Effect of N-acetyl-cysteine on the hypoxic ventilatory response and erythropoietin production: linkage between plasma thiol redox state and O₂ chemosensitivity

Wulf Hildebrandt, Steve Alexander, Peter Bärtsch, and Wulf Droge

Oxygen-sensing chemoreceptors contribute significantly to the regulation of the respiratory drive and arterial PO₂ levels. The hypoxic ventilatory response (HVR) decreases strongly with age and is modulated by prolonged hypoxia and physical exercise. Several earlier studies indicated that the regulation of the ventilatory response and erythropoietin (EPO) production by the respective oxygen sensors involves redox-sensitive signaling pathways, which are triggered by the O₂-dependent production of reactive oxygen species. The hypothesis that HVR and EPO production are modulated by thiol compounds or changes in the plasma thiol-disulfide redox state (REDST) was investigated. It was demonstrated that both responses are enhanced by oral treatment with N-acetyl-cysteine (NAC) and that HVR is correlated with plasma thiol level and REDST. Results suggest the possibility that age-related changes in plasma REDST may account for the age-related changes in HVR.

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Introduction

Oxygen tension regulates a series of important physiological responses in practically all cells and organisms. This is exemplified by the O₂-sensing arterial chemoreceptors that contribute significantly to regulation of the efferent respiratory drive and arterial PO₂. The hypoxic ventilatory response (HVR) is triggered by acute hypoxia and precedes a series of other hypoxic cellular responses that include changes in smooth muscle tone and the increased expression of hypoxia-inducible proteins such as erythropoietin (EPO). Earlier studies showed that the HVR decreases strongly with age and is modulated by prolonged exposure to hypoxia and by physical exercise. The O₂ sensitivity of the chemoreceptors may become a determining factor in the pathogenesis of diseases associated with hypoxia, including cardiorespiratory diseases.

The 2 types of O₂ sensors that regulate ventilation and EPO production in response to changes in arterial oxygen concentration are not completely understood and, to some extent, are still the subject of controversy. Although the oxygen sensors for the HVR in the carotid body and in certain other chemoreceptors have been identified as NADPH oxidase isoforms (reviewed in Droge), the regulation of EPO production was shown to involve activation of the hypoxia-inducible transcription factor (HIF), which depends on redox-sensitive stabilization of its α subunit. Oxygen-sensing involves in this case the oxygen-dependent hydroxylation of a proline residue (HIF-1α PS64) and the subsequent ubiquitination and degradation of HIF-1α. A more recent study in mice suggested that S-nitrosothiols may play a decisive role in the ventilatory effect of hypoxia at the level of the nuclei tractus solitarius. In spite of the conspicuous differences in the mechanisms involved, O₂ sensing involves typically redox-sensitive signaling processes that are likely to be altered by thiols or by changes in the thiol-disulfide redox state (REDST). We are, therefore, investigating the hypothesis that the reactivity of the various O₂ sensors in healthy human subjects may be modulated by thiols or by changes in the plasma REDST. As a first test of this hypothesis, we performed a randomized, placebo-controlled study of the effect of the thiold compound NAC on HVR and plasma EPO concentration.

Patients, materials, and methods

Double-blind clinical trial on the effect of NAC on HVR and on plasma EPO level

Eligible subjects were healthy normotonic male nonsmokers who had not consumed alcohol, caffeine, or any drugs for at least 20 hours and had not been engaged in intense physical exercise for at least 12 hours. Twenty-eight subjects were recruited and randomized by the Pharmacology Department of the University of Heidelberg. The sample size was estimated on the basis of an unblinded pilot study to achieve a significance of P < .05. The study was approved by the ethics committee and was conducted according to the principles of the Declaration of Helsinki. After randomization, one subject (placebo) had to be excluded because of an infection. Another subject (NAC) had to be excluded because of a blood donation. Anthropometric data are shown in Table 1. NAC (200-mg capsules; HEXAL AG, Holzkirchen, Germany) or placebo was administered in 3 doses of 600 mg/d at 8 AM, 4 PM, and 11 PM for 5 days (days 2-6).

Blood, plasma, and serum

Blood samples were drawn from a cubital vein and immediately placed in ice water. Within 10 minutes, the samples were subjected to centrifugation at 2000g and 4°C for 10 minutes. Aliquots of plasma were stored at −70°C. Plasma amino acid concentrations were determined with an amino acid analyzer, and the concentration of acid-soluble thiol in the plasma was determined photometrically within 1 hour of sampling, as described. Typically, 0.93 mL plasma samples from heparinized blood were incubated with 0.07 mL sulfosalicylic acid (50%) for 10 minutes at 4°C and subsequent centrifugation (4°C, 15 minutes, 7000 rpm, 110g). Acid-soluble...
Table 1. Anthropometric data

<table>
<thead>
<tr>
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<th>Placebo (n = 13)</th>
<th>NAC (n = 13)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (y)</td>
<td>24.1 ± 1.3</td>
<td>25.2 ± 1.0</td>
<td>.49</td>
</tr>
<tr>
<td>Body weight (kg)</td>
<td>73.4 ± 3.3</td>
<td>77.9 ± 4.1</td>
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<td>Body height (cm)</td>
<td>177.3 ± 2.7</td>
<td>177.7 ± 1.1</td>
<td>.90</td>
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<tr>
<td>Body mass index (kg m⁻²)</td>
<td>22.4 ± 0.4</td>
<td>24.5 ± 1.2</td>
<td>.12</td>
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Values are mean ± SEM.

thiols in the supernatant (acid-soluble fraction) were determined by mixing 0.4 mL supernatant with 0.4 mL pH 8.0 buffer (0.2 M phosphate plus 0.01 M EDTA). The increase of the absorbance at 412 nm was then determined photometrically before and after the addition of 0.02 mL 10 mM 5,5'-dithiobis-2-nitrobenzoate. Cysteine was used as a standard. The term [thiol][cystine]⁻¹ (μM) was defined as the plasma REDST. Serum levels of EPO were analyzed by the EPO enzyme-linked immunosorbent assay (IBL, Hamburg, Germany). Plasma total peroxide concentrations were determined photometrically in EDTA plasma by the plasma peroxide concentration assay (Immundiagnostik, Bensheim, Germany), which is based on the reaction of horseradish peroxidase with plasma peroxides using tetramethylbenzidine as a chromogen substrate (450-nm wavelength).

Ventilation, respiratory gas analysis, and pulse oximetry

Ventilation (V̇̇) and inspiratory and end-tidal CO₂ were measured breath-by-breath by the respiratory monitoring system Oxycobeta (Mijnhardt, Bunnik, The Netherlands) using the software version 3.12 with elimination of gliding averages. Each subject wore a nose clip and a mouthpiece connected to a flowmeter (Triple V) with an integrated gas-sampling capillary. The flowmeter was attached to a low-resistance T-shape valve system (Haward, Edenridge, United Kingdom) with a dead space of 95 mL. The inspiratory side was connected to a 110-cm tube (inner diameter, 4.5 cm) through which room air and hypoxic or hypoxic mixtures were inhaled. Oxygen saturation (SaO₂) was measured continuously by a pulse oximeter (3740 Biox Pulse Oximeter; Ohmeda Biox, Louisville, KY) using the finger probe placed at heart level.

Protocol of the study under normoxia

Resting minute ventilation, end-tidal PCO₂, and isocapnic HVR were determined on day 1 and day 5. Subjects ingested 400 mL water at 7:00 am and arrived at the laboratory in a fasting state at 7:45 am. Blood samples were drawn after 15 minutes of rest. For ventilatory measurements between 8:30 am and 12:00 pm, subjects equilibrated to the semi-reclined test for at least 20 minutes. Breath-by-breath values of V̇̇ and end-tidal PCO₂ (PetCO₂) were monitored for several minutes. When stable conditions were reached, values were recorded for 5 minutes, and HVR measurements were performed under isocapnic conditions. This procedure was repeated between 4:30 pm and 8:00 pm. Additional plasma thiols determinations were made at 0:00, 4:00, 6:00, and 8:00 pm. HVR in isocapnia was determined as described. Nitrogen was admixed to the inspiratory air reservoir to lower the FiO₂ level (initially 35%) in such a way that SaO₂ fell within 6 to 10 minutes in a linear fashion to 80%. The slope of the ventilatory response (ΔV̇̇/ΔSaO₂, L min⁻¹%⁻¹) was calculated by linear regression of breath-by-breath values. For isocapnic HVR measurements, CO₂ was added to the inspiratory air to maintain PetCO₂ at the initial individual level during poikilocapnic conditions (HVR poikilo) was calculated as Δ V̇̇/Δ SaO₂ (L min⁻¹%⁻¹) for each time point. Blood samples were drawn after 15 minutes and 5.5 hours in hypoxia, and 15 minutes, 1 hour, 2 hours, and 4 hours after hypoxia.

Statistics

Statistical procedures were performed by SPSS for Windows (version 6.1). Results are presented as mean ± SEM. The 2 treatment groups (Table 1) were compared by the Student t test for unpaired samples. Statistical differences within either group were assessed by one-factorial analysis of variance for repeated measures and post hoc test (paired Student t test). Linear regression analysis was used to determine correlations. A difference was considered significant if \( P < .05 \).

Results

The randomized double-blind trial showed that relatively moderate doses of NAC caused a significant increase in the plasma thiol concentration and a shift in the plasma REDST (thiol[cystine]⁻¹) to more reducing conditions (Figures 1 and 2). NAC treatment also caused a significant increase in the isocapnic hypoxic ventilatory response (HVRiso) and the plasma EPO level (Figures 1 and 2). Protocol of the study under normoxia

Resting minute ventilation, end-tidal PCO₂, and isocapnic HVR were determined on day 1 and day 5. Subjects ingested 400 mL water at 7:00 am and arrived at the laboratory in a fasting state at 7:45 am. Blood samples were drawn after 15 minutes of rest. For ventilatory measurements between 8:30 am and 12:00 pm, subjects equilibrated to the semi-reclined test for at least 20 minutes. Breath-by-breath values of V̇̇ and end-tidal PCO₂ (PetCO₂) were monitored for several minutes. When stable conditions were reached, values were recorded for 5 minutes, and HVR measurements were performed under isocapnic conditions. This procedure was repeated between 4:30 pm and 8:00 pm. Additional plasma thiols determinations were made at 0:00, 4:00, 6:00, and 8:00 pm. HVR in isocapnia was determined as described. Nitrogen was admixed to the inspiratory air reservoir to lower the FiO₂ level (initially 35%) in such a way that SaO₂ fell within 6 to 10 minutes in a linear fashion to 80%. The slope of the ventilatory response (ΔV̇̇/ΔSaO₂, L min⁻¹%⁻¹) was calculated by linear regression of breath-by-breath values. For isocapnic HVR measurements, CO₂ was added to the inspiratory air to maintain PetCO₂ at the initial individual level during hypoxic conditions. All HVR data were determined in duplicate and are presented as mean of both values. Whenever the lower HVR measurement was less than 50% of the higher value, the measurement was repeated.

Study under prolonged hypoxia after 5 days of medication

On day 6, blood samples were drawn at 8:00 am from fasted subjects in normoxia. At 10:00 am, after a small breakfast, subjects entered a 14-m² normobaric hypoxic chamber that provided a constant FiO₂ level of 12% by admixture of N₂-enriched air through a feedback O₂-sensor control of an air inlet valve (AGA, Hamburg, Germany). FiCO₂ levels were kept below 0.1% by admixture of fresh air to the chamber. Subjects stayed in the chamber for 6 hours in a comfortable sitting position and were occupied with reading or TV watching. O₂ saturation and heart rate were monitored continuously. Resting V̇̇ and PetCO₂ were recorded after 30 minutes, 2 hours, and 6 hours in hypoxia, and the hypoxic ventilatory response under these poikilocapnic conditions (HVR poikilo) was calculated as Δ V̇̇/Δ SaO₂ (L min⁻¹%⁻¹) for each time point. Blood samples were drawn after 15 minutes and 5.5 hours in hypoxia, and 15 minutes, 1 hour, 2 hours, and 4 hours after hypoxia.

Figure 1. Effect of NAC treatment on the plasma thiol, REDST, HVR, and EPO levels. Relative changes during medication between baseline and terminal examination expressed as percentage of baseline values. With EPO, baseline examination took place on day 1 at 8 am, and the terminal examination was on day 6 at 8 am (before hypoxia) or at 6 pm (2 hours after hypoxia), as indicated. In all other cases, the relative changes were computed from the means of all measured values on day 1 and day 5, respectively. Similar data (\( P = .05 \)) were obtained if the HVR data were normalized according to the individual body weight (not shown). Values are mean ± SEM (P = placebo, n = 13; N = NAC, n = 13). Significant differences between baseline and terminal values are indicated (#P < .05; ##P < .01).
values of the day (Figure 3C-D), implying that persons who happened to have a relatively high mean thiol level and REDST during the day also maintained a relatively high HVR value during hypoxia, even several hours after the temporary decline in thiol level and REDST. A significant correlation between $HVR_{poikilo}$ and mean plasma thiol level and mean REDST of the day was also seen within the NAC-treated group alone (Figure 3C-D), indicating that the correlation was not merely based on differences between treatment groups. Collectively, these data support the paradigm that the HVR is indeed modulated by the REDST and that a temporary increase in plasma REDST may cause a long-lasting effect. There was no significant correlation between EPO level and thiol concentration or REDST (not shown).

**Discussion**

Our study has shown that the HVR and the EPO concentration are enhanced by oral treatment with NAC. Given that HVR and EPO production are 2 distinct physiological functions rigorously controlled by oxygen sensors, the results suggest strongly that the response of the respective redox sensors is modulated by changes in the plasma thiol level or the plasma REDST. In view of the fact that the regulation of ventilation in the carotid body in response to changing $O_2$ concentrations involves the intermediate production of superoxide radicals,13-15 it is reasonable to assume that NAC or its biochemical derivatives, cysteine and glutathione, may act by scavenging the $O_2$-derived radicals or by direct reaction with redox-reactive components of the signaling cascade. Plasma REDST was previously shown to be correlated with the intracellular glutathione redox state, at least in some tissues;26 and NAC treatment was previously shown to cause a decrease in the production of superoxide anion by stimulated neutrophils.27-30 In view of recent evidence for a role of nitrosothiols in regulating the ventilatory response at the level of the nucleus tractus solitarius,23 there is also the possibility that oral NAC application or the endogenous concentration of thiols or REDST may modulate HVR by altering nitric oxide production.
Because the γ-glutamyl-cysteine synthetase reaction, the first and generally rate-limiting step in glutathione biosynthesis, is largely determined by the concentrations of its substrates glutamate and cysteine, it makes sense that the HVR was correlated not only with the REDST but also with the plasma glutamate and cystine concentrations. These correlations were more pronounced under the conditions of prolonged hypoxia at 3:30 PM on day 6 with the REDST but also with the plasma glutamate and cystine concentrations. The production of EPO may be mediated by direct interference of NAC or endogenous thiols with the hydroxylation of the proline residues of HIF-1α, an obviously redox-sensitive process that controls the activity of this transcription factor. An obviously redox-sensitive proline residues of HIF-1α also participate in the scavenging of reactive oxygen species and may be associated with an increased expression of other proteins under control of HIF-1α such as the vascular endothelial growth factor. These points require further investigation.

Earlier studies have shown that plasma thiol level decreases with age and that corresponding shifts in the plasma REDST may contribute to the process of age-related wasting and may be a suitable target for therapeutic intervention with NAC. Moreover, studies from 2 different laboratories have shown that the HVR of elderly subjects in the 7th and 8th decades of life is approximately 50% lower than that of healthy young subjects. The emerging paradigm that aging may result, at least in part, from dysregulation resulting from an oxidative shift in REDST may be seen as an extension of the free radical theory of aging. Oral treatment with NAC has served as a useful investigative tool and may be an effective pharmacologic option to increase the plasma thiol level, REDST, HVR, and plasma EPO concentration. This treatment may be useful for elderly subjects and for patients who have other conditions with an oxidative shift in plasma REDST, such as coronary heart disease and malignant diseases.

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References

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