Gas Analysis

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Several putative markers of inflammation have been studied in the gaseous phase and in condensate of exhaled air, mainly in adults. Among these markers are nitric oxide (NO), carbon monoxide (CO), and, in breath condensate, hydrogen peroxide (H_2O_2) and leukotrienes (LTs). The methodology for these measurements has not been standardized, although several guidelines for the measurement of NO have been and will be published. A rapidly growing body of literature supports the use of NO as an inflammatory marker in asthma, where it may be useful for diagnosis and perhaps monitoring of (compliance with) treatment. Equipment to measure exhaled NO (eNO) for medical purposes has become commercially available. Although this equipment is still expensive and clumsy, there seems to be increasing interest to buy NO analyzers for clinical purposes. However, even for NO, there is little if any literature supporting its value in clinical practice, especially in pediatric patients. This overview focuses on NO and other possible markers of inflammation in exhaled air and air condensate, briefly summarizes some of the published data, and indicates gaps in knowledge with respect to pediatrics.

WHAT DO WE KNOW?

Inflammation Markers in Exhaled Air

Nitric oxide: Measurement of NO. NO can be measured by means of chemiluminescence, a method that has long been used in monitoring air pollution. The equipment is robust, and measures NO by bringing it into contact with ozone, which leads to the formation of an unstable reaction product that disintegrates and produces a single photon. The photon is detected by a photomultiplier tube and the number of photons is equal to the number of NO molecules entering the analyzer. NO analyzers for medical use are fast and allow evaluation of changes in eNO within breaths. They usually display the NO value while taking the measurements. Calibration takes place with certified NO gases and NO-free gas, and there is a linear relation between NO concentration and output of the analyzer. Various conditions may affect NO measurements. The presence of water vapor may lower NO readings (1) and therefore it is recommended that an (NO-inert) water absorber be used in the collection system. Also, inhalation of L-arginine, the substrate of NO synthase, has some effect on eNO (2), but this seems to be of little practical importance. Alcohol traces may give a false positive NO reading (3), and therefore it is recommended that materials used for measurement of NO not be cleaned with alcohol.

Sampling of exhaled air for NO measurements. Extensive recommendations were published by a European Respiratory Society task force (4), and even more detailed guidelines were produced during an American Thoracic Society workshop in 1998 (5). Despite these publications, several aspects of exhaled NO sampling remain to be explored, and there are insufficient data from which to produce guidelines for all aspects of the measurements. Among these are measurements in young children and off-line techniques (6). To date, NO has been studied extensively in adults and school-age children but only a few studies involving preschool children and infants have been published.

NO diffuses from the walls of the airways, enriching the essentially NO-free alveolar air traveling to the mouth during expiration. Hence, eNO concentrations are flow dependent, and one way to standardize for this is to sample during slow exhalation at a constant flow. The added value of measuring exhaled CO_2 has not been demonstrated; some analyzers have built-in capnographs and recommend that NO is measured at the alveolar plateau of CO_2. However, as NO has no "alveolar plateau" and eNO is determined by factors entirely different from exhaled CO_2, the combination of the two measurements seems to make little sense (7).

Breath holding leads to accumulation of NO in the airways and should be avoided. NO is not only generated in the lower airways, but also in the nose (8), paranasal sinuses (9), and in the oropharynx (10). Contamination of eNO with much higher NO concentrations from the nose or stomach should be avoided, and this is generally achieved by applying a fixed positive mouth pressure during exhalation to close the soft palate. Grossly, two sampling methods can be distinguished: on-line and off-line sampling.

On-line sampling. With on-line sampling, gas is sampled by the analyzer at a fixed flow from a side port in an exhalation circuit. The subject is seated next to the analyzer and produces a constant flow against a fixed resistance to elevate oral pressure. Flow and pressure are regulated by means of a biofeedback signal. End-expiratory plateau values of eNO are recorded and selected for averaging to obtain eNO. The initial part of the expiration may show a peak in eNO, corresponding to NO accumulated in the dead space volume (7). Although this peak has been shown to correlate with plateau values, it has not gained acceptance, perhaps because its magnitude depends on breath holding and flow pattern. The relationship between peak eNO and area under the curve (AUC) of the whole tracing is not surprising as the peak often provides a large contribution to the AUC. A final possibility for on-line recording is that air can be sampled during quiet tidal breathing through a mouthpiece with or without resistor. NO in exhaled air, recorded by a fast analyzer, can then be calculated as an average signal over several breaths.

Exhaled NO depends on ambient NO concentration; in particular, the initial (dead space) volume may be contaminated by high ambient NO values. Formal studies evaluating the influence of ambient NO on eNO have been performed both in adults and children, and reach remarkably different conclusions (11–13). This may well be because of differences in methodologies. Devices that deliver NO-free air to the inspiration circuit have been developed, and are incorporated in some of the commercial analyzer systems. The effect of elevated ambient NO on end-expiratory NO values is probably

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minor. High ambient levels will prolong the exhalation time necessary to reach a plateau.

There are limited data on eNO measured on-line in babies (14, 15). Air sampling in this age group has been tried by using forced expiration and by collecting mixed expired air during quiet breathing. Published work suggests that contamination from the nose may not be as important as in older subjects (16). A single research group has tried sampling of nasal air, with and without occlusion, in premature and term infants. These measurements show an initial expiratory peak, depending on the occlusion time, suggesting some nasal NO production (17, 18).

Off-line sampling. Exhaled air can also be collected in an NO-inert storage bag (made of, e.g., Mylar Tedlar, or polyethylene; party balloons are excellent) and analyzed later. This method is attractive when subjects are studied in the home or school environment, where samples can be obtained without direct presence of an analyzer. NO has been shown to remain stable in suitable bags for several hours, with or without a facility to absorb water vapor (19). Bags can be transported to the analyzer and measured at a convenient time later in the day. The flow during balloon blowing can be standardized, by including a manometer and fixed resistor in the blowing channel (19), or can be uncontrolled (20). Sufficient resistance should be present to assure closing of the soft palate, which avoids nasal contamination during filling of the balloon (4, 21). Essentially, air can be collected during a single deep expiration, or during tidal breathing through a nonrebreathing valve, collecting the expired air in a balloon. Both methods have been shown to produce broadly similar results, and with suitable flows and resistance, correspond to those of on-line sampling (20, 21).

Off-line sampling in babies is possible but published data are scarce. In this age group, contamination may be less important than in older children, and collection of mixed nasal and oral exhaled air seems a promising diagnostic tool (22, 23).

Clinical applications of eNO. Several conditions have been shown to produce elevated eNO in adults and children. Among these are asthma (24–26), where NO is enhanced during exacerbations (27, 28) and by natural allergen exposure in adults (29) and children (16), viral infections of the upper airway (30, 31), and atopy. In asthmatic children, eNO correlates with the eosinophil percentage in induced sputum (32, 33) and, in a single study, with eosinophil cationic protein (ECP) in induced sputum and with urinary excretion of eosinophil protein X (33). Hence, eNO appears to reflect eosinophilic airway inflammation.

Slightly increased eNO levels were found in lung transplant recipients who developed rejection (34, 35) and in adults with fibrosing alveolitis (36). Other diseases have shown normal, varying or low eNO values: cystic fibrosis (37–39), ciliary dyskinesia (8, 23, 40), bronchiectasis, chronic obstructive pulmonary disease (41, 42) and smoking (43, 44), and adult respiratory distress syndrome (45). NO appeared to differentiate chronic cough due to asthma or nonasthmatic causes (46). Hence, eNO should be studied for its differentiating capacity as a new diagnostic tool. Data indicate that eNO may also be useful in differential diagnosis of lung disease in infants (23).

The possible use of eNO to guide antiinflammatory treatment should be further explored. Steroids reduce eNO in adults (47, 48) and children (49) with asthma. Data from adults indicate that inhaled steroids will reduce eNO to normal levels even before an effect on symptoms is apparent (50). This suggests that eNO shows a fast response. eNO may be used as a measure of compliance with steroid treatment, or perhaps as a predictor of relapse after discontinuation of antiinflammatory treatment.

Carbon monoxide. The possible use of exhaled CO as a marker of oxidative stress in the airways, including asthmatic airway inflammation, has been suggested (51). Horváth and coworkers (52) showed that individuals with asthma have, on average, slightly higher CO concentrations in their exhaled air than healthy subjects, especially when no steroids are taken. In another study, marginally increased CO values were also found in exhaled air of adult patients with bronchiectasis (53). The study showed that CO excretion was a consequence of increased expression of heme oxygenase in macrophages. No studies have been published on exhaled CO in children. One preliminary report showed no difference in exhaled CO in patients with asthma and healthy controls (54). These data suggest that it is unlikely that exhaled CO will be a useful marker.

Other markers. To date, no other markers in the gaseous phase have been studied in children. In adults, a number of studies have evaluated exhale volatile alkanes as markers of inflammation, by means of gas chromatography (55). In adults with asthma or obstructive sleep apnea, elevated levels of pentane, a marker of free radical activity, were found in nasal and oral exhaled air (56, 57).

Exhaled Markers in Breath Condensate

Collection of exhaled air condensate. Condensate can be obtained by passing exhaled air through a cold tube, the material of which should be appropriate for the retrieval of the substances under examination (glass in the case of hydrogen peroxide, Teflon for cysteinyl LTs). Glass tubes or vessels, cooled to 0° C with iced water, have been used (58–60), and condensate can also be obtained by blowing air through a storage vessel in ice or liquid nitrogen, capturing any water vapor in the exhalate as ice on the walls of the vessel (61). Frozen condensate can be stored until analysis, and remains stable for several months (at least shown for H_2O_2 [62]). The importance of collecting and storing the condensate in the dark to avoid breakdown of substances of interest has not been evaluated. Peroxide concentrations can be assessed by fluorimetry of the reaction product of peroxide, horseradish peroxidase, and parahydroxyphenylacetic acid, with concentrations determined from interpolation of a standard curve of H_2O_2 as described in detail elsewhere (59). A limitation is that the amount of condensate necessary for duplicate determination requires about 10 min of tidal breathing by a school-aged child, and is not feasible for most children younger than about 3 yr. Also, contamination of condensate by saliva, a rich source of peroxide, mediators, cytokines, and other proteins, should be avoided. The absence of saliva contamination can be checked by determining amylase in the condensates; amylase is abundant in saliva, whereas only traces are present in lower airway secretions. Finally, condensate has been collected from mechanically ventilated patients by draining the (cooled) expiratory ventilator tubing (63).

Hydrogen peroxide. Hydrogen peroxide is produced by inflammatory cells and macrophages, and can be detected in exhaled air condensate. Increased levels have been found in adult cigarette smokers (64), adults with asthma (65), and in children with unstable (58) and stable (59) asthma. In addition, increased levels of H_2O_2 have been demonstrated in individuals with common colds and in patients with cystic fibrosis (62). In cystic fibrosis, exhaled peroxide falls with antibiotic treatment (66). In adult patients with asthma, the H_2O_2 concentration in breath condensate correlates with sputum eosinophilia, but not with hyperresponsiveness (60). Exhaled H_2O_2 responds to antibiotic treatment in cystic fibrosis, and is therefore of potential use in monitoring cystic fibrosis treatment. Reference ranges have been published for school-aged children (62).

Leukotrienes. There have been few studies on levels of LTs in exhaled air condensate, mainly focusing on the chemotactic LTB_4 and the cysteinyl LTs (LTC_4, LTD_4, and LTE_4). One group, using condensate collected via Teflon tubing to avoid retention of LT by adsorption to glass, has found increased levels of LT in asthma (67). A weak point in these studies is that salivary contamination was not ruled out, and may account for the findings. Methodological differences in condensate collection (especially saliva contamination) may be responsible for discrepancies between studies. There are no published data for children.

Other substances in condensate. Oxidation metabolites of NO may be elevated in condensate, even if NO is not elevated in the gas phase, perhaps because of local metabolism in abnormal airways. This has been shown in patients with cystic fibrosis (68).

The presence of thiobarbituric acid-reactive products (TBARs) in condensate of exhaled air has been demonstrated in adults with asthma (65). TBARs are oxidation products of lipids that are thought to result from oxidant damage of the airway tissues. No data are available on TBARs in children.

In ventilated adults with respiratory distress syndrome, elevated levels of isoprostanes have been described in breath condensate (63). Isoprostanes are prostanoids that may reflect oxidant stress in the airways; they have not been studied in pediatric airway disease.

Another approach to assess exhaled substances is to perform two-dimensional polyacrylamide gel electrophoresis (PAGE) of condensates, and stain the resulting image for proteins. Characterization is possible on the basis of their place in the electropherogram, or by molecular techniques. Few data have been published (61), and as patients in these studies were encouraged to exhale forcefully, it seems likely that contamination with saliva may explain at least part of the results. Using PAGE and sensitive enzyme-linked immunosorbent assay (ELISA) techniques, we have been unable to detect significant amounts of protein in breath condensate that was not contaminated by saliva (our unpublished results, 1999).

WHAT DO WE NEED TO KNOW/HOW CAN WE ACHIEVE THIS?

NO Measurements: On-line Sampling

Can the procedure be standardized around desired flow with respect to, for example, equipment, target flow (dependent on lung size?), duration of plateau, and allowed variation around desired flow? Can on-line sampling be facilitated with special biofeedback software, and how important is ambient NO with different sampling techniques? We need to achieve active flow regulation, avoiding inaccurate blowing of target flow, and age-dependent reference values are needed with standardized methodology. eNO should also be validated against invasive (preferably biopsy) studies of airway inflammation in different patient groups and healthy children of different ages.

NO Measurements: Off-line Sampling

How important is flow standardization with off-line sampling and how can it be achieved? Is it advantageous to discard dead space volume from balloons? What ambient NO levels are acceptable and how can the effects of higher values be eliminated? Off-line methods need to be evaluated in infants and toddlers with respect to feasibility, methods of eliminating nasal NO, normal values, and applications. In older children, normal values are needed with standardized methodology.

Clinical Applications of eNO

The sensitivity and specificity of eNO need to be evaluated in differentiating airway diseases, using standardized methodologies in different age groups. The time course of changes in eNO with changes in treatment or disease activity should also be investigated. Can eNO be used as a marker for various pathologies, including nonrespiratory disease, and what is the predictive value of eNO with respect to treatment success or relapse after reduction/discontinuation of treatment? The added value of measuring eNO, above other common markers of disease activity (symptoms, lung function, hyperresponsiveness, etc.), should also be determined.

Carbon Monoxide

Is exhaled CO elevated in inflammatory airway disease in children? We need to investigate methodologies, normal values, and reproducibility.

Hydrogen Peroxide

Exhaled peroxide should be validated against the gold standard of airway inflammation (biopsies). What is the role of exhaled peroxide in the diagnosis and management of childhood asthma, cystic fibrosis, and other inflammatory conditions of the airways? Can the methodology be adapted for infants and toddlers, and is exhaled peroxide (unlike eNO) sensitive to changes in the dose of antiinflammatory medication? Furthermore, can exhaled peroxide be used to predict the response to or the risk of relapse after reducing/stopping treatment?

Other Markers in Condensate

Are these candidate markers detectable in exhaled air condensate of children, and are they elevated in inflammatory airway disease? Information is needed on appropriate methodology, normal values, and reproducibility.

CONCLUSIONS

Exhaled air is a newly recognized vehicle of several interesting markers of airway inflammation. Presently, a number of appropriate molecules have been identified, and now we need data on their validity, reproducibility, specificity and sensitivity, and normal ranges and on their possible use in research, diagnosis, and treatment of inflammatory airway disease in various age groups. Before such data are available, it makes little sense to promote application in daily practice.

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