ATS Workshop Proceedings: Exhaled Nitric Oxide and Nitric Oxide Oxidative Metabolism in Exhaled Breath Condensate

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CONTENTS

BACKGROUND TO THIS REPORT

In December 2002, a 2-day American Thoracic Society (ATS)–sponsored workshop was held in Toronto, Ontario, Canada, with the participation of scientists from the United States, Canada, and Europe working in the relevant field. Several companies who have developed technologies in the field were also present at all scientific sessions, although they were excluded from the session that dealt with revisions to the ATS statement.

The workshop believed that clinical application of exhaled nitric oxide (NO) was ready for asthma, whereas nasal NO could advance to clinical use in primary ciliary dyskinesias. However, exhaled breath condensate (EBC) was not considered ready for clinical application as the now-published recommendations for standardized methods were not ready at the time of the workshop, methodologic issues abounded, and evidence for application of specific markers in specific diseases was deemed inadequate.

AIMS OF THE WORKSHOP

Aim 1: To review developments in the field of fractional concentration of exhaled NO (FE_{NO}) measurement.

- New NO sensor technologies, facilitated by Serpil Erzurum
- Online FE_{NO} measurement, facilitated by Philip Silkoff
- Pediatric FE_{NO} measurement, facilitated by Eugenio Baraldi
- Offline FE_{NO} measurement, facilitated by Aaron Deykin
- New concepts in modeling NO output, facilitated by Steven George
- Measurement of FE_{NO} in ventilated patients, facilitated by Nandor Marczin
- Nasal NO measurement and clinical application, facilitated by Jon Lundberg
- FE_{NO} as a clinical tool, facilitated by Philip Silkoff

Aim 2: To review data on the assessment of NO redox status in EBC.

- EBC collection issues, facilitated by John Hunt
- Overview of issues under discussion at the European Respiratory Society (ERS)/ATS task force relating to standardization, facilitated by Ildiko Horvath
- Issues of dilution, anatomic origin, and volatile contribution, facilitated by Richard Effros
- Redox chemistry of nitrogen oxides, facilitated by John Hunt

Aim 3: To update the 1999 ATS statement on exhaled and nasal NO measurement (1) in light of recent developments presented at the workshops.

To highlight how this workshop proceedings document interfaces with the ATS/ERS 2005 statement on exhaled and nasal NO measurement, Aim 3 was realized in that statement (2). The discussions around the bullets in Aim 1 are captured at length in this ATS workshop proceedings document, giving much background to the 2005 updates to the ATS/ERS statement. Aim 2 above, dealing with EBC, overlaps very little with the recently published ATS/ERS task force on EBC, which deals with technical measurement issues and not redox status (3).

An Executive Summary of these Workshop Proceedings is published in the *American Journal of Respiratory and Critical Care Medicine* 2006;173:811–813 (http://ajrccm.atsjournals.org/cgi/content/full/173/7/811).

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SECTION 1: UPDATE ON F_{ENO}

Background

With over 1,000 peer-reviewed publications, research into F_{ENO} is well established, and the time has arrived for clinical application (4). The current online measurement technique is practical, and validated. For clinical use to occur, F_{ENO} monitors must achieve regulatory approval, and procedure reimbursement mechanisms must be developed. In June 2003, the U.S. Food and Drug Administration (FDA) approved the first F_{ENO} analyzer (Aerocrine, Stockholm, Sweden) for monitoring asthma. Technologic advances must allow the production of simple and reliable NO monitors if the cost is to be reduced. The workshop reviewed new sensor technologies, issues of measurement, and the evidence supporting clinical application of F_{ENO} and nasal NO.

Measurement Issues

Sensor technologies.

CHEMILUMINESCENCE. The most widely used approach to NO analysis is chemiluminescence. A number of commercial chemiluminescent NO instruments are available for the measurement of F_{ENO} (Aerocrine [Stockholm, Sweden, and Chicago, IL], Eco Physics and Eco Medics [Ann Arbor, MI, and Berne, Switzerland], Ionics Instruments [Boulder, CO]). Chemiluminescence is based on the reaction between NO in the sample and ozone, which is generated in the analyzer reaction cell as shown in the equations below. The reaction produces emission in the red and near-infrared region of the spectrum ($h\nu$), which is detected by a photomultiplier tube (PMT).

$$NO + O_3 \Rightarrow NO_2^{\cdot}$$

$$NO_2^{\cdot} \Rightarrow NO_2 + h\nu$$

The PMT signal is directly and linearly proportional to the NO concentration in the sample stream.

Newer sensor technologies. As clinical application of NO measurement develops, there will be a need for less complicated technologies and less costly equipment. There are basic requirements for new instruments to be applicable in different settings, such as physician offices and patient homes. These include simplicity, portability, real-time results, suitability for remote analysis, transmission of data, and lack of need to calibrate. The analyzer should, of course, conform to ATS/ERS guidelines. It will be necessary to develop new technology that enables these qualities. Standardization of the measurement and quality control of the results will be key features in equipment intended for primary care. Especially important is to consider facilitation of constant flow exhalation—for example, using dynamic flow control, rejection of nonvalid exhalations, control of ambient NO contamination, and automatic standardization. Analyzer development by several companies is ongoing, but needs to be coordinated with the progress of the methodology in clinical practice. New technologies will increase the availability of NO measurement, which will further reduce the cost as F_{ENO} measurement is incorporated into clinical practice. Eventually, it may be possible to extend F_{ENO} measurement to family practice, and into patients' homes, for self-monitoring.

Other NO analysis capabilities are in development and were briefly presented at the meeting:

EKIPS Technologies (Norman, OK) have employed a tunable diode laser absorption spectroscopy to measure NO and CO_2 in exhaled breath (http://www.breathmeter.com). This platform can also measure other gases of interest.

Aerocrine (Stockholm, Sweden, and Chicago, IL) presented a prototype portable NO analyzer, NIOX-Mino (http://www.niox-mino.com), which is based on a new electrochemical sensor technology that measures NO.

Online F_{ENO} measurement updates. The 1999 ATS statement made firm recommendations for online F_{ENO} measurement (1). Certain revisions to this statement were proposed at this workshop for joint ATS/ERS publication, and will be summarized briefly here. These 2005 ATS/ERS recommendations have now been published (2).

Exhalation flow rate. The exhalation rate of 50 ml/s appears to have met with acceptance in both adults and children, although there are only few publications so far that have used this flow rate, or even specified flow rate (5–19). In addition, there is no universal application of this flow rate by professional societies (1, 20) or NO analyzer companies. It is probably acceptable to use different flow rates for research purposes, as long as the flow rate used is described in the study report, and indeed for different diseases, different flow rates may be appropriate. However, for routine clinical application, a single flow rate will be desirable. The plateau concentrations have been reported to be highly reproducible at 50 ml/s compared with higher or lower flow rates in children (14). In addition, at flows above 100 ml/s, no true plateau exists and the tracing is down-sloping (14). Pedroletti and colleagues also found that the discriminatory power of F_{ENO} for asthma was best at 50 ml/s in children (14), although Deykin and coworkers reported that several flow rates between 47 and 250 ml/s had equivalent discriminatory power for diagnosing asthma (6).

Means of controlling flow. When controlling flow, it is possible to have subjects target airway opening pressure (which with a fixed resistance determines a fixed flow) or flow itself (16). Because flow is the prime determinant, it may be better to directly target flow. It is probably adequate that mean flow over a portion of the exhalation falls within a specified range, even if instantaneous flow deviates. There are many different ways to control flow: these include subject targeting of flow or pressure, dynamic or instrument-facilitated flow including dynamic resistors (21), starling resistors (15), and operator-controlled flow (5). These methods are able to compensate for subjects who are unable to precisely control their effort and may be of great use in young children.

Airway pressure. A fixed flow rate can be achieved by using different airway pressures with different resistances. Some investigators have reported that F_{ENO} levels are affected by airway pressure in animals (22), but not in humans (16), but a recent publication suggests the opposite (23). It is possible that in certain airway disease, airway pressure could recruit atelectatic lung, thereby affecting F_{ENO} values.

Exhaled NO plateau definitions. There has been some difficulty in using the plateau definitions as defined by the ATS statement from 1999 (1). The issues are as follows: (*1*) a first plateau may exist that conforms to the 1999 ATS standards to be followed by a second higher plateau that is probably the real plateau, (*2*) plateaus are difficult to identify at higher flows as the trace may show a steadily declining NO level (14), and (*3*) electronic interpretation of plateaus needs overriding by human interpretation on occasions. Plateau definitions need to be practical and easily achievable to avoid having to do an excessive number of exhalations. Although several companies have included software programs that analyze F_{ENO} profiles and identify plateaus that conform to ATS recommendations, visual inspection is also a valid way to determine the plateau. The section on plateau definition has undergone revision and has appeared in the revised 2005 statement (2) as follows:

> The duration of exhalation must be sufficient (at least 4 seconds for children$<$ 12 years old and $>$ 6 seconds for children $>$ 12 years

old and adults. This corresponds to an exhaled volume of at least 0.3 L in adults at an exhalation flow rate of 0.05 L/s) to allow the airway compartment to be washed out and a reasonable plateau achieved. In general, patients can exhale comfortably up to 10 seconds and this may be necessary for the achievement of a stable NO plateau. The plateau concentration in NO should be evaluated over 3 seconds (0.15 L) window of the exhalation profile according to the following guidelines. The plateau can be considered to begin at point A and end at point B. The plateau can be flat, positive sloping, or negative sloping. However, the magnitude of the slope should be minimized using the following criteria. Points A and B should be chosen to define the first 3-second window in the exhaled concentration profile such that the absolute magnitude of A–B is less than 10%. In addition, no point within the 3-second window should deviate from either the value at point A or point B by more than 10%. The plateau concentration, $F_{E_{NO}}$, is then defined at the mean concentration over this 3-second window. Once a 3-second plateau is achieved, there is no reason to continue the exhalation.

Number of required exhalations. Many workshop attendees believed that two reproducible $F_{E_{NO}}$ values (agreement within 10% of lower value) are adequate for clinical purposes, although for some research purposes, it may be preferable to continue to require three reproducible values. The revised ATS/ERS statement has incuded this change (2). If the patient is measuring $F_{E_{NO}}$ for the first time, however, a "training blow" is recommended.

Inhalation phase. The 1999 ATS statement recommends inhalation to total lung capacity (TLC), which is a standard maneuver used in spirometry and is familiar to patients. $F_{E_{NO}}$ levels are reduced when exhaling from lower inspiratory volumes (16). However, inhalation to TLC may be difficult for some subjects. It is now stated in the revised statement that inspiration to TLC is recommended but that inspiration *close* to TLC is acceptable and that subjects should be told to inspire "deeply" or "as deep as you can."

Calibration gases. There has been concern that calibration gases are in the ppm range, whereas human levels are in the ppb range with assumption of linearity. It has also been difficult to obtain stable, reliable ppb gases in the United States, although these are available in Europe. Eco Physics (Ann Arbor, MI) dilutes a ppm NO gas to the ppb range for calibration. Aerocrine uses a gas of approximately 200 ppb imported from Sweden, and Ionics Instruments uses a 45-ppm gas with linearity tested to the ppb range. Further discussion with industry is necessary to determine feasibility of producing stable ppb gas in the United States. Meanwhile, it is reasonable to continue using current calibration gases.

Normal range of $F_{E_{NO}}$ values. For both research and clinical purposes, it will be necessary to refer to published normal values in adults and children. There was consensus that such normal data with measures of central tendency and variability should be published in the revised statement or preferably published separately and referenced by the statement. There is a large variability in normal subjects similar to other physiologic parameters.

CLINICAL APPLICATION OF $F_{E_{NO}}$ MEASUREMENT

Asthma

Exhaled NO is well established as a research tool in asthma with over 400 publications regarding this marker. There is no currently recommended periodic assessment of airway inflammation in asthma. The time is ripe for clinical application, as recently reviewed (4). Leaving aside bronchoscopy, which is impractical for routine clinical use, the tests of inflammation available include induced sputum, blood eosinophils and eosinophil cationic protein, urinary leukotrienes, breath condensate, and $F_{E_{NO}}$. Exhaled NO is attractive as the test is completely noninvasive, the cost per test is low, and the result is immediate. This makes it ideal for clinical application. Evidence supporting the clinical utility of $F_{E_{NO}}$ in asthma was reviewed and is summarized in Table 1.

Impediments to the clinical application of $F_{E_{NO}}$ include the paucity of publications regarding the utility of this marker in predicting exacerbations and improving long-term asthma control or reducing health care costs. Although the FDA has cleared one $F_{E_{NO}}$ monitor, reimbursement mechanisms are not yet established. The equipment remains very expensive. Finally, the value of $F_{E_{NO}}$ monitoring is not widely appreciated in the medical community.

Other diseases. Exhaled NO may be a good diagnostic tool for primary ciliary dyskinesia (PCD) syndromes (45), but its clinical utility in chronic obstructive pulmonary disease (COPD), interstitial lung disease, cystic fibrosis (CF), and other pulmonary or extrapulmonary diseases remains to be established.

SECTION SUMMARY: ONLINE MEASUREMENT OF EXHALED NO

This section reviewed some advances in the technologies available for the detection of NO, which will facilitate the progress of this marker from bench to bedside. The 1999 ATS recommendations were reviewed and some changes were recommended to the online exhaled NO measurement technique, which were updated in the revised ATS/ERS statement on exhaled and nasal NO measurement that was published in 2005 (2). Finally, knowledge about the clinical application of exhaled NO was presented and discussed.

TABLE 1. RATING OF EVIDENCE FOR UTILITY OF $F_{E_{NO}}$ IN ASTHMA MANAGEMENT

Utility	Level of Evidence	Potential Application
$F_{E_{NO}}$ raised in asthma (24–29)	5	Diagnosis
$F_{E_{NO}}$ falls after steroids (27, 30–32)	5	Monitoring and titrating response
$F_{E_{NO}}$ falls after other antiinflammatory drugs (e.g., leukotriene antagonists, anti-IgE) (33–35)	2–3	Monitoring and titrating response
$F_{E_{NO}}$ rises on stopping medication (29)	5	Compliance check
$F_{E_{NO}}$ correlates with eosinophilic inflammation (36–39)	2–3	Monitoring the inflammatory component
$F_{E_{NO}}$ correlates with other inflammatory components of asthma (40)	0–1	Monitoring the inflammatory component
$F_{E_{NO}}$ rises with exacerbation (41, 42)	3	Assessment of exacerbation
$F_{E_{NO}}$ predicts exacerbation (43, 44)	1–2	Predicting exacerbation
Targeting therapy to inhibit $F_{E_{NO}}$ to improve control, QOL, health care costs, etc.	0	Improving long-term control
Targeting $F_{E_{NO}}$ to prevent remodeling, FEV_1 decline, etc.	0	The holy grail

Definition of abbreviation: QOL = quality of life.
For level of evidence, this scale is as follows: 5: > 10 publications; 4: 6–9 publications; 3: 3–5 publications; 2: 1–2 publications; 0: no publications.

SECTION 2: UPDATE ON NASAL NO MEASUREMENT

Background

There is continuous high production of NO in the human nasal passages and paranasal sinuses. Noninvasive measurements of nasal NO are easy to perform and have revealed altered levels in several respiratory disorders. At the 2002 ATS workshop in Toronto, we discussed the latest development in the nasal NO field with focus on two major issues: clinical indications for nasal NO measurements and methodology. In healthy subjects, a large proportion of NO found in exhaled air originates from the upper airways, with only a minor contribution from the lower respiratory tract and the lungs (46–48). The role of NO in the upper airways is not entirely known but may involve host defense functions, including direct toxic effects on microorganisms (49) and regulation of mucociliary function (50). Nasal NO is altered in several respiratory disorders—for example, primary ciliary dyskinesia (PCD) (47), cystic fibrosis (CF) (51, 52), and allergic rhinitis (53–55), and this has led to the proposal that a nasal NO test could be clinically useful in diagnosis and monitoring of these diseases. This section is a brief summary of discussions at the workshop. More extensive reviews of nasal NO are available elsewhere (56, 57).

The Role of Nasal NO Measurement

Nasal NO measurements are rapid, completely noninvasive, and can be performed easily even in infants. Because nasal NO is altered in certain airway disorders, the measurements could be clinically beneficial to aid in the diagnosis and monitoring of therapy. There is a great difference in background NO output between the upper and the lower airways (47, 48, 58). Background lower airway NO output is normally low, which makes it easy to find increases (e.g., asthma) but more difficult to detect decreases. In the upper airways, there is a high background output, and so an increase (e.g., in allergic rhinitis) can be obscured, whereas a decrease is usually easy to reveal. Adding to the complexity is the fact that swelling of the mucosa or secretions during inflammation may lead to less passage of NO from the mucosa of the paranasal sinuses to the nasal cavity where it is measured. In such cases, the net change in nasal NO will be variable and difficult to predict.

Conditions in which Nasal NO Might Be Clinically Applicable

The workshop participants concluded that, as of today, there is one indication for nasal NO measurements that could be considered almost ready to use in the clinic—namely, diagnosis of PCD. Several independent studies have now uniformly reported extremely low nasal NO in PCD. In recent trials, the sensitivity and specificity of this test in PCD has proven to be excellent (59–61). This makes the nasal NO test attractive for screening of PCD, as a guide to confirmatory testing (e.g., ciliary structure analysis). A simple, noninvasive test for PCD could indeed be very useful. Diagnosis of PCD is often delayed despite the presence of typical symptoms early in life (62). The present diagnostic procedure for suspected PCD is quite cumbersome and often involves ultrastructural examination of airway epithelial ciliary structure (63, 64). The use of nasal NO in diagnosis and monitoring of other respiratory disorders (e.g., allergic rhinitis, sinusitis, nasal polyposis, CF) is potentially of great interest, but more research is needed before we know how clinically useful this test can be for these disorders.

How Should Nasal NO Be Measured?

The ATS and ERS have agreed on a highly standardized procedure for measurements of lower respiratory tract F_{ENO} (*see* section on online NO). Such guidelines are extremely helpful for researchers when comparing results and they have been of great value in the process of moving F_{ENO} measurements into clinical application. For nasal NO measurements, however, one single standardized measurement procedure has not yet been defined. There are several reasons for this as follows: (*1*) there has been no well-defined major disease indication (such as asthma for F_{ENO}) where routine measurements of nasal NO can be easily foreseen, (*2*) the methodology is quite complex, and (*3*) measurement variability is often substantial. Because of these reasons, the nasal NO field has been driven more by scientific curiosity than by a need for rapid standardization.

The F_{ENO} test for orally exhaled NO is now moving into the clinical arena as a routine test in asthma and will likely also be used when handheld analyzers are available for home use. In parallel, novel, more sophisticated ways of measuring and analyzing F_{ENO} are emerging (*see* sections on NO models). Such tests add important information and could be particularly useful in specialized centers. A similar development could occur for nasal NO testing. As a first step, it would be desirable to standardize a simple method for a specific disease, and this will most likely be PCD. This would not preclude more sophisticated nasal NO procedures and analyses for other indications.

Several techniques for measuring nasal NO have been used. The most commonly presented way to measure nasal NO is to sample nasal air directly from one nostril. Using the intrinsic sampling flow of a chemiluminescent analyzer or an external pump, air is aspirated from (or insufflated into) one nostril (56, 65). Because of its simplicity, the aspiration method is still by far the most used technique for nasal NO. Another technique to measure nasal NO includes nasal single-breath exhalation using a facemask (66).

If the nasal NO test should become a part of future clinical practice, it will of course be of great importance to standardize the method carefully. However, the recommendation by the workshop at this time was to allow maximum freedom for nasal NO measurements. The chosen method must, however, be carefully described, including reporting of flow rate and ambient NO levels. It is likely that several methods for nasal NO will be used depending on the situation and the patient population. For example, breath-holding or single-breath measurements are not possible in noncooperating individuals (e.g., infants and sedated patients).

Nasal NO and Ostial Patency

One of the more recent findings in this field is the intriguing discovery that nasal NO increases dramatically (5- to 15-fold) during humming compared with silent nasal exhalation (67). The oscillating sound waves may speed up the exchange of gases over the sinus ostium, resulting in a rapid washout of NO from the sinuses (68). This NO peak is transient and the levels decrease gradually during repeated humming maneuvers. However, nasal NO levels fully recover after a short period of silence, which allows sinus NO to accumulate again. In a model of the nose and sinus, it was found that ostium size was the main determinant of the humming-induced increase in NO. In addition, in patients with computed tomography–proven complete sinus ostial obstruction (bilateral nasal polyposis), the nasal NO increase during humming was abolished (69). This suggests that the increase in nasal NO during humming correlates to ostial function. Ostial obstruction is central in the pathogenesis of sinusitis, and one goal in medical and surgical therapy of chronic sinusitis is to improve sinus ventilation. Future studies will show if a nasal NO humming test could be used to monitor ostial function in at-risk patients or in subjects with established sinus disease.

SECTION SUMMARY: NASAL NO

About a decade ago, it was found that NO is released in large quantities in the nasal passages of healthy humans. Especially large concentrations of NO have been found in the paranasal sinuses. The physiologic role of this NO still has not been clarified but may include important local host-defense mechanisms. Nasal NO can be measured online using different noninvasive techniques. Using these methods it has been found that nasal NO is altered in several airway disorders, including allergic rhinitis, PCD, CF, and sinusitis. For most indications, nasal NO is still to be regarded as an interesting research tool with potential clinical importance. However, in PCD, the situation is different because nasal NO is uniformly extremely low. The sensitivity and specificity of a nasal NO test in PCD are so good that this test now should be considered for routine use in specialized centers. To facilitate this process, we need to quickly establish guidelines for a simple, standardized nasal NO test in PCD.

SECTION 3: NO EXCHANGE MODELS

Background

NO was first discovered in exhaled breath of humans and other vertebrates in 1991 (70). This finding spurred an era of intense investigation aimed at determining the source and potential use of this noninvasive biological signal. In 1997, the concentration of NO in the exhaled breath was reported to be highly dependent on the exhalation flow rate (71, 72), differing significantly from other endogenously produced gases such as carbon dioxide and nitrogen. This finding necessitated the development of new models and analytic methods to understand the underlying physiology and gas exchange mechanisms.

A simplified two-compartment model of the lungs was presented in 1998 (73), which explained many of the unique features of NO exchange dynamics—in particular, the dependence on exhalation flow rate. The two-compartment model describes F_{ENO} as having significant sources from two compartments—namely, the airways and the alveolar region. Thus, NO exchange dynamics are described using three flow-independent exchange parameters. One describes the alveolar region: C_{ANO}, the steady-state alveolar concentration; and two describe the airway region: (*1*) the airway NO diffusing capacity (Daw_{NO}), alternatively described as the conductance for mass transfer or transfer factor of NO between the airway tissue and the gas phase, and (*2*) the maximum airway wall flux ($J'aw_{NO}$); $J'aw_{NO}$ is equal to $Daw_{NO} \times Caw_{NO}$ (the airway wall NO concentration).

Since the original description of the two-compartment model, research has focused in three areas: (*1*) developing experimental breathing and analytic techniques to accurately and reproducibly estimate the flow-independent NO exchange parameters, (*2*) estimating the flow-independent NO parameters in health and disease, and (*3*) further development and testing of the underlying assumptions of the simple two-compartment model. Together, these research thrusts have enhanced our understanding of NO exchange mechanisms and the pathophysiologic interpretation of the flow-independent NO exchange parameters.

The two-compartment model has been previously described in detail (73–76). Figure 1 depicts the basic features of the model. NO is produced by cells in the alveolar membrane producing a net flux of NO into the alveolar space. During an exhalation or breath-hold of more than approximately 8 to 10 s (73, 77–81), the concentration in the alveolar region, C_{ANO} (ppb), reaches a steady state. As alveolar air is convected through the airways toward the mouth during exhalation, additional NO is absorbed by the gas stream from the airway walls denoted by the flux of NO from the airways, Jaw_{NO} (rate of NO transferred per unit time in picoliters [pl]/s). Jaw_{NO} is expressed as a linear function of the airway gas phase NO concentration, C_{NO}, by the following equation (73):

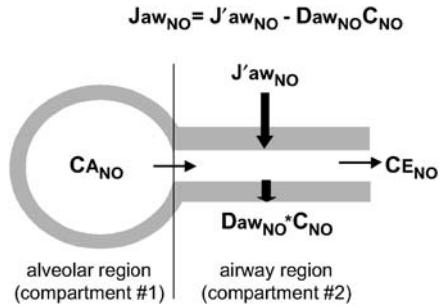

Figure 1. Schematic of two-compartment model used to describe nitric oxide (NO) exchange dynamics. Exhaled NO concentration, C_{ENO}, is the sum of two contributions—the alveolar region and the airway region—which depend on three flow-independent parameters: maximum total volumetric flux of NO from the airway wall ($J'aw_{NO}$, picoliters [pl] $\cdot$ s^{-1}), diffusing capacity of NO in the airways (Daw_{NO}, pl $\cdot$ s^{-1} $\cdot$ part/billion), and steady-state alveolar concentration ($C_{ANO,}$ part/billion). Jaw_{NO} is the total flux (pl/s) of NO between the tissue and gas phase in the airway, and is an inverse function of the exhalation flow rate, $\dot{V}_E$.

$$Jaw_{NO} = J'aw_{NO} - Daw_{NO}C_{NO}$$

or

$$Jaw_{NO} = Daw_{NO}(Caw_{NO} - C_{NO})$$

$J'aw_{NO}$ is the maximum flux of NO from the airway tissue that is equal to the airway compartment flux if airway gas phase NO, C_{NO}, was zero occurring at infinite exhalation flow, or alternatively, simply the product Daw_{NO}*Caw_{NO}.

Once the flow-independent parameters are known, the two-compartment model can be used to predict the exhaled concentration of NO, C_{ENO} (also represented symbolically as F_{ENO}), at any constant exhalation flow using the relatively simple exponential expression:

$$C_{ENO} = Caw_{NO} + (C_{ANO} - Caw_{NO}) \cdot \exp(-Daw_{NO}/\dot{V}_E)$$

where $\dot{V}_E$ is the constant exhalation flow rate.

The potential advantage of the flow-independent NO parameters lies in their ability to partition F_{ENO} into two important anatomic subdivisions of the lungs—namely, the airways and the alveolar region, each with their own pathologic involvment. To uniquely determine all three flow-independent parameters, both low ($<$ 50 ml/s) and high ($>$ 100 ml/s) exhalation flow rates must be sampled (74–76, 82). Three approaches have been described in the literature: multiple constant exhalation flows (73–76, 83), a dynamically changing flow within a single exhalation (82), and tidal breathing methods (84). Recently, the impact of axial diffusion of NO toward the alveolar region during exhalation on the estimated parameters has been described (85–87).

The flow-independent NO exchange parameters have been estimated in healthy adults, children, and infants, as well as in several diseases, including asthma (76, 88–93), allergic alveolitis (89, 91), CF (15), scleroderma (94), allergic rhinitis (88), and COPD (88), and in smoking (88, 95). Although asthma is a heterogeneous disease, it appears that steroid-treated subjects with asthma have an elevated Daw_{NO}, whereas subjects with asthma who are not treated with steroids have elevated Daw_{NO}, $J'aw_{NO}$, and C_{ANO}. Subjects with allergic alveolitis have elevated C_{ANO}, whereas subjects with scleroderma have an elevated C_{ANO} but a reduced $J'aw_{NO}$. Subjects with CF have an elevated Daw_{NO}

but a reduced Caw_{NO}. Allergic rhinitis appears to increase Daw_{NO}. For subjects with COPD, the CA_{NO} is elevated, and in smoking, Caw_{NO} and $J'aw_{NO}$ are both reduced.

SECTION SUMMARY: NO EXCHANGE MODELS

Exhaled NO has significant sources from both the airway and alveolar regions of the lungs. This feature has stimulated the development of new analytic techniques to understand this rich feature. To date, a two-compartment (airways and alveoli) model of the lungs has proven to be a simple and robust means of describing NO exchange dynamics. The two compartments are described by three flow-independent NO parameters, two of which characterize the airway compartment (Daw_{NO} and Caw_{NO}) and one which characterizes the alveolar compartment (CA_{NO}). Several analytic techniques have been used to estimate these parameters in both health and disease. Early reports suggest that the flow-independent NO parameters are uniquely altered in several inflammatory disease states and thus may provide pathophysiologic insight or assist in the clinical management of lung diseases. Future studies must address several important model simplifications, as well as improve our understanding of the clinical and physiologic significance of alterations in these parameters.

SECTION 4: EXHALED NO IN MECHANICALLY VENTILATED PATIENTS

Background

The anesthetic and critical care community has expertise with breath analysis; e.g., capnography and anesthetic gas monitoring during patient care. Extension of this expertise to exhaled breath markers of inflammation would provide this community valuable insight into metabolic alterations that potentially contribute to critical illness (96). Thus, parallel to increased understanding of the role of breath markers in spontaneously breathing patients, a number of marker molecules have been identified in the breath of mechanically ventilated patients that may be useful in identification of disease progression, or in monitoring therapeutic efficacy (97–102).

Despite this progress, the Toronto workshop participants believed that there was insufficient knowledge to make firm recommendations for guidelines for FE_{NO} measurement in ventilated patients for inclusion in the revised ATS/ERS statement on exhaled and nasal NO measurement. Therefore, the session on FE_{NO} in mechanical ventilation was used to overview available information on determinants of FE_{NO} and published measurement approaches.

Patient Safety

The ATS group stressed the need to address patient safety when measuring FE_{NO} for research purposes. Although outpatient measurement of FE_{NO} is considered a low-risk research procedure, the same in ventilated, critically ill patients involving alterations in the ventilation circuit, ventilator settings, and inhaled gases requires special consideration in the unique environment of the operating room or intensive care unit, where electrical hazards and inflammatory gases exist. Where measurement involves interrupting ventilation and disconnecting the ventilator circuit, caution is recommended not to use excessive inflation volumes, to prevent inadvertent hypoxia and infection, and accidental extubation.

Measurement Techniques and Confounding Factors

Several approaches have been developed for online NO testing, including tidal, mixed expired, and flow-controlled single-breath analysis.

Breath-to-breath (tidal) FE_{NO} measurements. Fast-response chemiluminescence analyzers are now able to resolve the NO concentration profile within one respiratory cycle, while ventilation is standardized over a measurement epoch. Breath-to-breath analysis results in a dynamically changing NO concentration versus time plot, reflecting NO production in the lower airways of intubated patients. It is likely that this pattern is related to continuously changing expiratory flow. Simultaneous recording of CO_2 concentration and flow profile greatly facilitates data analysis and interpretation where sample-tubing characteristics, lag phase of NO, CO_2 and flow measurements, and variable response times of each of these analyzers should be considered.

The NO versus time trace obtained by breath-to-breath analysis can be described in several ways, including the following: (*1*) the peak FE_{NO} concentration, (*2*) mean FE_{NO}, (*3*) the area under the concentration curve for an indicated time period, or (*4*) NO output rate $\dot{V}_{NO}$ (nl/min) (103).

The anatomic origin of FE_{NO} has great implications for data interpretation and the utility of this marker to monitor alveolar inflammation in critically ill patients. The participants agreed that very low alveolar NO concentrations (104–106) cause signal-to-noise ratio issues, and disease states causing a decrease in alveolar NO concentrations will likely face problems of lower detection limit (104, 107). In contrast, mechanisms that increase pulmonary microvascular and alveolar NO, such as endothelium-dependent vasodilators and NO donor drugs, appear to produce a readily detectable increase in FE_{NO} (108–112), which might be useful in probing this important compartment.

Determinants of breath-to-breath FE_{NO} profile. Animal and human studies indicate four major categories of determinants: (*1*) composition of inhaled gases, (*2*) ventilation parameters during controlled mechanical ventilation, (*3*) different ventilation modes, (*4*) pulmonary blood flow and intrathoracic blood volume.

Among inhaled gases, NO, O_2, and CO_2 should be carefully considered (106, 113–116). Within a single ventilation mode, FE_{NO} concentration in ventilated patients is affected by respiratory rate, tidal volume, and inspiratory/expiratory ratio, even when minute ventilation is held constant (104, 117). In addition, bias flow from the ventilator and the influence of positive end-expiratory pressure require special attention (118–120). It is also evident that different ventilation modes, such as pressure-controlled inverse ratio ventilation and high-frequency oscillatory ventilation, influence FE_{NO} through multiple mechanisms (121, 122). It has been shown that significant acute reduction in perfusion pressure, such as seen during hemorrhage, onset of cardiopulmonary bypass, occlusion of pulmonary vessels, and correction of left to right intracardiac shunts, alters FE_{NO} (107, 120, 123–126). However, moderate changes in either direction from normal blood flows do not alter FE_{NO} concentrations significantly (119, 127).

Breath-holding. This simple maneuver might be quite useful in some ventilated patients, especially when there are limitations of the tidal breath measurements. Gaseous NO exhibits exponential increase during breath-holding and reaches a plateau phase within 20 to 30 s (104, 106, 128). Because the plateau phase can be explained by a steady state between NO production/release to the gas phase, and consumption/removal from this phase presumably by pulmonary blood flow, different measures of FE_{NO} during breath-holding (slope and plateau phase) might provide useful information regarding NO production and consumption in the lung without the confounding effect of different ventilation parameters and modes.

Real-time analysis of mixed-expired gas. This alternative method relies on the measurement of $\dot{V}_{NO}$, the rate of elimination

TABLE 2. COMPARISON BETWEEN ONLINE AND OFFLINE NITRIC OXIDE MEASUREMENTS IN MECHANICAL VENTILATION

Online	Offline
Advantages	
Suboptimal measurements can be immediately identified and discarded or repeated	Samples can be collected from sites remote from the analyzer
Allows for simultaneous measurement of flow	Less dependent on analyzer response times
Allows precise timing of breaths	More efficient use of analyzer (less analyzer time per patient)
NO levels can be determined at different points in the respiratory cycle	Allows for measurement of other gases in the same sample
Disadvantages	
Requires more stringent analyzer specifications	Possible errors due to sample storage and transport
Patient and analyzer need to be at the same place at the same time	Suboptimal measurements cannot be immediately identified
Less efficient use of analyzer (more analyzer time per patient)	More likely to be affected by ventilator variables
	Provides NO level in a mixed-gas sample and not at a defined point in the respiratory cycle.

Definition of abbreviation: NO = nitric oxide.

of NO from the lungs. A mixing chamber is connected to the exhaust port of a ventilator, and a real-time NO analyzer is used to sample NO concentration in the gas exiting the mixing chamber. The minute ventilation is measured by means of an online spirometer connected with the mixing chamber. After standard adjustments for ambient temperature and pressure, $\dot{V}_{NO}$ is the product of the 1-min volume entering the chamber and the concentration in the chamber. This method may be used with any mode of ventilation.

Flow-controlled single-breath measurements. To obtain flow-independent parameters in intubated, nonparticipating patients, a method for multiple, single-breath exhalations at various flow rates has recently been proposed (128). This requires disconnection from the ventilator circuit and performing a manual deep inspiration followed by a flow-controlled exhalation using an aspiration suction device. This method allows for repeated measurements at various controlled exhalation flow rates, both in the intubated and the awake state of the same patient. A potential disadvantage might be alveolar collapse during disconnection from the respiratory circuit. Caution is recommended not to use excessive inflation volumes, to prevent inadvertent hypoxia, accidental extubation, and infection.

Offline methods. Here, exhaled gas is collected into an inert reservoir for detection at a different time or location. Offline techniques offer several added advantages compared with online measurements, including the following: (*1*) portability, (*2*) less dependency on analyzer response times, (*3*) more efficient use of analyzers, and (*4*) potential to measure other gases in the same sample (*see* Table 2) (129).

At the time of the workshop, there was limited published information on the offline measurement of $F_{E_{NO}}$ levels in ventilated individuals (103, 125, 126, 130, 131). These studies used various methods of gas collection (syringe aspiration, tidal breath collection, and controlled collection with a pump) and different ports of sampling (endotracheal tube, ventilator circuit, and exhaust port of the ventilator). Furthermore, single or multiple breaths were collected for analysis. It appears that variability of NO levels obtained by these different methods was very high, which underscores the need to standardize offline measurement issues.

Pathologic Mechanisms Contributing to Altered $F_{E_{NO}}$ in Critical Illness

Activation of the constitutively expressed NO synthase (NOS) isoforms, induction of type II NOS, and production of NO from acidified nitrite are the principal mechanisms contributing to increased NO production in the lung and potentially causing higher than normal $F_{E_{NO}}$ (132–134). The main mechanisms that would decrease NO bioavailability and concentrations include down-regulation of constitutive NOS expression, inability to induce type II NOS by inflammatory cytokines and consumption reactions of NO in the fluid phase. (135, 136).

Clinical Implications of Exhaled NO in Critical Care Medicine

Exhaled NO is elevated in various forms of pulmonary infection, including intensive care patients with developing ventilator-associated pneumonia and in lung transplant recipients with infective complications (137).

The characteristics of $F_{E_{NO}}$ in sepsis remain controversial, possibly due to species differences (138–140). In healthy volunteers, lipopolysaccharide administration produced only a modest increase in $F_{E_{NO}}$ (141). Little is known regarding $F_{E_{NO}}$ in patients with clinical sepsis, severe sepsis, or septic shock.

There are also species differences regarding $F_{E_{NO}}$ in acute lung injury (142, 143). In patients with established clinical adult respiratory distress syndrome, levels of $F_{E_{NO}}$ were decreased (144). Further studies are needed to delineate kinetics of gaseous and fluid phase levels of NO during the entire spectrum of the disease (145).

The relationship between $F_{E_{NO}}$ and lung injury associated with ischemia–reperfusion during cardiac surgery and transplantation has received particular attention. Dysfunction of NO pathways and reduced $F_{E_{NO}}$ have been demonstrated in children after cardiopulmonary bypass (125, 130). In adults, $F_{E_{NO}}$ was either increased or decreased or unaltered after cardiac surgery (103, 131, 146–148). In conditions of more severe lung ischemia and reperfusion, such as during lung transplantation, $F_{E_{NO}}$ was reduced in the majority of patients, which appeared to correlate with clinical outcome (149).

SECTION SUMMARY: EXHALED NO MEASUREMENT IN VENTILATED PATIENTS

In summary, the Toronto ATS meeting was an important step in our worldwide dialog toward international consensus and guidelines on measurement issues and on the value of $F_{E_{NO}}$ in critical illness. However, further studies are needed to clarify many of these initial suggestions before the technology can be considered as a diagnostic tool of inflammation in ventilated, critically ill patients.

SECTION 5: LUNG NITROGEN OXIDE AND REDOX ASSESSMENT USING EBC

Background

There are two principal discrete purposes for studying EBC. First is that this fluid can safely provide information about airway lining fluid (ALF) composition. Second is the potential for EBC

assays to provide evidence of airway disease, particularly in regard to inflammation and redox disturbance.

Many of the early studies of EBC related to redox monitoring. Hydrogen peroxide and nitrogen oxides have been studied more than other compounds. Because acidity is a critical determinant of many redox activities (chemical reactivities and enzyme function), EBC pH has also received attention. This workshop focused on the potential of EBC to provide information regarding the otherwise difficult to assess lung NO and redox balance. Studies of exhaled cytokines, leukotrienes, and other compounds of interest in EBC were left to other forums. Summaries of data presented and discussions held are presented below.

Anatomic Source and Potential for Oral Contamination of EBC

In contrast to endotracheal collections, when collecting samples of EBC orally there is likely some contribution from the upper airway. The oropharyngeal secretions contain substantial concentrations of many of the biochemical markers that have been identified in EBC, and slight contamination of a sample with oral secretions has potential to greatly affect EBC concentrations. The various EBC collection systems used by laboratories usually incorporate methods to limit gross salivary contamination.

Theoretically, regions of turbulent airflow, such as carinae and cartilage rings, should provide more particles to the exhaled airstream, although airway reopening after closure may aerosolize particles. The source of the gas phase (volatile) constituents is more likely considered the entire airway and the alveoli.

Issues of Dilution

To gain insight into precise concentrations of nonvolatile constituents of ALF, it is necessary to determine to what extent the particles of ALF are diluted by condensed water vapor. Several methodologies for this are becoming available.

When the substances of interest are volatile, the dilution issue becomes entirely different and in many ways irrelevant.

Importantly, just as has always been accepted for cellular comparisons in bronchoalveolar lavage fluid (BALF), ratios of substances in EBC can be enlightening without need for dilution markers. Ratios of nitrite (NO_2^-) to total nitrogen oxides (NOx), acids to bases (pH), oxidized glutathione (GSSG) to reduced glutathione (GSH), and others are therefore likely to be of interest. Of course, as for BALF, whether one compound is rising or the other is declining will not necessarily be clear.

Nitrogen Oxides

Multiple studies have identified differences in EBC NOx concentrations in various lung diseases. Initially undertaken in an effort to develop a surrogate assay for the more expensive F_{ENO} assay (150), it became clear that the complexities of nitrogen oxide chemistry make EBC NOx assays and F_{ENO} complementary, not equivalent.

Although NO in the airway can be formed by the NOS enzymes (151, 152) and released as gas in the exhaled air, only a small portion of NO thus formed is released. Some is oxidized to become reactive species with downstream signaling effects. Species such as peroxynitrite and peroxynitrous acid may then lead directly to nitration reactions forming nitrotyrosine. Some NO becomes incorporated in various S-nitrosothiols, which serve as storage molecules for NO activity, and have key signaling properties of their own, discrete from NO (153). Much NO is oxidized to NO_2^- and NO_3^-. These less reactive species may be reabsorbed by the airway, carried up the airway in the ALF and swallowed, or exhaled in particles. Nitrite may be consumed by eosinophil peroxidase (154) and neutrophil myeloperoxidase as substrate for enzymatic nitration. When protonated, nitrite will release NO (155). Likewise S-nitrosothiols also release NO (156). Bacterial and fungal enzymes can reduce NO_3^-, NO_2^-, and NO, reversing the normal eukaryotic pathway, and on complete reduction, form ammonia (157). Many of the intermediaries and final products listed above are found in EBC.

Lung NOx, including F_{ENO}, are best understood when considered as a group, and then especially if other chemical species are considered, such as superoxide and hydrogen peroxide. The bacterial load and the pH of the airway environment need to be considered. Inhalation or endogenous formation of oxidants may affect F_{ENO}, in the same manner that inhaled NO may alter exhaled H_2O_2 (158). Deviations of EBC NOx concentrations, or ratios among them, may reflect the oxidative and nitrogen oxide conditions of the airway, including not just formation of NO, but all the various inorganic, eukaryotic, and prokaryotic reactions that occur around NO. These assays then may reflect NOS activity, airway inflammation, innate immune responses, bacterial burdens, airway pH, and nitrergic neurotransmission.

Additional values of NOx assays include the potential for them to provide information regarding ventilator-induced lung injury and acute respiratory distress syndrome (ARDS) (159, 160). In this regard, Drs. Gessner and Wirtz presented data at this workshop from their studies of acute lung injury in humans, identifying strong correlations between EBC NO_2^- concentration and the tidal volumes used for ventilation. The ratio of NO_2^- to tidal volume correlated highly with injury severity scores. Nitrite may be formed in association with mechanical stress, with overexpansion of open respiratory units leading to increasingly high and readily measurable EBC NO_2^- output (159). This simple assay may allow titration of ventilatory volumes while monitoring objective evidence of evolving lung injury hour to hour.

The biochemical source of aqueous phase nitrogen oxides in EBC remains obscure. Nitrate and NO_2^- in EBC may be present because these ions are in the particles of ALF. Or they could occur from exhaled gas phase NO being oxidized *ex vivo* in the EBC (e.g., by hydrogen peroxide). Thus, NOx may need to be considered in part as indirectly volatile constituents of EBC.

Cautious techniques need to be used for EBC collection, storage, and assay when NO_2^- or NO_3^- are the ions of interest. These NOx are ubiquitous laboratory contaminants, abundant on fingertips that are carried in humidity and deposit on every available surface. Protection by covering lab surfaces is not sufficient because NO_2^- and NO_3^- are also formed from NO oxidation, and NO gas is in almost all labs and clinics at variable, but relevant, concentrations. NO can travel through plastic coverings as well. Latex gloves may have extremely high levels of NOx on their surface. NOx almost always substantially contaminates microcentrifuge tubes and other test tubes. This is an important issue, particularly for NO_2^-, because it is found in low to submicromolar ranges in EBC, and thus contamination, though unavoidable, can overwhelm the signal from the subject. In addition, NO_2^- can be oxidized to nitrate in aqueous solution, or be converted to NO (through protonation and release of NO from decomposed nitrous acid). These processes can decrease nitrite levels during storage over time.

EBC pH Assays

Most redox reactions are very sensitive to the local pH. Acids tend to be volatile from acidic fluids, and bases volatile from basic solutions. One theoretic underpinning for studying EBC pH is that volatile airway acids could be trapped in EBC, and that they would be exhaled to a greater extent from an acidic source fluid. Thus, a low EBC pH would reflect a low ALF pH, which in turn would influence redox chemistry. Relative to controls, the pH of EBC has been found to be significantly

low in diverse respiratory diseases, including asthma, COPD, bronchiectasis (161, 162), CF (163), and exacerbations of these diseases, as well as in ARDS (164). Correlations of pH are present with relevant cellular inflammation in induced sputum, EBC cytokines, nitrogen oxides, hydrogen peroxide, and 8-isoprostane (161, 164).

The pH of EBC can be measured before or after gas standardization. Gas standardization involves bubbling a CO_2-free gas through the EBC sample for several minutes. Gas standardization removes carbon dioxide and bicarbonate from the EBC (although complete removal is not certain), and the pH rises and then stabilizes during the process. Without gas standardization, the EBC pH is affected by ambient and exhaled carbon dioxide and is not stable, and therefore should be measured immediately on collection. Gas standardization is useful when samples cannot be assayed immediately, when one is not interested in exhaled carbon dioxide levels, or when the EBC pH is expected to be particularly acidic (below a pH of 5, removal of carbon dioxide has little affect on pH, consistent with pH being a logarithmic scale).

Gastroesophageal reflux of acidic fluid, with or without microaspiration into the trachea, is one potential mechanism of EBC acidification that has been considered and is under active investigation in several laboratories. Alternate explanations for EBC acidification include lower airway acid production by numerous described pathways (165). In likelihood, any process that acidifies any level of the airway could lead to EBC acidification if anions present in the fluid become protonated and volatilized.

Ammonia concentrations in EBC range up to 3 mM (166), derived predominantly, but not completely, from the upper airway (167, 168). It has been noted that a low EBC pH is always accompanied by low EBC ammonia concentration, although the obverse is not true. There are reports of isolated lower airway samples of EBC being acidic in various diseases, but not in healthy controls. Although there is general consensus regarding the utility of EBC pH as a simple marker of intuitive value in airway disease, this consensus is not unanimous, as alternative explanations for EBC acidification other than lower airway pH deviation have been presented. There are reasoned theoretic concerns that ammonia derived from the mouth might interfere with EBC pH assays (169), but these are not borne out as yet by empiric investigation. A recent study collecting EBC from a cuffed endotracheal tube from 32 subjects, altogether bypassing the mouth and its ammonia contribution, revealed EBC pH values unchanged from same-subject oral collections (170), despite the expected substantial declines in the levels of ammonia (168). Although this provides support to the concept that EBC pH is a valid measure of lower airway pH, also conceivable is the notion that bypassing the mouth may prevent oral acid(s) from entering the EBC, which could lead to the pH from the isolated lower airway samples being coincidentally the same as the oral collections. Data will assuredly emerge over time that will clarify these issues.

It will help to have more invasive pH measurements of the lower airway during active disease states to correlate EBC pH with airway pH deviations. However, it is possible that EBC acids may be volatilized from airway sources too low to access with other methods, or that discrete small areas of substantially low airway pH may lead to sufficient volatilization of acids to acidify the EBC. Extensive studies of EBC pH in the isolated lower airway of endotracheally intubated patients are ongoing and should clarify the anatomic source and chemical and clinical relevance of this noninvasive effort to gain an understanding of lower airway chemistry.

Interactions of pH and NOx

In animal models (171), experimental airway acidification leads to production of NO measurable in exhaled breath, and the speed with which this process occurs after instillation of acid (seconds) is consistent with inorganic decomposition of protonated nitrite (nitrous acid = HNO_2) forming, in the end, NO. This has been proposed to occur in acute asthma, where the most profound declines in EBC pH have been noted (162). Also, breakdown of s-nitrosoglutathione leads to increased exhalation of NO (172). It seems likely that there are multiple pathways of NO formation in addition to up-regulation of NOS isoforms, each with relevance to certain conditions.

Dr. Redington examined the relationship between EBC pH and F_{ENO} in patients with mild, stable allergic asthma, patients with stable CF, and control subjects. F_{ENO} was measured online at a flow rate of 250 ml/s and non–gas standardized EBC pH was measured immediately after collection. These findings demonstrate dissociation between non–gas standardized EBC pH and F_{ENO} in these conditions (173, 174). The chemistry of the NOx remains complex. Oxidative burdens in CF may lead to more NO_3^- and less NO_2^- formation from NO, thus trapping NO, while limiting NO_2^- sources for inorganic NO formation.

Hydrogen Peroxide

One of the first markers found in EBC in various disease states was H_2O_2. Several assays have been used, and normative values for children have been published (175). As with other chemical processes in the lower respiratory tract, *in vivo* assessment of the importance of H_2O_2 in disease states had been nearly impossible until the advent of EBC assays. Indeed, EBC has provided the strongest evidence to date of oxidative disturbance in lung diseases.

However, concerns about H_2O_2 flow dependence (176) and chemical reactivity support efforts to standardize EBC collection and assay techniques when this compound is of interest. Similar to F_{ENO}, evidence exists that lower controlled (nontidal breathing) exhalation flows lead to significantly higher EBC H_2O_2 levels than higher controlled flows. Levels of H_2O_2 in EBC decline over time in storage. It is unclear how rapidly H_2O_2 is consumed in EBC, although it is likely that some is consumed even during the time of collection. Hydrogen peroxide will react with NOx, and can react with ammonia also, forming various compounds, including hydroxylamine, peroxynitrite, and NO_3^-. To simplify understanding of H_2O_2 in the airway, the development of online measurement techniques may prove valuable as a standardized assay technique. In this regard, Dr. Becher presented data on a new instrument designed to provide rapid H_2O_2 assay immediately on collection of sample. The instrument, designed and manufactured by FILT in Germany, uses a biosensor for assaying small volumes of EBC, and completes the assay within several minutes.

The issue of flow dependence for EBC H_2O_2 is very different than for NO, as tidal breathing during EBC collection incorporates multiple different flows in each breath. Standardizing to many minutes of consistent-flow controlled exhalation may not be reasonable to demand from a patient.

An alternative to performing the H_2O_2 assay immediately has been presented. This involves addition of a reagent to the just-collected EBC (177), or to the collection device in advance of collection. This reagent reacts rapidly and specifically with hydrogen peroxide and allows for delayed spectrophotometric analysis, as much as 24 hours subsequently.

H_2O_2 is volatile, although less so than water, and the concentration in EBC may be derived both from gaseous H_2O_2 as well as ALF particles. The relative contribution of each source remains incompletely understood.

Evidence of Oxidation by Measurement of Larger Molecules in EBC

Dr. Corradi and colleagues presented data from their investigations of biomarkers of lipid peroxidation (aldehydes and 8-isoprostane) and an antioxidant (glutathione, GSH) in the EBC of children with asthma during acute asthma exacerbation and after 5 days of therapy with prednisone (178). Aldehydes and GSH were analyzed with liquid chromatography and detected with the most sensitive detection systems available today—namely, fluorescence detection and mass spectrometry techniques. 8-Isoprostane was measured by enzyme immunoassay. The data showed that the levels of malondialdehyde and 8-isoprostane were significantly higher in EBC of children with acute asthma than in control children. On the contrary, the levels of GSH were significantly lower in children with acute asthma compared with healthy control subjects. After 5 days of systemic corticosteroid treatment, GSH levels rose, whereas malondialdehyde and 8-isoprostane fell. After oral prednisone therapy, 8-isoprostane levels remained higher in children with asthma than in healthy children, suggesting that corticosteroids may not be fully effective in reducing oxidative stress during acute asthma exacerbation.

The relative changes in the reduction/oxidation chemistry in biomarker levels during acute asthma (highly turbulent airflow) and after treatment with systemic corticosteroids (reducing airway obstruction and turbulent airflow) support that changes in the concentration of biomarkers in EBC samples obtained from subjects with airflow limitation do not purely reflect increased aerosolization of ALF particles. Indeed, the biochemical changes in exhaled breath parallel current theory regarding redox disturbance in the lung, and their measurement now serves as a means to allow such theories to be tested.

Standardization Issues

There are currently two commercial EBC collection systems available, plus numerous homemade systems for sampling EBC. The various devices and techniques likely differ in many respects in regard to condenser materials, temperature of collection, efficiency of condensing water vapor, and efficiency of collecting ALF droplets. There may be temperature-dependent issues that affect the ability of different devices to trap volatile substances. Because EBC is collected from mixed air arising partially from deep airways but also from anatomic dead space and upper airways, breathing pattern, frequency, tidal volume, inspiratory breath holding, and minute ventilation may influence constituents. These issues need to be considered when interpreting EBC concentrations of redox-relevant substances. Standardization of collecting techniques and assays may assist in interlaboratory data sharing. However, the various substances in EBC have different reactivities with other EBC constituents, and differing stabilities. Thus, it would appear unlikely that one collection technique will be optimal for all compounds of interest in EBC. Some investigators urge standardization now. Others suggest that standardization too early may inhibit the development of newer and more effective techniques for studying exhaled breath constituents.

In this regard, Drs. Gessner and Wirtz presented data that EBC volume is strongly correlated to total expired air volume, and that EBC volume does not correlate with measures of airflow obstruction. The percentage of total water vapor removed by the condensation process was calculated to be approximately 40% using the Jaeger collection system. They reported protein concentrations of approximately 12 μg/ml of EBC both in subjects with COPD and control subjects. There was tighter correlation of protein with volume in control subjects than in patients with COPD. Having extensively studied EBC during mechanical ventilation, they reported normal or increased concentrations of protein and urea, despite larger volumes of EBC collected from intubated subjects than from subjects spontaneously breathing. This suggests either that the additional condensate volume collected while intubated is paralleled by increased ALF trapping, or that protein levels in ALF increase during mechanical ventilation.

Dr. Corradi has shown that EBC malondialdehyde and GSH concentrations are essentially independent of expiratory flow rate in the range of 50 to 200 ml s^{-1}. Dr. Hunt has shown that the pH of EBC is not affected by ventilation pattern (hyperventilation/hypoventilation), methacholine-induced airflow limitation, temperature or duration of collection, condenser material, or duration of storage (170). Hydrogen peroxide levels have been reported to be dependent on exhaled flow rates (176) in a manner reflective of exhaled NO (lower flow, higher concentrations) (72).

It may be prudent, and of use in the near future, to begin the standardization process by following some of Dr. Becher's suggestions. Specifically, he recommends presampling inspection of mouth, teeth, tongue, and throat to identify inflammation and spirometry to correlate with EBC assay results, unless it has been shown that expiratory flow issues are not relevant to the substance of interest. It may be useful to rinse the mouth (e.g., two times with 30 ml water) before collection, although again this needs to be studied for each marker of interest.

Although the use of nose clips for F_{ENO} measurements has been discouraged because they can enhance nasal contamination of the orally exhaled airstream with NO, this logic does not extend directly to the process of EBC collection. Samples of EBC are collected over many minutes, involving multiple, full respiratory cycles. This contrasts with the single exhalation-only technique usually used for F_{ENO} measures. For EBC collections, nose clips might increase entrained NO during exhalation, while decreasing it during inhalation. The data are lacking to support an unequivocal recommendation regarding nose clips during EBC collection, but as this issue is considered, it will be necessary to note that extension of F_{ENO} logic to EBC sampling is not straightforward.

Other early recommendations include encouragement for the subject to breathe at normal tidal volumes and avoidance of hyperventilation, and removal of collector from mouth during coughing or burping. There may be advantages to constant monitoring of airflow with biofeedback during collection, as it may assist the subject in performing the collection maneuver consistently. Such online registration of breathing frequency, tidal volume, mean expiratory flow, and minute volume might be helpful for comparing EBC constituents over time in one patient. It was suggested that forced lung function maneuvers should not be performed within 10 min before the collection. There is no consensus in these recommendations. Data to support any mandates in this regard are lacking. We stress the importance of examining the potential confounders for each individual substance of interest, and encourage continued empiric investigation to determine which potential confounders indeed are sufficiently problematic to warrant adding complexity to the collection technique.

The optimum temperature in which to store EBC samples is an issue that serves to underscore the diverse characteristics of EBC constituents. Nitrate is considered a stable compound in the matrix of EBC, and therefore will not react away at any reasonable temperature. However, NO_2^- has the potential to be oxidized to NO_3^- or, for that matter, reduced to NO over time in the chemical matrix of EBC. The reduction of NO_2^- occurs more aggressively at low pH, and therefore the NO_2^- stability is pH dependent. Hydrogen peroxide will react with

lipids and inorganic molecules, and can be expected to decline over time. However, data examining storage at −70°C suggest that this may be acceptable for even up to 40 days (179). The pH of EBC in storage at –20° did not change significantly over periods longer than 1 year (165). For the less stable compounds, it seems wise to store the samples in as cold a temperature as possible and assay soon after collection.

To assist with interlaboratory communications, it will be helpful if reports of EBC assays include as many methodologic details as reasonably possible regarding the type of condenser, temperature and duration of collections, and any other breathing patterns used other than normal tidal respiration.

An ERS/ATS joint task force on EBC has begun to address these issues. One key advantage of EBC assays is the simplicity of the collection process. Although many factors may affect concentrations of substances in EBC, empiric investigations into these factors need to be performed. For now, the dependence on collection technique of each disease marker studied needs to be evaluated by the investigators involved.

SECTION SUMMARY: EBC, REDOX STATUS, AND NITROGEN OXIDES

Still clouded by uncertainties regarding anatomic sources and complex chemistries, EBC nonetheless allows us to gain cautious insights into ALF constituents in disease states and in health. Lung redox information has become obtainable through this technology, through assays of pH, nitrogen oxides, hydrogen peroxide, glutathione, and markers of the presence of disturbances in these compounds, such as nitrotyrosine, isoprostane, and aldehydes. EBC has the potential to complement F_{ENO} to more fully elucidate the nitrogen oxide chemistry of the airways and lung. As a marker of disease activity, EBC assays may monitor acid stress, oxidative stress, and inflammation, separate but often-related entities to which many of our therapies are directed.

OVERALL SUMMARY OF WORKSHOP

This meeting was convened to encourage cooperation among international investigators interested in assessment of airway nitrogen oxides and redox chemistry. Methodology of exhaled NO measurements was extensively discussed. Updates to NO sensor technologies were presented, the ATS current online method for NO measurement was reviewed in light of recent experience, and changes were proposed that are currently being incorporated in a revision to the ATS statement. The role of nasal NO measurement was discussed and the new fields of NO excretion modeling and NO measurement in ventilated patients were presented. In addition, the less technically defined methodology of EBC collection and assay were discussed, stressing primarily redox relevant chemistries. Although assays of large molecules in EBC, such as cytokines, suffer from insufficiently sensitive assays, the sensitivity of the assays available for the redox-relevant compounds of central interest to this workshop are clearly sufficient for the purpose. Issues of dilution remain critically important for an understanding of precise chemical constituents of the airway, although ratios of compounds nonetheless can provide insights into overall lung redox status. Potential for contamination from the upper airway remains a concern in all oral exhaled biomarker measurements. Nonetheless, the data supporting the clinical utility of F_{ENO} measures now are substantial. Although EBC studies are being rapidly published, there are numerous biomarkers present, and thus technical validation remains less advanced than for F_{ENO}. It is reasonable to conclude that, as the technical work is performed, concurrent measures of F_{ENO}, EBC nitrogen oxides, pH, hydrogen peroxide, and isoprostanes and other markers of redox activity in the lung will enhance our understanding of nitrogen oxide balance and the pathologic process we call "oxidative stress."

THIS OFFICIAL ATS WORKSHOP PROCEEDINGS WAS DEVELOPED BY AN AD HOC SUBCOMMITTEE OF THE ASSEMBLY ON ALLERGY, IMMUNOLOGY, AND INFLAMMATION.

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