or respiratory bronchioles of each lung section and, therefore, there were 500 immunoreactive cells from each group. We estimated the numbers of cells showing different grades of immunostaining: (–) no immunostaining; (+) scarce brown immunostaining; (+) 1/2 plasma area was immunostained brown; (++) 3/4 plasma area was immunostained brown; and (+++) all of the plasma area was immunostained brown.

Western blot analysis

All BALF supernatants were lyophilized and the total protein concentrations were measured by the BCA assay. Protein (10 µg) from each sample was electrophoresed on 15% sodium dodecylsulfate-polyacrylamide gels.^{13,14} Western transfer to a nitrocellulose membrane (0.45 µm) was performed at 80 V

for 1 h at 0°C. The membrane was incubated with 5% non-fat dry milk/Tris-buffered saline containing 0.1% Tween 20 (TBST) at room temperature for 2 h, and then washed three times with TBST, followed by incubation at 4°C overnight with goat polyclonal anti-mouse CC16 antibody (diluted 1:1000 in 5% BSA). After washing three times with TBST, the membrane was incubated with alkaline phosphatase-conjugated rabbit anti-goat IgG (diluted 1:1000 in 5% BSA) for 1 h at room temperature. The membrane was again washed three times with TBST. Detection of CC16 was performed using a BCIP/NBT kit. The results were analyzed on a multiimager.

Statistical analysis

All results are reported as means ± standard error of the mean. Significance was determined by Student's *t*-test using Microsoft Excel software. The intensity of Clara cell immunostaining was analyzed as a contingency table using SPSS version 10.0 for Windows (SPSS Inc., Chicago, IL, USA). A *P*-value <0.05 was considered statistically significant.

RESULTS

Immunohistochemistry

The immunohistochemistry showed that CC16 was localized in non-ciliated cells lining the terminal and respiratory bronchioles, corresponding to Clara cells (Fig. 1a). No staining was observed in alveolar tissue or in ciliated epithelial cells of the airways (Fig. 1b). In the OVA group, the abundance of immunoreactive cells and the intensity of immunostaining were reduced compared to that in the control group (Fig. 1c). In the NAC group, the abundance of immunoreactive cells did not change, but the intensity of immunostaining was increased compared to the OVA group (Table 1, Fig. 2).

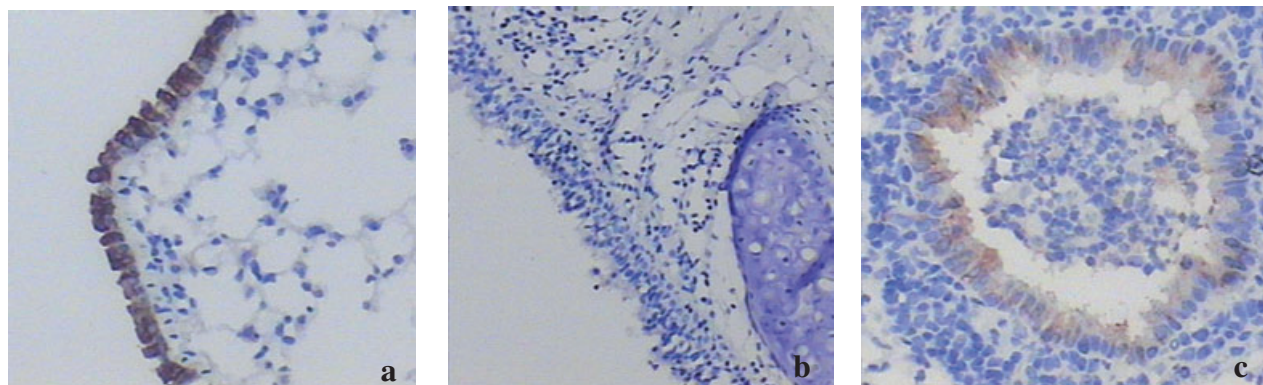


Figure 1 Immunohistochemistry (× 100) of murine distal airways. (a) In the control group, there were many Clara cells with brown immunostaining in the terminal bronchiole; no staining was observed in alveolar tissue. (b) In the control group, no staining was observed in ciliated epithelial cells of the airways. (c) In the ovalbumin challenged group, the number of Clara cells decreased; the abundance of immunoreactive cells and the intensity of immunostaining were both reduced.

Table 1 Percentage of Clara cells in terminal and respiratory bronchioles (mean $\pm$ standard deviation)

Group	Cases	Terminal bronchioles		Respiratory bronchioles	
		Total cell number	Clara cells (%)	Total cell number	Clara cells (%)
Control	10	2148	74.54 $\pm$ 5.81	2037	78.46 $\pm$ 1.68
OVA	10	2363	58.05 $\pm$ 3.75*	2216	63.70 $\pm$ 1.79*
NAC	10	2236	62.74 $\pm$ 1.72**	2341	67.47 $\pm$ 3.53**

* $P < 0.01$ versus the control group; ** $P > 0.05$ versus the OVA group.

NAC, N-acetylcysteine; OVA, ovalbumin.

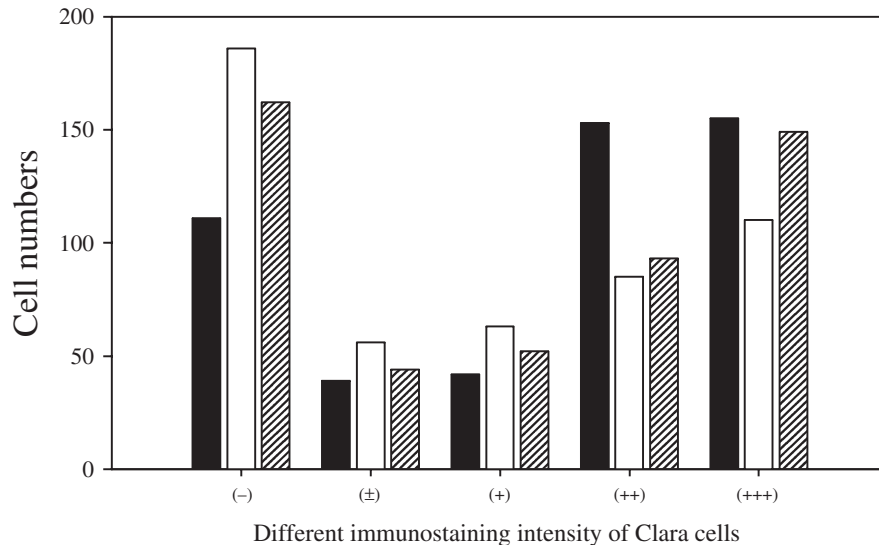


Figure 2 Immunostaining intensity of Clara cells was determined by manually grading 500 cells from 10 sections of airway from control mice ($n = 10$), mice challenged with ovalbumin (OVA; $n = 10$), and mice challenged with OVA and treated with N-acetylcysteine (NAC; $n = 10$). The intensity of immunostaining ranged from (-) to (+++). Comparison of the OVA group (□) versus the control group (■), $\chi^2 = 53.25$, $P < 0.01$; the NAC group (▨) versus the OVA group (□), $\chi^2 = 10.38$, $P < 0.05$.



Figure 3 Clara cell 16 kDa protein (CC16) levels in bronchoalveolar lavage fluid of the control, ovalbumin (OVA) challenged and with N-acetylcysteine (NAC)-treated groups of mice. The molecular weight of CC16 on sodium dodecylsulfate-polyacrylamide gel electrophoresis is approximately 10 kDa. 1,2, the OVA group; 3,4, the control group; 5,6,7, the NAC group; 8, protein marker. CC16 levels were reduced in the OVA challenged groups and partially restored by treatment with NAC.

Clara cell 16 kDa protein levels in bronchoalveolar lavage fluid

The CC16 levels in BALF were 54.7 ± 5.0 , 35.6 ± 4.0 and 44.1 ± 3.2 in the control, OVA and NAC groups, respectively. By Student's *t*-test, the CC16 level in the OVA group was significantly lower than that in the control group ($P < 0.01$), while CC16 in the NAC group was significantly greater than that in the OVA group ($P < 0.05$; Fig. 3).

White cell counts and cell differential analysis in bronchoalveolar lavage fluid

Total white cell count and the percentage of eosinophils in BALF were significantly increased in the OVA

group compared to the control group. NAC reduced the total number of white cells and the percentage of eosinophils in the OVA group (Table 2).

Histological findings

Figure 4 shows representative histological findings. Marked bronchial inflammation was detected in the OVA group (Fig. 4b). There was an infiltration of inflammatory cells (mostly eosinophils) into the peribronchial interstitial area and into the mucosal and submucosal areas of the bronchus. Goblet cell formation was observed in the epithelium. Hypertrophy of the smooth-muscle cell layer was also observed. In the control group, these phenomena were not observed (Fig. 4a). Inflammation in the NAC group

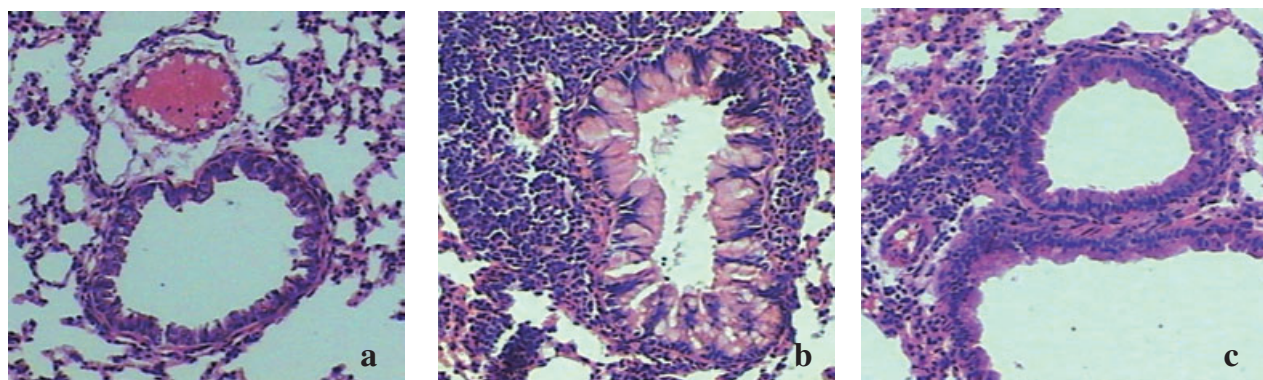


Figure 4 Haematoxylin and eosin staining ($\times 100$) of murine airways. (a) The control group showing a normal, non-inflamed state. (b) In the ovalbumin (OVA) challenged group the bronchial inflammation was significant. There was an infiltration of inflammatory cells (mostly eosinophils) into the peribronchial interstitial area and into the mucosal and submucosal areas of the bronchus. Goblet cell formation was observed. Hypertrophy of the smooth-muscle cell layer was also observed. (c) In the N-acetylcysteine-treated group, inflammation was significantly reduced compared to the OVA group.

Table 2 White cell counts and cell differential analysis of bronchoalveolar lavage fluid (mean $\pm$ standard deviation)

Group	Cases	Total cell number		Differential cell count ($\times 10^7/L$)			
		($\times 10^7/L$)	Eosinophils	Neutrophils	Macrophages	Lymphocytes	Epithelial cells
Control	10	2.62 ± 1.36	0.03 ± 0.02	0.28 ± 0.12	2.15 ± 0.63	0.40 ± 0.12	0.26 ± 0.09
OVA	10	$7.85 \pm 1.46^{**}$	$4.61 \pm 1.37^{**}$	1.07 ± 0.18	1.04 ± 0.51	0.58 ± 0.12	0.31 ± 0.07
NAC	10	$5.77 \pm 1.24^*$	$3.13 \pm 0.60^*$	1.25 ± 0.15	0.22 ± 0.13	0.87 ± 0.29	0.30 ± 0.05

Total cell number and differential cell counts were all expressed as numbers/concentration. $^*P < 0.05$ versus OVA; $^{**}P < 0.01$ versus control.

NAC, N-acetylcysteine; OVA, ovalbumin.

was significantly reduced compared to the OVA group (Fig. 4c).

DISCUSSION

The purpose of the present study was to investigate the effect of allergen-induced airway inflammation on Clara cells and CC16 production. The number of Clara cells as well as the production and secretion of CC16 decreased in the OVA group. However, after NAC treatment, the production and secretion of CC16 increased compared to the OVA group.

Clara cells can be found in all mammalian lungs, and CC16 is the main protein secreted by these cells. CC16 has anti-inflammatory activity, including inhibition of the key enzyme in inflammation, PLA_2 .¹⁵ PLA_2 is a rate-limiting enzyme in the production of arachidonic acid (AA),¹⁶ which is the precursor of pro-inflammatory lipid mediators such as platelet-activating factors, leukotrienes, prostaglandins and thromboxanes. Thus, the inhibition of PLA_2 activity is likely to result in a reduction in inflammatory mediators, and a reduction in extracellular inflammatory activity in the lung. CC16 also directly inhibits the production and activity of inflammatory cytokines, such as tumour necrosis factor (TNF)- α ,

IFN- γ ¹⁷ and IL-1 β ,¹⁸ which are all relevant to airway inflammation.

Immunohistochemically, the number of Clara cells on terminal and respiratory bronchioles in the OVA group were significantly reduced compared to those of the control group. The intensity of immunostaining in Clara cells was also decreased. Therefore, the decreased CC16 concentration in BALF could be due to a reduction in Clara cell numbers as well as reduced production of the protein.

IFN- γ may also alter the rate of production of CC16 protein at both the transcriptional and post-transcriptional levels.^{19,20} *In vitro* and *in vivo* studies have verified that the binding regions for IFN- γ and hepatocyte nuclear factor-3 β (HNF-3 β) are both localized at the 5'-prime region of the CC16 promoter. IFN- γ may promote CC16 expression directly through binding to its response element or indirectly through an interferon regulatory factor-1-mediated increase in HNF-3 β . CC16 promoter activity might also be markedly down-regulated by TNF- α treatment, through regulation of the same promoter element.²¹ Ohkawara *et al.*²² observed that when compared to control mice, the level of IFN- γ decreased, while TNF- α increased in BALF in a murine OVA model of allergen-induced airway inflammation. We speculate that the cooperative effects of decreased IFN- γ and

increased TNF- α on the CC16 gene during allergen-induced airway inflammation inhibits the production of CC16 protein in Clara cells, but further experiments are needed to confirm this. As a result, the inhibitory effect of CC16 on the activity of PLA₂ would be reduced. The release of high levels of AA may eventually lead to an increased production of pro-inflammatory lipid mediators, causing migration of inflammatory cells and T cells into the pulmonary mucosa. These cells secrete inflammatory cytokines and further reduce CC16 production. Thus, a vicious cycle could ensue, initiating and propagating an inflammatory response in the airways or aggravating existing inflammatory processes.

NAC is a derivative of the thiol-containing amino acid, L-cysteine.²³ It may act directly as an oxygen radical scavenger.²⁴ Moreover, since NAC is a cell-permeable sulphhydryl compound, which can enter cells and promote the production of glutathione,²⁵ it has become one of the more widely investigated antioxidants, and has shown some beneficial effects in disease states in which oxygen radicals are involved.^{23,25,26} There is increasing clinical and experimental evidence that an excessive production of reactive oxygen species and defective endogenous antioxidant defence mechanisms may be present in asthma.^{7,27} Therefore, NAC may be of clinical benefit in the treatment of asthma. In the present study, NAC elevated CC16 expression in Clara cells, increased CC16 levels in BALF, and markedly decreased the number of eosinophils in BALF compared to the OVA group. Moreover, NAC substantially reduced the allergen-induced influx of inflammatory cells into peribronchial tissue.

Many studies have shown that NAC can abolish the activity of the redox-sensitive transcription factor, nuclear factor (NF)- κ B, *in vivo*.^{28,29} There is experimental evidence that the activation of NF- κ B is important for the expression of various inflammatory genes, such as IL-1 β , TNF- α and IL-6, in models of allergen-induced airway inflammation.³⁰ Therefore, NAC may elevate CC16 gene expression by inhibiting the production of inflammatory cytokines such as TNF- α , through inhibition of NF- κ B. Some experiments have shown that increased levels of CC16 protein inhibited PLA₂ activity, thereby inhibiting airway inflammation.³ Therefore, NAC may relieve airway inflammation through elevating the expression of CC16, which can be seen with HE staining, as well as in the white cell counts and cell differential analysis of BALF in the NAC group. We conclude that in addition to reducing reactive oxygen species, the therapeutic effects of NAC on allergen-induced airway inflammation are partly mediated through promotion of the production and secretion of CC16.

Our data has demonstrated that allergen-induced airway inflammation induced a decrease in the number of Clara cells as well as in the production and secretion of CC16. NAC may have effects that are partly due to promotion of the production and secretion of CC16. The antioxidant NAC may provide a new therapeutic approach for treating asthma. However, the exact mechanisms involved require further investigation.

ACKNOWLEDGEMENTS

The authors wish to thank Chaoping Fang (Changhai Hospital) and Zongbin Li (Second Military Medical University) for their excellent technical assistance.

REFERENCES

- 1 Umland TC, Swaminathan S, Furey W, Singh G, Pletcher J, Sax M. Refined structure of rat Clara cell 17kDa protein at 3.0 Å resolution. *J. Mol. Biol.* 1992; **224**: 441–8.
- 2 Bernard A, Dumont X, Roels H *et al.* The molecular mass and concentrations of protein 1 or Clara cell protein in biological fluids: a reappraisal. *Clin. Chim. Acta* 1993; **223**: 189–91.
- 3 Levin SW, Butler JD, Schumacher UK, Wightman PD, Mukherjee AB. Uteroglobin inhibits phospholipase A₂ activity. *Life Sci.* 1986; **38**: 1813–19.
- 4 Lensmar C, Nord M, Gudmundsson GH *et al.* Decreased pulmonary levels of the anti-inflammatory Clara cell 16 kDa protein after induction of airway inflammation in asthmatics. *Cell. Mol. Life Sci.* 2000; **57**: 976–81.
- 5 Arsalane K, Broeckaert F, Knoops B, Wiedig M, Toubreau G, Bernard A. Clara cell specific protein (CC16) expression after acute lung inflammation induced by intratracheal lipopolysaccharide administration. *Am. J. Respir. Crit. Care Med.* 2000; **161**: 1624–30.
- 6 Paredi P, Kharitonov SA, Barnes PJ. Analysis of expired air for oxidation products. *Am. J. Respir. Crit. Care Med.* 2002; **166**: S31–7.
- 7 Henricks PAJ, Nijkamp FP. Reactive oxygen species as mediators in asthma. *Pulm. Pharmacol. Ther.* 2001; **14**: 409–21.
- 8 Malm-Erfjelt M, Persson CG, Erfjelt JS. Degranulation status of airway tissue eosinophils in mouse models of allergic airway inflammation. *Am. J. Respir. Cell Mol. Biol.* 2001; **24**: 352–9.
- 9 Kung TT, Jones H, Adams GK 3rd *et al.* Characterization of a murine model of allergic pulmonary inflammation. *Int. Arch. Allergy Immunol.* 1994; **105**: 83–90.
- 10 Blesa S, Cortijo J, Martinez-Losa M *et al.* Effectiveness of oral N-acetylcysteine in a rat experimental model of asthma. *Pharmacol. Res.* 2002; **45**: 135–40.
- 11 To Y, Dohi M, Tanaka R, Sato A, Nakagome K, Yamamoto K. Early interleukin 4-dependent response can induce airway hyperreactivity before development of airway inflammation in a mouse model of asthma. *Lab. Invest.* 2001; **81**: 1385–96.
- 12 Nilaver G, Kozlowski GP. Comparison of the PAP and ABC immunocytochemical techniques. In: Bullock GR, Petrusz P (eds). *Techniques in Immunocytochemistry*. Academic Press, London, 1989; 199–215.
- 13 Halatek T, Hermans C, Broeckaert F *et al.* Quantification of Clara cell protein in rat and mouse biological fluids using a sensitive immunoassay. *Eur. Respir. J.* 1998; **11**: 726–33.
- 14 Elia J, Aoki A, Maldonado CA. Response of bronchiolar Clara cells induced by a domestic insecticide. Analysis of CC10 kDa protein content. *Histochem. Cell Biol.* 2000; **113**: 125–33.
- 15 Hayward A. Who's to blame for asthma? *Lancet* 1995; **346**: 1243.

- 16 Drazen JM, Israel E, O'Byrne PM. Treatment of asthma with drugs modifying the leukotriene pathway. *N. Engl. J. Med.* 1999; **340**: 197–206.
- 17 Dierynck I, Bernard A, Roels H, De Ley M. The human Clara cell protein: biochemical and biological characterisation of a natural immunosuppressor. *Mult. Scler.* 1996; **1**: 385–7.
- 18 Johnston CJ, Mango GW, Finkelstein JN, Stripp BR. Altered pulmonary response to hyperoxia in Clara cell secretory protein deficient mice. *Am. J. Respir. Cell Mol. Biol.* 1997; **17**: 147–55.
- 19 Yao XL, Ikezono Z, Cowan M, Logun C, Angus CW, Shelhamer JH. Interferon-gamma stimulates human Clara cell secretory protein production by human airway epithelial cells. *Am. J. Physiol.* 1998; **274**: L864–9.
- 20 Ramsay PL, Luo Z, Magdaleno SM *et al.* Transcriptional regulation of CCSP by interferon-gamma in vitro and in vivo. *Am. J. Physiol. Lung Cell Mol. Physiol.* 2003; **284**: L108–18.
- 21 Harrod KS, Jaramillo RJ. *Pseudomonas aeruginosa* and tumor necrosis factor- α attenuate Clara cell secretory protein promoter function. *Am. J. Respir. Cell Mol. Biol.* 2002; **26**: 216–23.
- 22 Ohkawara Y, Lei XF, Stampfli MR, Marshall JS, Xing Z, Jordana M. Cytokine and eosinophil responses in the lung, peripheral blood, and bone marrow compartments in a murine model of allergen-induced airway inflammation. *Am. J. Respir. Cell Mol. Biol.* 1997; **16**: 510–20.
- 23 Ventresca GP, Cicchetti V, Ferrari V. Acetylcysteine. In: Braga PC, Allegra L (eds). *Drugs in Bronchial Mucology*. Raven Press Ltd, New York, 1989; 77–102.
- 24 Aruoma OI, Halliwell B, Hoey BM, Butler J. The antioxidant action of N-acetylcysteine: its reaction with hydrogen peroxide, hydroxyl radical, superoxide, and hypochlorous acid. *Free Radic. Biol. Med.* 1989; **6**: 593–7.
- 25 Cotgreave IA. N-Acetylcysteine: pharmacological considerations and experimental and clinical applications. In: Sies H (ed). *Antioxidants in Disease: Mechanisms and Therapy (Advances in Pharmacology, Vol. 38)*. Academic Press, New York, 1997; 205–27.
- 26 Tepel M, van der Giet M, Schwarzfeld C, Laufer U, Liermann D, Zidek W. Prevention of radiographic-contrast-agent-induced reductions in renal function by acetylcysteine. *N. Engl. J. Med.* 2000; **343**: 180–4.
- 27 Barnes PJ. Reactive oxygen species in asthma. *Eur. Respir. Rev.* 2000; **10**: 240–3.
- 28 Ho E, Chen G, Bray TM. Supplementation of N-acetylcysteine inhibits NF- κ B activation and protects against alloxan-induced diabetes in CD-1 mice. *FASEB J.* 1999; **13**: 1845–54.
- 29 Blackwell TS, Blackwell TR, Holden EP, Christman BW, Christman JW. In vivo antioxidant treatment suppresses nuclear factor- κ B activation and neutrophilic lung inflammation. *J. Immunol.* 1996; **157**: 1630–7.
- 30 Liu SF, Haddad EB, Adcock I *et al.* Inducible nitric oxide synthase after sensitization and allergen challenge of Brown Norway rat lung. *Br. J. Pharmacol.* 1997; **121**: 1241–6.