ORAL N-ACETYLCYSTEINE REVERSES HYPEROXIA-RELATED COUGH SUPPRESSION IN GUINEA PIGS

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Hyperoxia-induced lung injury is well known in animal and human studies. We have previously shown that hyperoxic exposure of guinea pigs is associated with suppression of cough reflex. The goal of this study was to determine the effects of oral N-acetylcysteine (NAC) on hyperoxia-induced oxidative stress in lung tissue directed on cough reflex. The experimental group was pretreated with NAC daily for 7 days and subsequently exposed to 100% O\textsubscript{2} for 60 h. Hyperoxic group inhaled 100% O\textsubscript{2} only. The control group was exposed to normoxia. Cough was induced by inhalation of citric acid aerosol before and after exposure to oxygen. Cough was also induced by mechanical stimulation of airways in anesthetized animals just after the end of oxygen exposure. Our results showed a significant decrease (P=0.002) in citric acid-induced cough in hyperoxic animals and reversal of that effect in animals pretreated with NAC. In addition, there was a significant interaction between antioxidant therapy and hyperoxia (P=0.005). NAC also reversed the hyperoxia-induced inhibition of mechanically-induced cough. In conclusion, our results indicate that NAC attenuated hyperoxia-induced down-regulation of chemically and mechanically-induced cough.

Key words: antioxidants, citric acid-induced cough, hyperoxia, N-acetylcysteine, mechanically-induced cough

INTRODUCTION

Hyperoxia-induced lung injury is of great clinical interest due to the use of oxygen therapy in the care and management of many patients. Oxygen therapy has been implicated in the development of chronic obstructive pulmonary
disease, adult respiratory distress syndrome or bronchopulmonary dysplasia in the newborn (1, 2). Although oxygen supplementation has a beneficial effect, increasing concentrations of oxygen are required with progressing pulmonary dysfunction, which increases the likelihood of additional pulmonary injury due to toxic effects of oxygen (3, 4). Exposure to hyperoxia is a well-established model of lung injury characterized by the development of pulmonary edema and inflammation (5, 6). The development of hyperoxic lung injury has been clearly shown to require the generation of reactive oxygen species (ROS), which leads to alveolar epithelial and endothelial damage through their ability to react with and damage essential biomolecules, including enzymes, membrane lipids, and nucleic acids (7).

Because the lungs are directly exposed to high levels of oxygen, there is no doubt that respiratory epithelium is a major target for oxidative injury that manifested in lung function changes including cough. Information about hyperoxia related cough response is still limited. On the basis of available information we supposed that hyperoxia alone or in combination with primary lung tissue injury should have a damaging effect on lung tissue, including the airway nerve endings, with the changes in the sensitivity of the central and peripheral neuronal pathways regulating cough (8, 9).

We have previously reported on the inhibitory effect of 100% oxygen breathing for 60 h on cough in awake guinea-pigs (10). The inhibitory effect of pure normobaric oxygen breathing on citric acid-induced cough has been shown in other experiments with experimental airway damage (11, 12). From our previous results it follows that 100% oxygen plays a key role in the development of airway and lung damage, which brings about a down-regulation of cough reflex. Because oxidative stress is involved in the pathophysiology of inflammatory airways diseases, we can surmise that supplemented antioxidants could mitigate the oxidant status, inflammatory cytokines or gene expression, and thus could be of clinical benefits in hyperoxia-induced lung injury (13, 14).

More recently, animal and human studies on N-acetylcysteine have shown it to be a powerful antioxidant and a potential therapeutic agent in the treatment of respiratory diseases, cancer, heart disease, heavy metal toxicity, and other diseases characterized by oxidant-antioxidant imbalance. NAC is a thiol compound endowed with antioxidant properties that reduces lung damage produced by oxidant stress in different experimental models and exerts beneficial effects in pulmonary diseases in which oxidant stress appears pathogenetically relevant (15). Historically, NAC has been used as a mucolytic and expectorant agent in chronic respiratory illnesses. NAC is the acetylated precursor of both the amino acid L-cysteine and reduced glutathione (GSH). The biological activity of NAC is attributed to its sulfhydryl group, while its acetyl substituted amino group affords protection against oxidative and metabolic processes (16).
In the present study, the effects of NAC on airway function in guinea pigs after long-term oxygen therapy were examined. The cough reflex was regarded as an indicator of airway dysfunction.

MATERIAL AND METHODS

Animal model and experimental groups

Twenty four adult male Trik guinea-pigs (250-350g) were supplied by the Department of Experimental Pharmacology of the Slovak Academy of Science (Dobra Vodá, Slovakia), kept in the animal house with food and water ad libitum and with a standard air conditioning system before starting the experiment. The experimental protocols were approved by the institutional Ethics Committee and complied with Slovakian and European Community regulations for use of laboratory animals.

The animals were randomly assigned to one of the following three protocol groups: NAC-hyperoxia group (n=8) - pretreated with NAC for 7 days and subsequently exposed to 100% O₂ for 60 h; hyperoxia group (n=8) - received vehicle, saline instead of NAC and was exposed to the same 100% O₂ for 60 h, and control group (C, n=8) - exposed to ambient air under the same condition as the hyperoxic group.

NAC (200 mg/kg body weight, ACC 200, Salutas Fahlberg – List Pharma GmbH, Barleben, Germany) in distilled water (0.5 ml) was administered orally by a micropitte as a single daily dose for 7 days before and 2 days during hyperoxia.

Exposure either to oxygen or air was performed individually in a sealed glass chambers. Oxygen concentration was periodically monitored by an oxygen analyzer (Permolyt 3, Veb Junkalor, Germany). Other biophysical parameters of the chamber environment were the following: temperature 22-24°C, humidity 55-65%, CO₂ concentration ~0.2 vol% (Capnograph), and O₂ concentration in ambient air ~21%. Respiratory rate was monitored in all animals during exposure to hyperoxia or normoxia.

Citric acid-induced cough

Unanesthetized animals were individually placed into a double-chambered body plethysmograph box (type 855, Hugo Sachs Elektronic, Germany) and were exposed to citric acid aerosol (Lachema) in doubly increasing concentrations (from 0.05 to 1.6M) with 30 s inhalation of each concentration of the tussigen. The interval between exposures was 1 min. To expose an animal to the aerosol, the head chamber was connected to a jet nebulizer (Pari Provocation Test I, Pari Starneberg, Germany; manufacturer’s specification: output of aerosol – 5 l min⁻¹, particle mass median diameter - 1.2 µm). Cough was distinguished on the basis of plethysmograph airflow changes and measured using a pneumotachograph (Godart, Germany) with a Fleish head (Gould Godart Statham BV, type 18515, No 1) connected to the head chamber of the plethysmographic box. The airflow was directly registered with the moving pen recorder (Multiscriptor Hellige 21). The appearance of cough was also detected with a microphone placed in the roof of the head chamber and connected to a tape recorder. The airflow signal and cough sound were simultaneously recorded in PC for off-line analysis. The cough response was evaluated on the basis of sudden enhancement of expiratory airflow accompanied by a typical cough sound. The cough sound was analyzed from a self-developed software system according to Xiang et al (17). Differentiation between cough and sneeze was based on spectral analysis of respective sounds (digitalized at a sampling frequency of 11025 Hz). As was shown previously, cough sound differs from sneeze sound by shorter duration,
lower frequency of maximal spectral power peak, and a steeper increase of sound intensity at the
beginning of sound (17). To quantify the intensity of cough reaction, the cough response was
expressed as a total number of coughs during all citric acid challenges.

A control cough challenge was done in all groups of animals (1st cough challenge) at the onset
of the experiment. The next provoked of cough was performed after antioxidant treatment before
hyperoxia (2nd cough challenge), and a final cough challenge was performed at 60 h of exposure to
100% O2 or ambient air (3rd cough challenge).

Mechanically-induced cough

Cough also was induced by mechanical stimulation of laryngopharyngeal (LP) and
tracheobronchial (TB) mucosa using a nylon fiber during 5 s in anesthetized animals (Urethane, 1.1
g/kg, i.p.) just after surgical procedure with tracheostomy 1 h after the end of exposure to hyperoxia
and chemically-induced cough challenge (12). The number of coughs was then counted from the
trace of intrapleural pressure recorded by an electromanometer (Multiscriptor Hellige 21) using
intrapleural cannula. Tracheobronchial and laryngopharyngeal cough was analyzed separately. The
number of cough efforts (NE) during a cough bout and the intensity of a cough bout (ICB = the sum
of all positive deflection of intrapleural pressure during all cough efforts in the cough bout) were
used to quantify cough (18).

Histological specimens

At the end of the experiment, anesthetized animals were killed by an overdose of anesthesia and
samples of tracheal, bronchial and lung tissue were removed, fixed in 10% formalin, dehydrated in
sequential alcohol concentrations, and embedded in paraffin. Cross-sectional specimens were made
and stained with hematoxylin and eosin. Histopathological assessment was performed by light
microscopy.

Statistical analysis

The number of coughs is presented as median and interquartile range. Data of the intensity of
cough bouts are expressed as means ±SD. The inter-group differences in chemically and
mechanically-induced cough response were assessed with one-way analysis of variance
(ANOVA). If a significant difference was detected, multiple comparisons of groups were made
using the Duncan multiple range test. To compare the effects of antioxidant therapy and
hyperoxia, two-way ANOVA for repeated measures was employed. Significance was accepted at
the P< 0.05 level.

RESULTS

Effects of N-acetylcysteine supplementation on citric acid-induced cough
in guinea pigs exposed to 100% O2

Our findings demonstrate that hyperoxic exposure for 60 h caused a
significant inhibition in citric acid-induced cough in hyperoxia-alone group of
animals without NAC [1(0-1.5) vs. 5(4-6.5); P=0.002] (Fig. 1). In contrast, in the
NAC-hyperoxia-treated group, the number of coughs did not drop significantly
after the hyperoxic period [4(2.5-7) vs. 6(3.5-7.5); P=0.07] (Fig. 1). Comparison
of the effects of hyperoxia in both tested groups revealed a significant interaction (P=0.005) between antioxidant therapy and hyperoxia.

When cough responses were analyzed in all animal groups after three cough challenges, according to the protocol, we found no significant difference in the number of coughs between the normoxic group and NAC-hyperoxia-treated group (not shown). A significant decrease in cough vs. the baseline level was only found in the hyperoxia-alone group, as above mentioned (Fig. 1).

Effects of supplementation with N-acetylcysteine on mechanically-induced cough in guinea-pigs exposed to 100% O₂

There were similar differences among the control and two tested groups of animals in mechanically-induced cough from LP and TB mucosa. Oxygen exposure significantly decreased cough induced from LP in the hyperoxia-alone group compared with the control group [1(1-1) vs. 3(2-5); P<0.01], the decrease was significantly less in the NAC-hyperoxia-treated group [2(1-3) vs. 3(2-5) P<0.05] (Fig. 2A). Likewise, we found significant decreases in the intensity of cough bouts from the LP region expressed in kPa in the hyperoxic [5.3 (1.5) vs. 16.7(4.5); P<0.01] and NAC-hyperoxic animals compared with controls [9.5 (3.4) vs. 16.7(4.5); P<0.05] (Fig. 2B).

Hyperoxic exposure significantly reduced the number of coughs provoked from TB mucosa in both tested groups, in either hyperoxia-alone [1.5(1-2) vs. 3.5(2.5-4.5); P<0.01] or NAC-hyperoxia group [2(2-3) vs. 3.5(2.5-4.5); P<0.05] compared with control (Fig. 3A). At the same time, the results revealed a significant decrease in the intensity of TB cough bouts in the same tested groups; in the hyperoxic animals [5.7 (4) vs. 14(2.5); P<0.01] and in the NAC-hyperoxic animals [8.6 (4.1) vs. 14(2.5); P<0.05] compared with the normoxic controls (Fig. 3B).
Changes in mechanically-induced laryngopharyngeal (LP) cough expressed as the number of coughs (Panel A) and the intensity of cough bouts (Panel B) in the control (normoxia), antioxidant-hyperoxia (NAC+O₂), and the hyperoxia-alone (100% O₂) groups. The number of coughs is expressed as median and interquartile range and the intensity of cough bouts as mean ±SD. *P<0.05 and **P<0.01 vs. control.

Changes in mechanically-induced tracheobronchial (TB) cough expressed as the number of coughs (Panel A) and the intensity of cough bouts (Panel B) in the control (normoxia), antioxidant-hyperoxia group (NAC+O₂), and hyperoxia-alone (100% O₂) groups. The number of coughs is expressed as median and interquartile range and the intensity of cough bouts as mean ±SD. *P<0.05 and **P<0.01 vs. control.

**Histology**

The histological examination of samples taken from the larynx, trachea, bronchi, and the lungs did not reveal any appreciable differences in the intensity and quality of morphological changes between the animals of the NAC-hyperoxia and hyperoxia-alone groups. There were some pathological changes including hyperplasia and desquamation of the epithelium, dilation of mucosal lymphatic and blood vessels, with the appearance of lymphocytes. More intense histopathological changes were seen in lung tissue. There was emphysematous infiltration with non-specific inflammatory cells, characteristic of interstitial...
pneumonitis, and a reduction of alveolar spaces. Other changes included diffused excessive vascular dilation, congestion, oedema, and inflammation, with aggregates of lymphocytes (not shown).

**DISCUSSION**

Recent clinical and experimental studies have emphasized the upregulation (sensitization) of cough in pathological conditions of airways. The peripheral mechanisms of increased cough sensitivity during airway diseases include the potential role of inflammatory mediators, neutrophins, and changes in airway mucosal structure (19). However, in some situations, reflex cough can be downregulated (20). We previously reported the inhibitory effect of hyperoxia-induced oxidative stress on cough reflex (11, 12). The results of our present study are consistent with those previous experiments.

Hyperoxia-induced lung injury (normobaric 100% oxygen exposure) has been used as a well-established model of lesions mimicking acute respiratory distress syndrome (21, 22). Oxygen-induced lung injury is characterized by extensive alveolar damage leading to disruption of the alveolo-capillary barrier, pulmonary edema, and pleural effusion (4-6, 21). It is generally assumed that lung damage results essentially from a direct action of increased intracellular reactive oxygen species (ROS) and from the accumulation of inflammatory mediators within the lungs (21). Both apoptosis and necrosis have been described in alveolar cells (mainly epithelial and endothelial) during hyperoxia.

It has been postulated that antioxidants may influence the expression of hyperoxia-induced lung injury. Many recent studies demonstrate that antioxidants, by virtue of anti-inflammatory effects protect the lung in a model of oxidative lung injury (21-23, 27, 28). The relationship between airway oxidative damage and supplementation of antioxidant substances is still unclear and many findings are controversial (23).

In the present study we tested the hypothesis that hyperoxia-induced downregulation of cough would be prevented by NAC that has antiinflammatory and antioxidant properties. Several animal and human studies have explored NAC’s effectiveness as a therapeutic agent for various types of respiratory illness. While results vary, NAC administration facilitates mucuc expectoration, decreases cough severity, and improves pulmonary function (24). NAC may protect against oxidative injury by providing cysteine for GSH biosynthesis or by reducing the level of ROS (25).

In this study we induced a guinea pig model of hyperoxia-induced oxidative stress, and we studied the cough reflex in hyperoxia alone or in NAC-hyperoxia condition. The doses of NAC used in the study were in accordance with the literature data (27, 29). Our results showed a significant decrease in chemically-induced cough after exposure to hyperoxia, which was consistent with our
previous reports (10-12). Likewise, hyperoxia resulted in a significant decrease in laryngopharyngeal and tracheobronchial mechanically-induced cough. These results support a suggestion that the intensity of hyperoxia-induced oxidative stress was strong enough to develop functional and morphological changes in airway mucosa and airway nerve endings responsible for mediation and modulation of cough. Histological findings are in accordance with this supposition, because there were signs of injury to airway mucosa and lung tissue in hyperoxic animals.

There is increasing evidence that oxidative stress is implicated in the development of airway inflammation and ROS might play an important role in the modulation of airway inflammation induced by hyperoxia. Budinger and Sznajder (26) reported that hyperoxic lung injury involves damage to membrane receptors, channels, and mitochondria (26). Morphological changes accompanying hyperoxia, manifested in airway and lung damage, can contribute to decreased cough. ROS overproduction is liable to be involved in cough attenuation by hyperoxia. At the present state of knowledge, however, it is unknown what level of ROS or any other components of oxidative stress are involved in the regulation of cough, with its depression as a consequence. Antioxidant depletion may contribute to oxidative stress in the hyperoxia-induced cough attenuation observed in the present study.

In contrast, we showed no significant changes in citric acid-induced cough in hyperoxia after NAC pretreatment. In addition, we found a significant interaction between NAC pretreatment and hyperoxia. In mechanically-induced cough, however, our results revealed a significant decrease in cough induced from laryngopharyngeal or tracheobronchial region in both tested group, but the animals of the NAC-hyperoxia group showed, statistically, a somewhat smaller reduction in cough (P<0.05) than those in the hyperoxia-alone group (P<0.01).

To elicit cough, we used two different methods in this study: chemical and mechanical stimulation, with different mechanisms of pathways regulating cough in either. While chemically-induced cough was evoked in awake animals, mechanical stimulation was performed in anesthetized ones. Another difference between the two methods was that citric acid aerosol (chemical) affected the whole surface of airways, while mechanical stimulation was restricted to confined areas. Although antioxidants did not cause any obvious changes in the morphological picture, they reversed the hyperoxia-induced inhibition of cough, particularly in response to chemical stimulation. Other clinical and experimental studies support a beneficial effect of NAC against oxidative stress through enhancement of GSH level or improvement of lung function (13, 27, 28), but other studies report no effect of NAC on type II cell injury or lung function in condition of oxidative stress (29, 30).

We conclude that NAC may have a potential role in the treatment of oxidative stress-related lung diseases (or lung pathology) accompanied by changes in sensitivity of cough reflex.
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