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Exhaled Carbon Monoxide is not Flow Dependent in Children with Cystic Fibrosis and Asthma

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Abstract
Study objectives: Exhaled nitric oxide (eNO) and carbon monoxide (eCO) concentrations are elevated in inflammatory airway diseases like asthma and have been investigated as potential diagnostic markers. For eNO concentrations knowledge about the inverse flow dependency is essential for reproducibility and comparability of measurements.

The aim of this investigation was to evaluate a possible expiratory flow dependency of eCO in children with different inflammatory airway diseases.
Design: ENO and eCO concentrations were measured electrochemically and via chemiluminescence in the exhaled air of 20 healthy children, 17 stable cystic fibrosis (CF)-patients and 15 steroid-naive asthmatics in a combined analyzer at five different expiratory flows (10, 20, 45, 86, 184 ml/sec).
Results: ECO was not flow dependent in any of the three groups. At 45 ml/sec the mean eCO-concentration of healthy children was 3.72 ± 0.23 ppm, of CF-patients 3.67 ± 0.37 ppm and of asthmatics 4.99 ± 0.45 ppm. Elevated eCO ($p<0.0122$) was found in asthmatics but not in CF-children. There was no age dependency and no correlation between eNO and eCO.
Conclusions: In contrast to CF-patients in the exhaled air of steroid-naive asthmatics elevated eCO concentrations are found that may serve as non-invasive inflammatory marker. In contrast to eNO, eCO did not show any expiratory flow dependency.

Key words: carbon monoxide, nitric oxide, flow dependency, asthma, cystic fibrosis

Abbreviations: CF: cystic fibrosis; (e)CO: (exhaled) carbon monoxide; (e)NO: (exhaled) nitric oxide; FEV_1: forced expiratory volume in the 1st second; FVC: forced vital capacity; HO: heme oxygenase; MEF_{25}: maximal expiratory flow at 25% vital capacity; MMEF: maximal mid-expiratory flow; NOS: nitric oxide synthase; ppm: parts per million; ppb: parts per billion

Introduction

The assessment of inflammation by bronchoalveolar lavage or bronchial biopsy is too invasive to be performed on a regular basis in the management of children with chronic airway disease. As repeated spirometry, home peak-flow measurements and symptom scores are only indirect measures of the degree of airway inflammation [1], non-invasive techniques, especially exhaled gas measurements, have attracted increasing interest due to an easy performance by direct exhalation into an analyzer.

Elevated concentrations of exhaled nitric oxide (eNO), produced by various lung cells from the amino-acid L-arginine via different iso-enzymes of NO synthase (NOS), can be found in the exhaled air of steroid-naive asthmatic adults [2] and children [3-5] but not in CF-patients [4-6]. An inverse flow dependency with low eNO concentrations at high expiratory flow rates has been described and is essential for reproducibility [4]. ENO has been characterized extensively but still has not proven its usefulness as a parameter to guide anti-inflammatory treatment in clinical studies [7].

In steroid-naive adult [8] and pediatric asthmatics [9] elevated levels of exhaled CO (eCO) were demonstrated. Endogenous CO is generated in humans by two subtypes of the enzyme heme oxygenase: the constitutive isoform HO-2 (brain, liver) and the inducible isoform HO-1, which is produced by many cell types in the lower airways and is up-regulated by oxidative stress, pro-inflammatory cytokines and NO [10] as a cytoprotective mechanism against oxidative cellular injury. Some authors have presented evidence, that in steroid-naive adult asthmatics, raised levels of HO-1 protein in airway macrophages correspond to elevated eCO, while in steroid-treated patients lower eCO is detectable [11]. Others however found similar HO-1 expression and eCO in asthmatics regardless of steroid treatment [12]. Regarding stable CF- patients normal [13] and elevated [14] baseline eCO has been described, but high eCO has been found after exercise [13] and with pulmonary exacerbation [15].

These findings suggest a role for endogenous CO in inflammatory airway diseases and therefore eCO may provide a valuable non-invasive measurement of inflammation suitable for use in children. Before the power of eCO for diagnostics and as a practical tool for the assessment of inflammation during therapy of airway diseases may be evaluated in clinical studies, more work needs to be accomplished to further describe this variable.

The aim of this study was to characterize the influence of expiratory flow on eCO as well as its correlation to lung function measurements and eNO.

METHODS

PATIENTS

15 steroid-naive asthmatics aged 11.6 ± 0.7 years (9 female) were recruited from the Pediatric Pneumology and Allergy outpatient clinic. Their asthma was diagnosed before treatment according to the recommendations of the National Heart, Lung and Blood Institute (NHLBI) Expert Panel Report [16]. Patients with oral or inhaled steroid treatment during the last two months, acute upper airway infection and serious other illness (cardiovascular disease, interstitial lung disease, tuberculosis) were not included.

CF-patients are seen on a regular basis in the Cystic Fibrosis outpatient clinic. 17 of them, aged 12.9 ± 0.9 years (11 female) were in stable condition at the time of the investigation and agreed to participate. In all participants CF had been diagnosed by at least two different sweat tests or DNA analysis for CF mutations. 11 children were chronically infected with Pseudomonas aeruginosa, the other six grew intermittent Staphylococcus aureus in their sputum or throat swab.

20 healthy, non smoking individuals aged 11.5 ± 0.7 years (9 female) were recruited from relatives, friends and a local sport club and served as controls. Children with known allergies, a history of chronic respiratory tract disease as well as children with acute respiratory symptoms or symptoms for 3 weeks prior to the test were not included in the study.

The study was approved by the local ethics committee and informed consent was obtained from all patients and their parents.

MEASUREMENTS

CO and NO concentrations were measured simultaneously electrochemically and via chemiluminescence in the combined LR 2000 analyzer (Logan Research, Rochester, Kent, UK) sensitive to eNO at concentrations of 1-5000 parts per billion (ppb, by volume) and with a CO detection limit of 0.1 ppm. The analyzer was calibrated daily using certified NO mixtures (100 ppb) in nitrogen (BOC Gases, Guildford, UK) and 50.000 vpm CO in nitrogen (Linde, Munich, Germany). The investigation was basically conducted as recommended by the European Respiratory Society Task Force "Measurement of Nitric Oxide in Exhaled Air" [17] before regular spirometry as described earlier [18]. Briefly, the children were asked to perform a slow vital capacity manoeuvre over 15-20 s into widebore Teflon tubing without wearing a nose clip. To isolate the nasopharynx from the oropharynx by a closed soft palate (NO), the subjects exhaled against a defined resistance while maintaining a mouth pressure of 5 cm H_2O displayed on the computer. Five different expiratory flow velocities were achieved by exchanging the resistances specially calibrated to yield flows of 10, 20, 45, 86 and 184 ml/sec according to the capacities of the measuring unit. For every resistance a new maneuver was performed, while the order in which they were measured was changed from patient to patient randomly. Exhaled gas concentrations were continuously monitored. Measurements were performed at least twice at 3 min intervals and only those were accepted in which the subject had produced an eNO-plateau (± 0.5 ppb) of at least 3 sec, eCO had reached a peak, CO_2 concentration recorded simultaneously was >4.5% and the exhalation pressure was constant at 5 cm H_2O. NO was measured at the final phase of the exhalation to obtain an endexpiratory NO value while CO peak values were recorded, both visually determined from the computer screen. Ambient CO was recorded daily (always less than 0.5 ppm) and subtracted from the individuals peak. To account for environment CO exposure, patients had to spend at least two hours inside the outpatient clinic before the measurement was conducted. The mean of the two measurements was taken. All measurements were made by a well experienced technician.

Spirometry was performed in a Jäger MasterLab (Jäger, Würzburg, Germany) and FEV_1, FVC, MMEF and MEF_{25} were measured according to the recommendations of the ATS [19]. Reference values of Zapletal and co-workers were used [20]. Short acting β2-agonists were not taken up to eight hours before lung function testing. Results are given as % of predicted (lung function at rest).

STATISTICAL ANALYSIS

The statistical analysis was done using Graph Pad Prism software (San Diego, CA, USA). All variables analyzed were normally distributed. Deviations from Gaussian distribution were checked by using the Kolmogorov-Smirnov test with a p value from Dallal and Wilkinson´s approximation to Lilliefors´s method [21] calculated by Graph Pad Prism. Statistical results are reported as mean ± standard error of the mean (sem) in n subjects if not mentioned differently. Flow dependency was evaluated by repeated measurement ANOVA (followed by Newman-Keuls post test if further evaluated) and linear regression analysis. Differences between healthy individuals and asthmatics/CF-patients were analyzed by one way ANOVA, followed by the Dunnett post-hoc test with the healthy subjects as control group. Comparisons between groups were done with the data of 45 ml/sec, as similar expiratory flow velocities have been used by other authors [22].

RESULTS

PATIENT CHARACTERISTICS

Patient characteristics are described in Table 1. There were no differences between the three groups regarding age. Among asthmatics and CF-patients slightly more females had been recruited. Overall, CF-patients and asthmatics had worse FEV_1, FVC, MMEF and MEF_{25} compared to healthy controls, however only in CF the difference was significant (Table 1).

FLOW DEPENDENCY

A strong flow dependency of NO concentrations in exhaled air was shown for all investigated groups, including CF by repeated measurements ANOVA ($p<0.0001$). Compared to healthy children the NO

Table 1. Subject Characteristics*.

	ANOVA p	asthma	Dunnett p	healthy controls	Dunnett p	cystic fibrosis
Number		15		20		17
age, years		11.6 ± 0.7		11.5 ± 0.7		12.9 ± 0.9
gender (male/female)		6/9		11/9		6/11
FEV_1, % predicted	<0.0001	92.9 ± 5.3	>0.05	104.0 ± 2.7	<0.01	70.1 ± 6.3
FVC, % predicted	0.001	91.3 ± 3.8	>0.05	97.0 ± 2.4	<0.01	75.9 ± 5.1
MMEF, % predicted	0.0076	80.4 ± 10.7	>0.05	103.6 ± 7.4	<0.01	59.5 ± 10.7
MEF_{25}, % predicted	0.0006	72.9 ± 12.4	>0.05	103.1 ± 10.2	<0.01	39.1 ± 10.2
Staph. Aureus						6
P. aeruginosa						11

*Values are mean ± sem. Statistical analysis of overall differences between the three groups was done by the one way ANOVA test, while differences between the groups healthy individuals and asthmatics/CF-patients were analyzed by the Dunett post-hoc test.

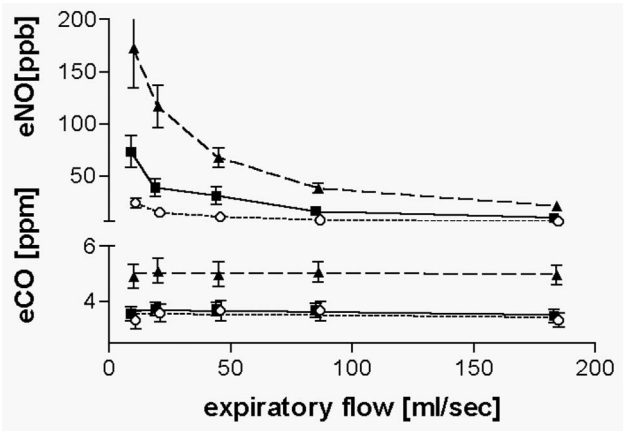

Fig. 1. Flow dependency of eNO and eCO obtained at five different flow rates. Upper part: flow dependency of eNO [ppb] in healthy children (n = 17; closed boxes), asthmatics (n=15; closed triangles) and CF-patients (n = 17; open circles). Data sets visualized by connecting lines. Values are means ± sem of n values. Lower part: lack of flow dependency of eCO [ppm] in healthy children (n =1 7; closed boxes), asthmatics (n = 15; closed triangles) and CF-patients (n=17; open circles). Data are given in mean ± sem of n values.

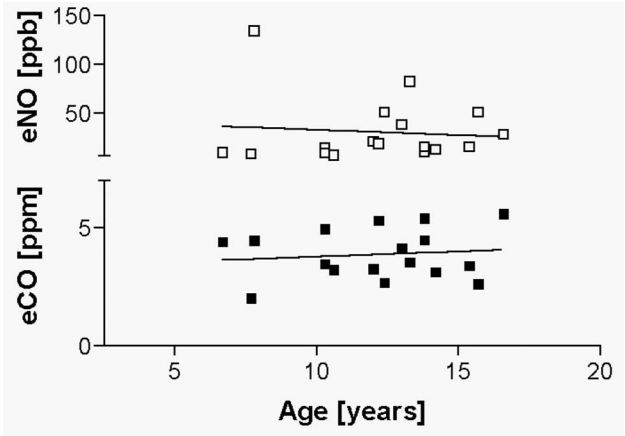

Fig. 2. Lack of an age dependency of eNO [ppb] (open boxes) and eCO [ppm] (closed boxes) in 17 healthy children at an expiratory flow rate of 45 ml/sec. Linear regression analysis; eCO: p = 0.6446, r^2 = 0.01456; eNO: p = 0.7237, r^2 = 0.008576.

levels were significantly higher at all five flows in asthmatic patients (p<0.01) but not in CF (>0.05) (Fig. 1, upper part).

In none of the groups a CO flow dependency was shown by repeated measurement ANOVA (p>0.05), linear regression analysis (p>0.05) or correlation analysis (p>0.05). Elevated CO concentrations were found in the exhaled air of asthmatics (p<0.0187) but not in CF-patients (p>0.05) regardless of the expiratory flow velocity (Fig. 1, lower part).

Age Dependency

There was no correlation between the CO concentration in exhaled air and the age of the children participating at any expiratory flow velocity in any of the groups investigated. The same was observed for eNO. Figure 2 outlines data obtained with healthy controls at an expiratory flow velocity of 45 ml/sec.

Correlation Analysis

No significant correlation between both exhaled inflammatory markers became evident at an expiratory flow velocity of 45 ml/sec (Fig. 3) nor at any other expiratory flow velocity. The same was observed for healthy individuals and CF-patients (data not shown). Neither for healthy individuals nor for asthmatics or CF-patients a correlation between any lung function parameter (FEV_1, FVC, MMEF and MEF_{25}) and eNO or eCO at any expiratory flow velocity could be established.

Discussion

Although eNO is the non-invasive marker of airway inflammation being most intensively investigated at

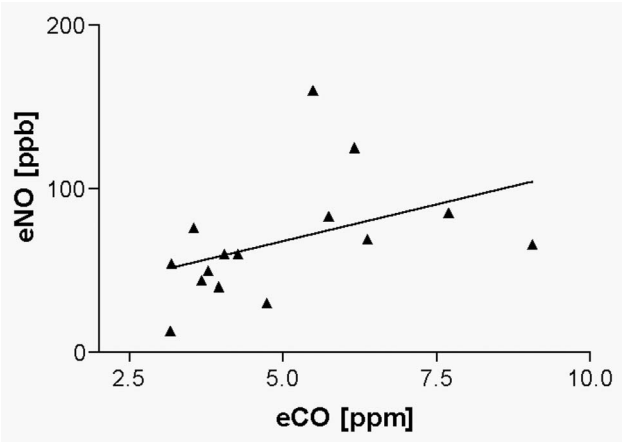

Fig. 3. Lack of correlation between eNO [ppb] and eCO [ppm] in 15 asthmatic children at an expiratory flow velocity of 45 ml/sec. Linear regression analysis, $p = 0.113$, $r^2 = 0.1833$.

the moment, it has not been established as a diagnostic and therapeutic tool to be used on an everyday basis by the pediatric pulmonologist. This is partly due to its flow dependency and the possibility of nasal contamination, which makes its measurement especially in infants very demanding [23]. Therefore other non-invasive markers such as eCO need to be further evaluated. Elevated eCO has been reported in adult and childhood asthmatics by almost all investigating groups so far [8, 9, 11, 24], only Zetterquist et al. reported normal eCO levels in a mixed pediatric/adult group of steroid-naive asthmatics [22]. Similar to the majority of studies we also observed elevated eCO. The elevation of eCO in steroid-naive asthmatic children is thought to be caused by an increased expression of HO-1 in airway cells by oxidant stress and cytokines [10, 11, 25]. NO, which is also elevated in asthma caused by an up-regulation of iNOS [26], has been shown to directly stimulate HO-1 but not HO-2 expression in human primary airway epithelium cells independent of the cytokine-induced elevation of both enzymes [10].

In patients with CF, most authors [14, 15, 27, 28] have found elevated eCO in adult patients, while the very few studies [13, 22] investigating children report normal eCO in stable patients. This is similar to our results. Increasing lung destruction in older CF-patients may offer an easy explanation for this phenomenon, but there was no age dependency in our small group of patients. In addition, the age ranges of children, adolescents and adults investigated by different authors were close and individual severity of disease may vary widely independently of age. Explanations for normal eCO in CF-children include less production of CO in the airway epithelium, increased loss during exhalation or altered diffusion of the gas from the epithelial surface into the airstream. Unlike experiments showing less production of NO in CF-lungs by absent iNOS in bronchial epithelium [29], HO activity in biopsies has not been investigated so far. As different observations have been made by different authors, it is very important to outline very clearly patient characteristics, study design, methodology and biological factors. Not all publications point out clearly e.g., whether CO peak or baseline values were obtained for further evaluation.

Here we show, that in contrast to eNO the concentration of eCO is independent of expiratory flow velocity. There are several explanations for the lack of flow dependency of eCO. Exhaled CO is the sum of CO produced in the airways and of CO produced somewhere else in the body and transported by the bloodstream into the lungs. The alveolar origin of eCO causes this lack of flow dependency, because eCO mainly produced in the airways would be expected to be markedly increased with a decrease in expiratory flow velocity. The elevation of eCO in inflammatory airway disease may originate from the respiratory epithelium of the bronchi via induction of HO-1 [11] and from an increased expression of HO-1 in alveolar macrophages. The latter has recently been reported in steroid naive asthmatics [25]. Donnelly et al. showed, that HO-1 in cells harvested from healthy adult volunteers, may also be up-regulated by inflammatory cytokines and NO in human airway epithelium cells [10]. However the contribution of this mechanism to the elevation of eCO in asthmatics is yet unknown.

In this study steroid-naive asthmatic children had elevated levels of eNO and eCO. In patients with CF, eNO was lower and eCO similar to healthy volunteers. In all groups investigated eCO but not eNO was independent of expiratory flow velocity possibly due to its alveolar origin. This lack of flow dependency of eCO opens up several new perspectives in monitoring inflammatory airway disease. For pediatric patients, measurements of eCO can be performed more easily than those of eNO, since it is not necessary to keep the expiratory flow rate at preset level and thus the mouth pressure. As apparently there is no significant nasal production of CO [30], there is no need to perform maneuvers such as exhaling against a pre-set resistance in order to close the soft palate. Although we are able to show, that eCO is not flow dependent, before eCO may be used in clinical practice, its role in assessing airway inflammation and treatment response in pediatric asthma needs to be further explored.

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