

N-acetylcysteine exerts therapeutic action in a rat model of allergic rhinitis

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Background: The pathophysiologic mechanism of allergy is dependent on the action of many redox-sensitive proinflammatory mediators. However, even though redox disturbances are believed to be a hallmark of inflammation, little is known of the effect of redox imbalance to the pathophysiology of allergic rhinitis. We thus opted to investigate the relation of oxidative stress and allergic rhinitis, through the utilization of a potent antioxidant substance (N-acetylcysteine [NAC]) in a rat model of allergic rhinitis and the evaluation of its action on specific markers of inflammation.

Methods: NAC (50 mg/kg and 250 mg/kg) was intraperitoneally administered to ovalbumin (OVA)-sensitized rats prior to intranasal challenge with OVA. Mucosal congregation of inflammatory cells (eosinophils and mast cells), mucosal expression of redox-sensitive enzymes (inducible nitric oxide synthase [iNOS] and cyclooxygenase 2 [COX-2]), and the blood levels of a key proinflammatory mediator (tumor necrosis factor- α [TNF- α]) were evaluated.

Results: Intranasal OVA challenges lead to mucosal inflammation, induction of the mucosal expression of iNOS and

COX-2 and elevation of TNF- α blood levels. NAC significantly inhibited accumulation of inflammatory cells and downregulated iNOS expression and TNF- α serum levels. The role of COX-2 appeared to be 2-fold and its expression was divergently modulated by NAC.

Conclusion: Our findings suggest that redox balance is involved in the pathophysiology of allergic rhinitis in rats and that NAC can potentially suppress the allergen-induced nasal inflammatory cascade. The investigation of the role of oxidative stress in atopy could help in the evaluation of the therapeutic potential of antioxidant substances in allergic diseases. © 2013 ARS-AAOA, LLC.

Key Words:

allergic rhinitis; N-acetylcysteine; inflammation; cyclooxygenase-2; TNF- α ; experimental protocol

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Allergic rhinitis arises from a complex allergen-induced inflammatory process, which is heavily depended on various redox-sensitive proinflammatory mediators. Redox state disturbances are a major cause of cell damage and are

one of the hallmarks of inflammation, inasmuch as oxidative stress interferes with the normal function of membranous systems and results in structural and functional degenerative changes.^{1,2} However, even though there is increasing evidence of a correlation between allergic diseases and reactive oxygen species (ROS), little is known of the involvement of oxidant-antioxidant imbalance in the pathophysiology of allergic rhinitis.³

N-acetylcysteine (NAC) is a sulfhydryl-containing compound that exerts its antioxidant action through direct scavenging of ROS and by furnishing cysteine for the synthesis of glutathione (GSH).³ Inasmuch as GSH has a pivotal antioxidant role, the induction of its biosynthesis by NAC renders NAC a likely candidate for use in redox-related experimental models.^{1,2,4,5} Nevertheless, evaluation of NAC's action in the nasal mucosa, in an in vivo experimental protocol of allergic rhinitis is, to our best knowledge, novel in the literature. Thus, in order to shed more light on the pathophysiological mechanism of experimental allergic rhinitis and its relation to oxidative

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stress, we opted to investigate the potential therapeutic effect of NAC in a rat allergic rhinitis model.

Materials and methods

Animals

Eight-week-old male Sprague-Dawley rats (200–225 g) were used. The animals were allowed food and water ad libitum, and kept at $22 \pm 2^\circ\text{C}$. All experiments were conducted in accordance with the Institutional Animal Ethics EL 54 BIO 20, 13/174.

Sensitization and nasal challenges

Nasal allergic inflammation was induced according to Fu et al.,⁶ with slight modifications. Briefly, throughout the first phase (sensitization phase: days 1 to 15), rats were actively sensitized by intraperitoneal administration of 1 mL of saline containing ovalbumin (1 mg) (Grade V; Sigma Chemical Co., St. Louis, MO) and alum (10 mg) (aluminum hydroxide hydrate gel; Sigma Chemical Co., St. Louis, MO) on day 1 and on every other day for 8 times. Throughout the second phase (antigen exposure phase: days 16 to 21), intranasal challenges were performed on a daily basis, by instillation of OVA-saline solution (10 $\mu\text{g}/10 \mu\text{L}$ /nostril, or saline alone for the control group) into the bilateral nasal cavities using a micropipette.

The animals were randomly allocated into 4 groups: (1) the OVA group ($n = 10$) consisted of rats that were sensitized and intranasally challenged via administration of OVA and were given saline instead of NAC intraperitoneally (i.p.); (2) the NAC50 group ($n = 10$) and (3) the NAC250 ($n = 10$) group consisted of rats that were sensitized, intranasally challenged with OVA, and given NAC 50 mg/kg and 250 mg/kg i.p., respectively, 1 hour before the daily intranasal challenges; and (4) the control group ($n = 10$), consisted of rats that were sensitized with OVA, but were challenged intranasally with saline alone and were given saline i.p. instead of NAC.

Histopathological evaluation

At 24 hours after the last intranasal challenge, rats were sacrificed (i.p. administration of ketamine and xylazine), then their heads were removed, fixed in 10% neutral buffered formalin for 48 hours, and decalcified (ethylene diamine tetra-acetic acid [EDTA, (ReAgent, Runcorn, England)] solution) for 15 days. Afterward, the sinonasal structures were sectioned coronally and tissue blocks that correspond to respiratory epithelium sites were embedded in paraffin, thin-sectioned at 4 μm and stained with hematoxylin-eosin (H&E) and Giemsa stain. In H&E-stained tissue, the degree of inflammation was assayed semiquantitatively by light microscopy in a blinded manner, was evaluated by the density and pattern (focal or diffuse) of the inflammatory infiltrate, and was scored as mild, moderate, or severe. In the Giemsa-stained samples, inflammatory cells (eosinophils and mast

cells) were counted per 10 high power fields (HPF-10 fields $\times 40$ magnification).

Measurement of serum tumor necrosis factor- α by enzyme-linked immunosorbent assay

Before the start of the experiment (day 0) and upon sacrifice, a blood sample was obtained from the jugular vein of the rats, centrifuged at 1800g, and the serum was frozen at -70°C . Concentration of tumor necrosis factor- α (TNF- α) was determined from a standard curve with a commercially available enzyme-linked immunosorbent assay (ELISA) kit (R&D Systems, Minneapolis, MN) and was expressed as picograms per milliliter (pg/mL).

Immunohistochemistry

Formalin-fixed, paraffin-embedded sections were used. Antigen unmasking was performed by microwave heating in a solution of 0.01 M sodium citrate buffer (pH 6.0) and sections were incubated overnight with primary antibodies (1:100 for monoclonal cyclooxygenase 2 [COX-2]; (Spring Bioscience Pleasanton, CA); and 1:100 for polyclonal inducible nitric oxide synthase [iNOS]; Santa Cruz Biotechnology, Santa Cruz, CA) at 4°C . The avidin-biotin-peroxidase complex method was employed (REALEnvision Detection System; Dako, Glostrup, Denmark). Tissues were counterstained with hematoxylin and a semiquantitative analysis of the immunohistochemical findings was made according to Soslow et al.⁷ The protein expression was graded by combining the percentage of immunoreactive cells (density of immunostaining) with an estimate of the intensity of the immunostaining. Staining density was rated as follows: 0% to 10% of cells demonstrating staining scored as 1; 11% to 50% scored as 2; 51% to 80% scored as 3; and 81% to 100% scored as 4. Staining intensity was rated on a scale of 0 to 3 as follows: 0 = negative; 1 = weak; 2 = moderate; and 3 = strong. Through the multiplication of the staining density and intensity scores, each section received an immunohistochemical score from 0 to 12. The final immunohistochemical grades were formed as follows: score 1 to 3 = grade 1 (mild); score 4 to 6 = grade 2 (moderate); score 7 to 9 = grade 3 (significant); and score 10 to 12 = grade 4 (strong).

Statistical analysis

For the immunohistochemical analysis and cell counts, the Kruskal-Wallis test for ordinal data was used and all pairwise comparisons were conducted by the Mann-Whitney U statistic. Type I error was controlled by the Holm's sequential Bonferroni method and the computation of each p value of the pairwise comparisons was done by the Monte Carlo method. The 4 groups' sample distributions of the numbers of eosinophils and mast cells were depicted in box-plots and it was observed that none of them was symmetric. Serum TNF- α levels were determined with paired and unpaired t tests, analysis of variance (ANOVA), and Bonferroni post hoc test. The analysis was performed with

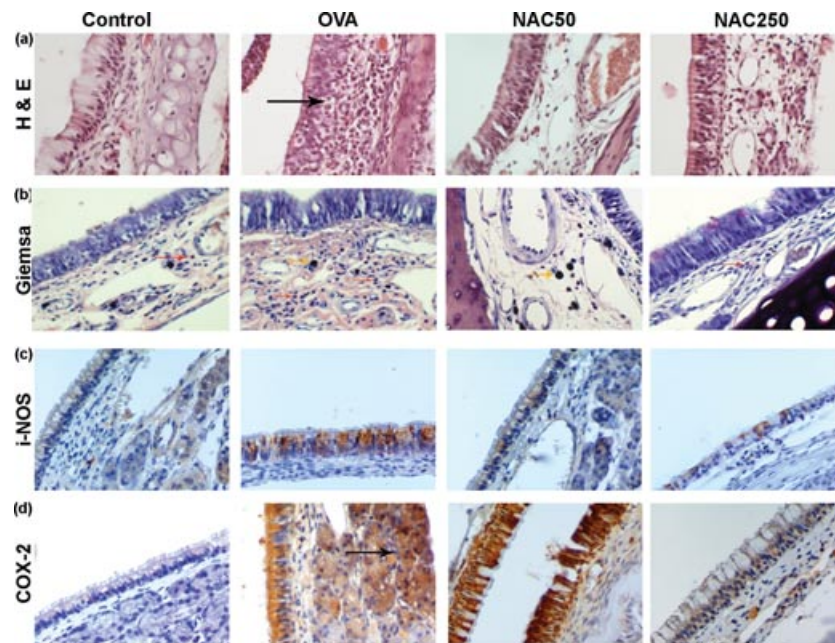


FIGURE 1. Effect of antigen challenge and NAC administration on the rat nasal mucosa. (A) Hematoxylin and eosin stain. Very mild mucosal inflammation in the control, NAC50 (50 mg/kg), and NAC250 (250 mg/kg) rats. Dense inflammatory infiltrate and intraepithelial expansion of the inflammation (arrow) in the OVA group. (B) Giemsa stain. Rare inflammatory cells in the nasal mucosa of the control, NAC50, and NAC250 rats. Increased numbers of mucosal inflammatory cells in the rats of the OVA group (red arrow = eosinophils, orange arrow = mast cells). (C) iNOS immunostaining. Very mild expression of iNOS confined mainly in the epithelium in the control and NAC50 groups. Moderate to significant expression of iNOS in the OVA and NAC250 groups. (D) COX-2 immunostaining. Intense and diffuse staining in the epithelium, the lamina propria and the submucosal glands (arrow) in the OVA and NAC50 groups. Mild staining in the control and NAC250 groups. COX-2 = cyclooxygenase 2; iNOS = inducible nitric oxide synthase; NAC = N-acetylcysteine; OVA = ovalbumin.

IBM SPSS Statistics 19.0 (IBM, Armonk, NY); $p < 0.05$ was considered statistically significant.

Results

NAC attenuated mucosal accumulation of inflammatory cells

In the OVA group, allergen challenge resulted in the presence of large inflammatory infiltrates in the lamina propria, that were occasionally seen to involve the overlying epithelium (Fig. 1A). The number of eosinophils and mast cells in the OVA group was significantly enhanced in comparison to the control group ($p = 0.008$ and $p = 0.01$, respectively) (Figs. 2–5). Conversely, inflammation in NAC-treated rats was mild (Fig. 1A, B). NAC in both doses (50 mg/kg and 250 mg/kg) demonstrated a marked capacity for inhibiting eosinophil accumulation ($p < 0.001$) (Figs. 2 and 3). Administration of NAC also reduced the numbers of mucosal mast cells, albeit not as robustly as eosinophils ($p = 0.02$ for 50 mg/kg and $p = 0.006$ for 250 mg/kg) (Fig. 4 and 5). The results of the semiquantitative essay in the H&E-stained samples were comparable to the Giemsa findings, hence revealing a statistically significant inhibition of inflammation via the action of NAC (data not shown).

NAC inhibited serum TNF- α levels induction

The end levels of TNF- α were increased in all groups, in comparison to the initial levels (Fig. 6). Nevertheless, in

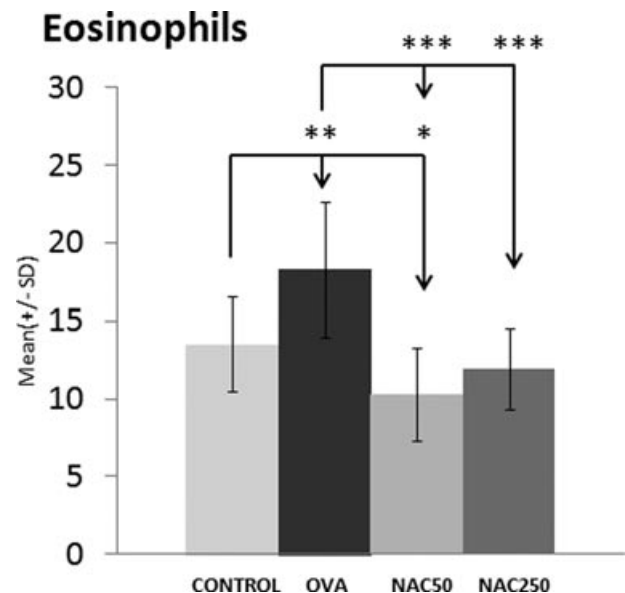


FIGURE 2. Mean values of eosinophils with SD per group and significant associations. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. SD = standard deviation.

the control and NAC50 groups this increase was not statistically significant ($p > 0.05$), whereas in the OVA and NAC250 groups it was significant, albeit marginally ($p < 0.05$). There was no significant difference in the TNF- α levels among the 4 experimental groups ($p > 0.05$) (Fig. 6).

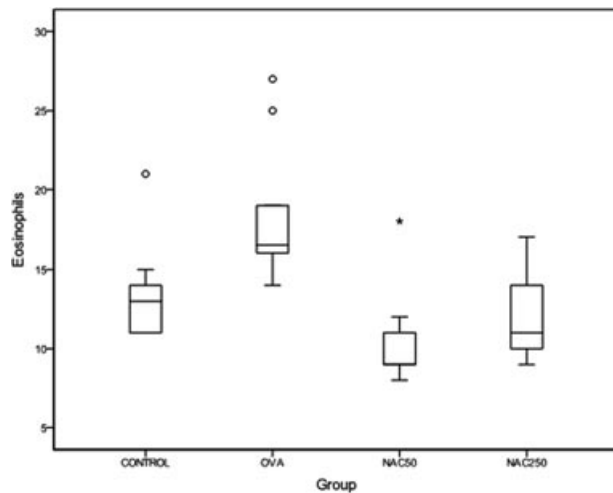


FIGURE 3. Sampling distributions of eosinophils per group.

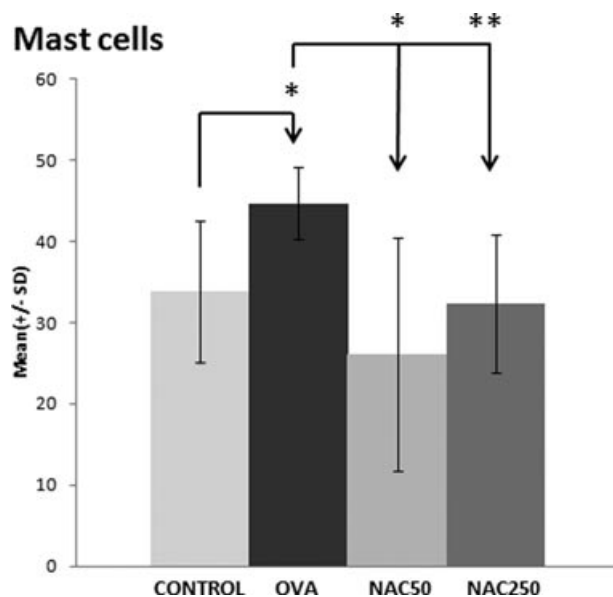


FIGURE 4. Mean values of mast cells with SD per group and significant associations. * $p < 0.05$, ** $p < 0.01$. SD = standard deviation.

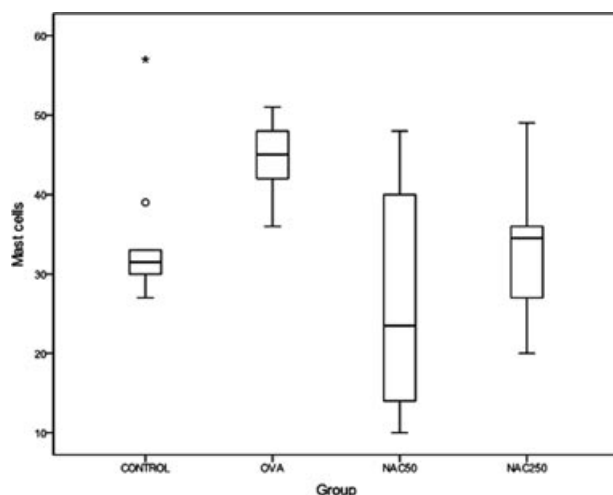


FIGURE 5. Sampling distributions of mast cells per group.

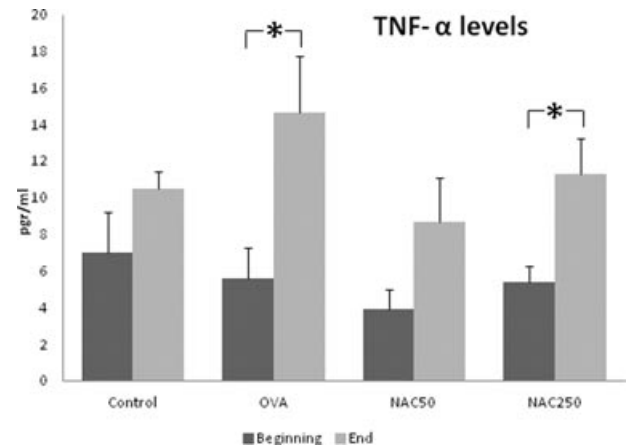


FIGURE 6. TNF- α serum levels at the beginning and at the end of the experimental protocol and significant associations. * $p < 0.05$. TNF- α = tumor necrosis factor- α .

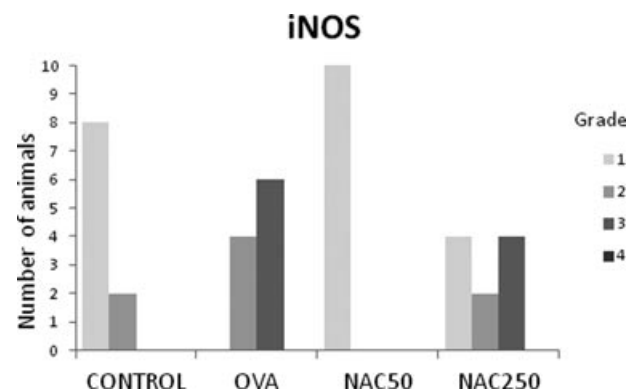


FIGURE 7. Expression of iNOS in the nasal mucosa, shown as number of rats of each group that were assigned to each of 4 grades of expression, depending on the density and intensity of iNOS immunostaining. Grade 1 = mild expression; Grade 2 = moderate expression; Grade 3 = significant expression; and Grade 4 = strong expression. iNOS = inducible nitric oxide synthase.

NAC inhibited iNOS expression

Allergen challenge significantly upregulated the mucosal expression of iNOS in the OVA group (“significant,” grade 3, 60%) in comparison to the control group (“mild,” grade 1, 80%) ($p < 0.001$) (Figs. 1C and 7). Low-dose NAC (50 mg/kg) demonstrated a substantial capacity in inhibiting the upregulation of the iNOS expression ($p < 0.001$). On the contrary, high-dose NAC (250 mg/kg) did not exert such a robust inhibitory effect on iNOS expression and 40% of the NAC250 rats were assigned to the “significant” grade (grade 3) (Figs. 1C and 7). Extremely intense and/or widespread protein expression (“strong,” grade 4) was not observed.

NAC diversely affected COX-2 expression

Antigen challenge caused a considerable induction of the protein expression, as demonstrated by the substantial difference in the density and intensity of COX-2 staining between the control and the OVA groups ($p < 0.001$).

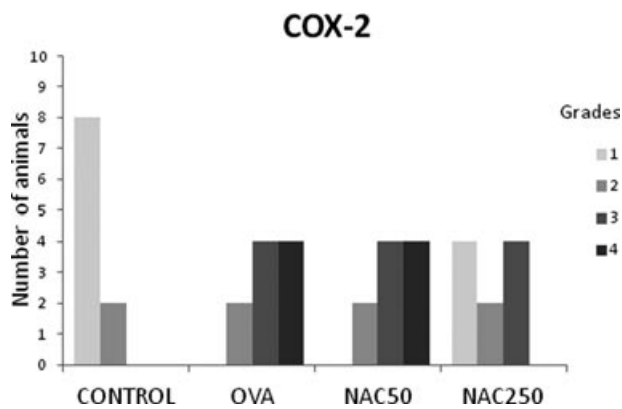


FIGURE 8. Expression of COX-2 in the nasal mucosa, shown as number of rats of each group that were assigned to each of 4 grades of expression, depending on the density and intensity of the COX-2 immunostaining. Grade 1 = mild expression; Grade 2 = moderate expression; Grade 3 = significant expression; and Grade 4 = strong expression. COX-2 = cyclooxygenase 2.

Indeed, intense and diffuse staining (grade 3 and 4), which was evident in the respiratory epithelium, the lamina propria, and the submucosal glands, was noted in the majority of the OVA rats (80%) that were subsequently assigned to the “significant” and “strong” expression (grade 3 and 4) (Figs. 1D and 8). On the contrary, minimal COX-2 expression (grade 1), which was confined to the epithelium, was detected in the majority of the control rats (80%) (Figs. 1D and 8). The intensity of the staining in the NAC50 rats, as opposed to the NAC 250 rats, was substantial and almost identical to that of the OVA group ($p > 0.05$) (Figs. 1D and 8).

Discussion

In our protocol, the nasal mucosa of the OVA rats was markedly infiltrated by inflammatory cells (ie, eosinophils and mast cells), a condition well-established in the literature.^{8–10} NAC in both doses efficiently suppressed inflammation, congruent with other studies.^{3,11,12} In fact, fewer eosinophils were counted in the NAC50 rats, even in comparison with the control rats ($p = 0.015$). Plausible explanations for the mild inflammation in the control rats could be nonspecific irritation of mucosal receptors by the liquid vehicle, potential challenge-related mechanical stress, or irritation by common airborne pollutants.^{13,14}

Various lines of evidence suggest the involvement of oxidative stress in airway inflammation.^{3,12} Indeed, ROS modulate inflammatory cell trafficking¹⁵ via their effect on cell adhesion molecules, and directly regulate eosinophils due to the latter’s high activity of nicotinamide adenine dinucleotide phosphate (NADPH) oxidase and low availability of intracellular glutathione.^{16,17} The oxidant-sensitive transcription factor NF- κ B has also been linked to allergen-induced eosinophilia¹⁸; its expression is in fact regulated by NAC.^{3,19–21} Moreover, eosinophil apoptosis is facilitated by NAC in the presence of proinflammatory cytokines common in allergic diseases.^{3,22} Therefore, NAC’s robust

anti-inflammatory effect could be related to its antioxidant properties.^{23,24}

A dependency of allergen-induced nasal eosinophilia on TNF- α and an inhibition of TNF- α production by glutathione have been documented.^{10,11} Therefore, the robust anti-inflammatory effect of NAC could potentially be ascribed to downregulation of this oxidant-sensitive proinflammatory cytokine, which extends eosinophil survival and is important for cell adhesion molecules and transcription factors.^{10,19,25} To test this hypothesis, we measured the levels of TNF- α in the blood serum on day 0 (before initiation of the experiment) and after sacrifice. Although its levels were increased in all groups, this increase was significant only in the OVA group (suggesting upregulated production in allergic rhinitis) and in the NAC250 group (suggesting a potential inability of NAC to inhibit TNF- α release in this dosage). Conversely, the nonsignificant upregulation of TNF- α in the NAC50 group implies that low-dose NAC did suppress the cytokines production, possibly via its antioxidant action.¹¹ This finding is consistent with previous reports.^{10,11,19,25,26} These results suggest that, in our protocol, TNF- α inhibition by low-dose NAC could have contributed to NACs anti-inflammatory action. Conversely, the inability of NAC250 to downregulate TNF- α production could conceivably relate to an auto-oxidation mechanism that diverts the behavior of thiols toward a pro-oxidant profile^{1,5,27–30}; such action could also apply to NAC in high doses.^{1,5,31} In any case, these associations—albeit significant—were not considerably robust, implying that NAC’s anti-inflammatory effect was also mediated through additional means, such as its documented ability to regulate other cytokines and transcriptional factors (eg, NF- κ B).^{3,11,12,19,20}

Because nitric oxide (NO) is associated with rhinitis,^{32,33} we investigated NAC’s potential action on the redox-sensitive enzyme, iNOS. We observed mild expression of iNOS in the control rats, as frequently reported for noninflamed tissue.^{32–34} Conversely, iNOS is likely upregulated during nasal allergy,^{33,35–37} a notion corroborated by our findings. Induction of iNOS is regulated by redox-sensitive transcriptional factors; therefore, it could likely be suppressed by NAC.^{11,25} Indeed, NAC downregulated iNOS expression, with the low dose demonstrating greater efficacy. These findings are in accord with the thiol auto-oxidation mechanism and further suggest that, in our model, NAC’s antioxidant properties could have contributed to its anti-inflammatory action.

Inasmuch as prostanoids can be generated in nasal mucosa and contribute to eosinophilia, the redox-sensitive enzyme cyclooxygenase-2 (COX-2) could conceivably be involved in rat nasal allergy.³⁸ Consistent with its inducible nature,^{27,39} the enzyme’s expression in the control rats was considerably mild. Conversely, COX-2 is upregulated during inflammation,^{38,40} a notion corroborated by our findings. Regarding NAC’s effect on the enzyme’s action, the results were rather unexpected. Specifically, COX-2 expression in the NAC50 rats (in the absence of distinct

inflammation) was robustly induced. This is not an anticipated effect on the basis of the—presumably—proinflammatory properties of COX-2. Indeed, enzyme expression was also markedly upregulated in the OVA rats (ie, in overtly inflamed tissue). To interpret these controversial findings, we propose that administration of NAC caused the action of COX-2 to switch from proinflammatory (OVA group) to anti-inflammatory (NAC groups). Such diverse COX-2 effects are, indeed documented in the literature. Gilroy et al.⁴¹ reported a switch of COX-2 from a proinflammatory to an anti-inflammatory profile. Accordingly, Peebles et al.⁴² postulated that COX-2 may exert anti-inflammatory action in their rat model of asthma. In fact, Margalit et al.⁴³ have documented an intriguing—and highly relevant to our findings—COX-2/GSH synergy in a mouse model of gout. In their study, elevation of GSH lead to a marked upregulation of the COX-2 expression and—importantly—unexpected alterations of its activity. Capdevila et al.⁴⁴ also reported a GSH-driven modulation of arachidonate catabolism.

Regarding NAC's effect on the expression of COX-2 (as compared to its action), conflicting findings are also frequently reported. Several investigators have suggested

a NAC-induced downregulation of COX-2 expression, through inhibition of the redox-sensitive mitogen-activated protein kinase (MAPK) pathways (which presumably catalyze the enzyme's induction).^{2,39,45} Conversely, other groups have suggested a glutathione-induced upregulation of the MAPK pathways and COX-2 expression.^{4,27,43} Therefore, the NAC/COX-2 link is a field of controversy, as it is likely dependent on both enzyme localization and underlying inflammatory conditions.^{40,43}

Conclusion

To our best knowledge, this is the first report of the anti-inflammatory action of NAC on experimental allergic rhinitis and of its effect on the expression of iNOS and COX-2 in this experimental setting. Collectively, our findings suggest that NAC can suppress the allergen-induced nasal inflammatory cascade. A NAC-induced downregulation of iNOS and TNF- α could potentially be part of this mechanism. The action of COX-2 could conceivably be altered by NAC, causing the enzyme to act in a therapeutic manner; the details of such a mechanism remain to be elucidated. 

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