LMR spectroscopy: a new sensitive method for on-line recording of nitric oxide in breath

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LMR spectroscopy: a new sensitive method for on-line recording of nitric oxide in breath. J. Appl. Physiol. 86(3): 1075–1080, 1999.—Laser magnetic resonance spectroscopy (LMRS) is a sensitive and isotope-selective technique for determining low concentrations of gaseous free radicals with high time resolution. We used this technique to analyze the nitric oxide (NO) concentration profile while simultaneously measuring the flow and expired volume during several single breathing cycles. Eight healthy, nonallergic volunteers were investigated. An initial NO peak was found in all breathing cycles before the NO concentration dropped to a relatively stable plateau in the late phase of expiration. The nasal NO peak was significantly higher than the oral NO peak. The nasal NO plateau was always higher than the oral NO plateau. The height of the initial nasal and oral NO peak rose with increasing duration of breath hold, whereas the late expiratory NO plateau changed only little for either the nasal or the oral breathing cycles. Our findings demonstrate, in line with other reports using other techniques, that the nose is the primary source for NO within the airways.

laser magnetic resonance; nitric oxide concentration; exhaled air; nitric oxide synthase

SINCE NITRIC OXIDE (NO) has been detected in exhaled air (9), the physiological role of NO within the airways and its exact origin are still a matter of controversy, especially during normal breathing cycles. A variety of different techniques for the detection of NO in exhaled air has been used. The standard method for measuring NO concentration in breath so far has been the O3/NO3-chemiluminescence method. In several studies it was found that, in healthy subjects, the upper airways, especially the nose and the nasopharynx, are the main source for NO in human breath (3, 5, 8, 13–15, 22). However, under pathological conditions the site of NO generation may vary. Indeed, patients suffering from various lung diseases exhale more NO, which is released mainly by the epithelium of the lower airways (1). NO is generated by NO synthases (NOS). All three known isofoms of this family of enzymes have been detected in the nasal mucosa (7). The inducible form (iNOS) is primarily expressed under inflammatory conditions and, on expression, can produce large quantities of NO (4). In healthy humans, iNOS is found in the epithelium of the paranasal sinuses and is also partly present in the epithelium of the nasal cavity (9, 16). The other, constitutive NOS isoforms, endothelial and neuronal NOS, are found preferentially in subsuperficial structures. In consideration of the short half-life of NO in tissues as well as its inhibition by steroids, it is most likely that iNOS is the main source for luminal NO generation (5).

The purpose of the present work was to establish laser magnetic resonance spectroscopy (LMRS) as a new, highly sensitive technique that is suitable for on-line detection of low NO levels in exhaled air. Because this method uses the magnetic properties of the NO molecule, it uniquely detects this particular molecule and is not affected at all by other absorptions possibly present in the wavelength region near 5 µm. The established method of measuring NO in breath with the O3/NO3-chemiluminescence technique has two main disadvantages: limited specificity (28) and lack of selectivity of isotopomers. Thus signal contributions from molecules like ethylene or H2S may arise (see Ref. 5a). Moreover, quenching of chemiluminescence by water in the exhaled air cannot be excluded. LMRS has a fast time response, permitting continuous recording of changes in NO concentration during the whole normal breathing cycle. It has to be mentioned that on-line recording with a chemiluminescence analyzer is also possible (2). In the present work we show data of on-line NO measurements in eight healthy subjects by using LMRS. Furthermore, we prove that the nose is the main source of NO generation without using invasive techniques, e.g., intubation.

MATERIALS AND METHODS

Experimental Design

Subjects. Eight young volunteers (4 male and 4 female students, nonsmokers), aged between 21 and 30 yr, were
selected according to the following requirements: 1) the anamnesis showed no hint of an atopic predisposition; and 2) subjects were free of diseases, especially acute and chronic alterations of the upper and lower respiratory tract, such as rhinitis and bronchial asthma. Furthermore, subjects had no medication for at least 3 days before the experiments.

Breathing cycles. The experiments were performed at the Institute of Applied Physics, University of Bonn. The subjects performed defined breathing cycles through the nose and the mouth by using NO-free synthetic air (see Fig. 1). Cycles of mouth breathing were performed with subjects not wearing noseclips, because this led to slightly increased NO content during the whole exhalation.

The duration of inhalation and exhalation was kept constant to 2.5 s, and the duration of breath hold was set at 0, 5, 10, and 15 s.

An additional measurement was performed with ambient air with a cycle equal to a breath hold of 5 s.

The flow was measured with a pneumotachometer (modified rhinomanometer, model A440, Allergopharma, Hamburg, Germany) with an analog-to-digital converter; the data were recorded digitally by means of a personal computer. Portions of the exhaled air were directed to LMRS via a lateral adapter. The subjects carried out five test cycles before the measurement, controlling their times for inhalation and exhalation and breath hold on a computer display. These test cycles served the dual purpose of practice and washout of the exogene NO because the NO-free air was used.

Fig. 1. Measurement of nitric oxide (NO) concentration and breath flow for nasal respiration (A) and oral respiration (B). Positive flow values, exhaled air; negative flow values, inhaled air. ppb, Parts/billion.
Technical Principles and Arrangement of the LMRS

The LMRS method is well established for spectroscopy of paramagnetic molecules (for principles and reviews, see Refs. 6 and 18). The advantage of this technique is that NO is detected selectively and at the same time with extreme sensitivity [sub-parts/billion (ppb) level]. The NO measurement is not interfered with by any other gas, and different NO isotopomers can be distinguished. Moreover, a time resolution adequate for time-resolved measurements of breathing cycles can be achieved.

For detection of NO molecules, a CO laser (25, 26) serves as the light source. The well-known resonance between $X^2\Pi_{3/2} R(1.5)$, $v = 1\rightarrow 0$ rovibrational transition of the $^{14}\text{N}^{16}\text{O}$ molecule, and the CO laser transition $P(13)$ $v = 9\rightarrow 8$ (1,884.349 cm$^{-1}$, 400 mW laser power, single mode) at a magnetic flux density of 0.1490 Tesla was used. More details about the corresponding signal, Zeeman component, and line shape are given elsewhere (16). The extreme sensitivity is achieved by polarization detection, which is one of the most sensitive spectroscopic methods in the midinfrared wavelength region. This particular technique called "Faraday LMRS" uses the Faraday effect to produce the signals and was first developed in our laboratory (11, 27).

A block diagram of the LMRS setup is given in Fig. 2. The central part of the apparatus is a liquid-nitrogen-cooled CO flow laser with internal single-line selection. It is stabilized on the gain maximum of a particular laser line by means of a standard frequency-modulation technique (uncertainty: $\pm 10$ MHz). The laser output is deflected into the bore of the magnet (solenoid, flux density: 0.1490 ± 0.0030 T). The total length of the detection zone is 200 mm. This is the homogenes part of the magnetic field; it is surrounded by a small coil for Zeeman modulation (at 8 kHz). A glass tube with glass fittings defines the NO-sample volume (active volume is some 10 cm$^3$; working pressure inside the cell is 27 mbar). A polarizer in front of the magnet entry ensures that the incoming laser beam is well polarized. The sample volume is sealed by plane CaF$_2$ windows. The polarization of the emerging beam is analyzed by a Rochon prism that is almost crossed with respect to the polarization of the incoming laser beam. With this setup, the Faraday rotation of a linearly polarized laser beam is detected. The infrared radiation is detected by a liquid nitrogen-cooled InSb detector and fed into a lock-in amplifier. The signal output is processed by a personal computer for averaging and quantitative signal-strength analysis. To avoid undefined reactions between NO and the wall material, such as adsorption or desorption, the gas-handling system was made of stainless steel, perfluoroalcoxy, and glass. Moreover, the experiments were done under flow conditions. The flow rate of the gas stream through the cell was 800 ml/min. Under these conditions, no significant change in the NO signal because of surface effects was observed.

The system was calibrated each day before and after a series of measurements by detecting the signal strength for different defined gas mixtures of NO calibration gas (1 part/million NO in N$_2$, Messer-Griesheim) and NO-free synthetic air (Messer-Griesheim, NO < 4 parts/trillion) at constant total gas pressure. We used a calibration gas with NO at $^1$This was measured with a parts/trillion chemiluminescence analyzer (ECO Physics CLD 770AL).
the parts per million level because these mixtures remain stable for months. Within the tested range (0–500 ppb NO), the relationship between signal strength and NO content is linear. Measurement errors of about ±3% are caused by instabilities of laser power. The drift rate of the system is less than some ppb during 1 h. However, the signal drift is corrected during each breathing cycle with the help of the signal level corresponding to the inhalation of the NO-free synthetic air (diffusion of NO from the mouth is negligible). We achieved a sensitivity of ~1 ppb at a lock-in time constant of 0.3 s. The corresponding response time, which is determined by both the lock-in time constant and by the gas flow conditions, was 0.8 s. Thus the contribution to the response time by both effects is on the same order. Faster lock-in time constants (average signal times) decrease the sensitivity. For example, it is possible to choose a lock-in time constant of 0.03 s (corresponding response time is 0.4 s) if a sensitivity of only 3 ppb is required. The gas dynamic time constant is principally determined by the connecting tubing and the gas flow rate. The gas exchange might be improved by using thinner and shorter tubing. Flow rates >800 ml/min did not improve the response time of the system.

Statistical Methods and Analysis of Data

To gain information about the site of prominent NO release and, for statistical reasons, the NO concentration was referred to the physiological volumes of the compartments of the respiratory tract. Therefore, the expiratory volume was divided into 150-ml fractions (see Fig. 3). The NO concentration (in ppb) in each 150 ml was analyzed statistically. The data from the first (150 ml) and third (450 ml) fractions were transferred to a table (Table 1). Normally, inhalation and exhalation volume did not exceed 1,000 ml. Thus only the NO data of the first 750 ml were taken. To investigate the significance of the data, the paired Student’s t-test was chosen.

RESULTS

In all breathing cycles a sharp initial NO peak was observed within the first 150 ml of expired volume. A relatively stable plateau of lower NO concentrations (by a factor of 1.2–2.6) was found within the 300- to 450-ml expiratory volume.

Nasal NO peak was significantly higher (by a factor of 3.3–7.6 depending on the type of breathing) than the oral NO peak (see Table 1). For example, for a 10-s breath hold, the nasal NO peak (126.6 ± 80.3 ppb) was 7.6-fold higher (P < 0.001) than the oral NO peak (16.6 ± 8.0 ppb). A strong correlation between the peak concentration and breath-holding time was observed. Five subjects showed saturation of the NO concentration after a 10-s breath hold.

Similar results were found for the plateau concentrations. The nasal NO plateau was always higher (by a factor of 2.9–4.3) than the oral NO plateau, but the height of the plateau concentration scarcely changed with increasing durations of breath hold.

An increase in NO concentration in the late phase of expiration (450- to 750-ml total expiratory volume) could be observed in some cases; this was always accompanied by a decrease in breathing flow.

All evaluated NO data for breathing with ambient air showed slightly, but insignificantly, increased levels compared with the same breathing type in synthetic air (see Table 1). A slight increase in NO is expected with ambient air, because the ambient air in our laboratory had a NO content of 2–7 ppb during the experiments.

DISCUSSION

Our results demonstrate for the first time that LMRS is an adequate method for NO measurements in breath. This method fulfills all expected requirements of sensitivity, specificity, and high time resolution. Indeed, on-line recording of breathing flow and NO concentration could be done simultaneously. Thus LMRS is an interesting alternative to the commonly used chemiluminescence analyzer. For direct comparison, the properties of both analyzers are given in Table 2. All of the specifications fulfilled by an up-to-date, state-of-the-art chemiluminescence analyzer, especially sensitivity, response time, and accuracy, are also performed by LMRS. The response time of LMRS in its present setup is of the same magnitude as the Sievers NOA 280 NO

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2 The electronic response time of the LMRS system is worse than that of chemiluminescence analyzers by a factor of 2–3. However, gas-flow conditions, which limit response time of the systems in practice, are better for LMRS because of better gas exchange due to a higher flow rate through the analyzing cell (800 ml/min at 27 mbar). Chemiluminescence analyzers work at atmospheric pressure and at a gas flow of ~100–300 ml/min. Under these conditions, electronic response time of the system is reduced by a factor of ~3 at least, if no external tubing is connected (22a). LMRS permits much higher flow rates of the gas sample through the cell because the system requires only a low working pressure (only a fraction of the exhaled air flows through the cell of the LMRS apparatus, even at high flow rates).
can distinguish between different NO isotopomers (e.g., of water in the exhaled air may arise. Second, LMRS with this technique, quenching problems because estimation is in line with previous reports using the pharmaceuticals. An increase in breath-hold duration leads to higher NO concentrations throughout the whole breathing cycle, in agreement with Persson and co-workers (21). Significant NO production has been postulated also for the distal airways, whereas the lowest NO release has been proposed for the alveoli (5, 15, 24). The studies yielding these results were performed by using invasive techniques, e.g., intubation, tracheostomy, closure of the nasopharynx, or bronchoscopy, to abolish addition of nasal air. In contrast, our experiments were performed without any of these invasive techniques and without subjects wearing noseclips, because this led to a slight increase in NO content during exhalation through the mouth. This indicates that there is a relevant mixture with nasal air in the nasopharynx under conditions of increased resistance in the nasal airways.

NO uptake in the lungs generated by conducting airways has recently been proposed (23). Although we cannot reach any conclusions in our present study with regard to such NO uptake by lung tissue, it is likely that NO diminishes the tone of smooth muscle cells of bronchioli and of blood vessels (12, 19, 20). This may be an important mechanism to counteract pulmonary hypertension. We are therefore planning for a future investigation in which breathing is modified with inspiration through the nose and expiration through the mouth. The comparison with data on mouth breathing may allow us to gain insight into the consumption of NO in the lower airway tract. Thus we may be able to evaluate changes in NO concentration under physiological and pathophysiological conditions in different parts of the airway tract during normal breathing cycles. Because of the capability to quantify the rare isotopomer 15N16O selectively (16) and to detect the very low biological 15N16O concentration of 0.3% of 14N16O in natural abundance, LMRs may be the method of choice to elucidate the kinetics of NO metabolism via application of 15N-labeled NO donors.

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Received 30 April 1998; accepted in final form 10 November 1998.

Table 1. NO in exhaled air from humans

<table>
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<tr>
<th></th>
<th>Synthetic Air</th>
<th></th>
<th>Ambient Air</th>
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<tbody>
<tr>
<td>Breath hold, s</td>
<td></td>
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<td></td>
</tr>
<tr>
<td>0</td>
<td>20.4 ± 11.6</td>
<td>68.9 ± 35.7</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>53.5 ± 22.1</td>
<td>14.9 ± 8.9</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>126.6 ± 80.3</td>
<td>31.4 ± 10.4</td>
<td></td>
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<tr>
<td>15</td>
<td>121.0 ± 34.5</td>
<td>7.2 ± 4.9</td>
<td></td>
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</tbody>
</table>

Values are means ± SD in parts/billion (ppb); n = 8 subjects. NO, nitric oxide; NOpeak,nasal, NOpeak,oral, NOplateau,nasal, and NOplateau,oral; peak and plateau nasal and oral NO exhalation, respectively. Peak levels (between 0 and 150 ml) and plateau levels (at 450 ml) for oral and nasal exhalation are shown. Observations were obtained for increasing duration of preceding breathholding and a constant duration of inhalation and exhalation time.

Table 2. Specifications of the LMRs in comparison with a present state-of-the-art chemiluminescence analyzer like the Sievers NOA 280

<table>
<thead>
<tr>
<th></th>
<th>LMR (Present Setup)</th>
<th>Chemiluminescence Analyzer (Sievers NOA 280)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sensitivity</td>
<td>1 ppb (at response time of 0.8 s)</td>
<td>&lt;1 ppb</td>
</tr>
<tr>
<td>Electronic response time (90% of full scale)</td>
<td>0.5 s (lock-in time constant of 0.3 s)</td>
<td>0.2 s</td>
</tr>
<tr>
<td>Response time in practice (90% of full scale)</td>
<td>0.8 s</td>
<td>&gt;0.75 s</td>
</tr>
<tr>
<td>Flow rate</td>
<td>800 ml/min</td>
<td>100 - 300 ml/min</td>
</tr>
<tr>
<td>Working pressure</td>
<td>27 mbar</td>
<td>≈1,000 mbar</td>
</tr>
<tr>
<td>Accuracy</td>
<td>3%</td>
<td>3% on average for 5–100 ppb</td>
</tr>
<tr>
<td></td>
<td></td>
<td>7% on average for 2–5 ppb</td>
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<tr>
<td></td>
<td></td>
<td>&gt;16% for &lt;1.5 ppb</td>
</tr>
<tr>
<td>Repeatability</td>
<td>±1 ppb</td>
<td>±1 ppb</td>
</tr>
</tbody>
</table>

LMRS, laser magnetic resonance spectroscopy.
REFERENCES


