

Is nitric oxide in exhaled air produced at airway or alveolar level?

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ABSTRACT: The aim of this study was to determine whether the nitric oxide (NO) measured in exhaled air is produced at airway or alveolar level.

Exhaled NO was measured using a chemiluminescence analyser, and carbon dioxide (CO₂) concentration using a Morgan capnograph in single exhalations in 12 healthy subjects (mean age 32 yrs; 6 males and 6 females). For each subject, five exhalations were made directly into the NO analyser and five were made through a T-piece system, which allowed measurement of expiratory flow rate.

The peak NO levels measured *via* the T-piece system were 41.2 (SEM 10.8) parts per billion (ppb), significantly lower than direct levels 84.8 (14.0) ppb ($p < 0.001$). The levels of NO tended to rise to an early peak and plateau, while the CO₂ levels continued to rise to peak late in the exhalation. The mean times to reach peak NO levels were 32.2 s (direct) and 23.1 s (T-piece), which were significantly different from that of peak CO₂ levels 50.5 s (direct) and 51.4 s (T-piece) ($p < 0.001$). At peak NO level, the simultaneous CO₂ level, mean 4.9% (SEM 0.14)%, was significantly lower than the peak CO₂ reached, 5.8 (0.21)% ($p < 0.001$).

We conclude that peak nitric oxide levels are dependent on measurement conditions. There are significant differences between the time to peak of carbon dioxide and nitric oxide. Therefore, most nitric oxide, unlike carbon dioxide, is produced in airways and not at alveolar level.

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Nitric oxide can be measured in exhaled air [1, 2], and has been shown to be produced by airway epithelial cells, vascular endothelial cells, macrophages, neutrophils and fibroblasts [3, 4]. The source of nitric oxide (NO) within the respiratory tract continues to be debated. Initially, BORLAND *et al.* [5] suggested that the NO measured in exhaled air from a single full exhalation and during tidal breathing was of alveolar origin, like carbon dioxide (CO₂). PERSSON *et al.* [6] suggested that NO was formed preferentially in the small airways, such as the terminal and respiratory bronchioles. By comparing the levels of NO exhaled during tidal breathing either through the nose or the mouth, ALVING *et al.* [7] suggested that the major contribution came from the nasal space, with a minor addition from the lower airways. LUNDBERG *et al.* [8] demonstrated a decreasing concentration of exhaled NO when sampling progressively down the respiratory tract at the nose, mouth and, in four tracheostomized patients, below the vocal cords. By isolating the nasal passages from the rest of the respiratory tract with the use of voluntary closure of the soft palate, KIMBERLY *et al.* [9] showed that the release of NO in the nasal passages was approximately seven times that of the rest of the respiratory tract. Extremely high levels of NO have now been demonstrated in the paranasal sinuses, with an age-dependent increase in keeping with sinus pneumatization [10]. It may be the sinus production of NO which communicates with the

nasal spaces, that gives rise to the high levels of NO measured in the nasal cavity.

The aim of this study was to ascertain the level of the source of NO in exhaled air within the respiratory tract.

Methods

Subjects

Twelve healthy volunteers aged 17–42 years (mean 32 yrs; 6 males and 6 females) were recruited. They were nonsmokers, nonatopic, with no history of respiratory or cardiac disease and receiving no medication. They had normal spirometry (Compact Vitalograph; Vitalograph Ltd, Buckingham, UK).

Equipment

Exhaled NO was measured using a chemiluminescence analyser (Model 2107; Dasibi Environmental Corporation, Glendale, CA, USA), sensitive to NO from 2–4,000 parts per billion (ppb), and adapted for on-line recording of NO concentration. The NO analyser sampled at 240 mL·min⁻¹. The CO₂ levels were measured with a Morgan capnograph PK Morgan, Kent, UK. The CO₂ analyser sampled at 200 mL·min⁻¹. Both the NO and CO₂ analysers were calibrated before and after each

subject, with known amounts of the appropriate gases. Mouth pressure was measured using a Medex Straingauge pressure transducer (Medex Medical Inc., Rossendale, UK) through a Galtec pressure amplifier (Galtec Ltd, Isle of Skye, UK) in mmHg. Flow was measured *via* a Platon Flow Meter to a low flow pneumotachograph (CT Platon Ltd, Basingstoke, UK) and a GM Electrospirometer (GM Instruments Ltd, Kilwinning, UK). The signal for each of these four separate measurements was then displayed on a Linseis chart recorder (Linseis Ltd, Cambridge, UK).

Preliminary experiments were conducted to determine the delay time (time for the sample to get down the tube to the analyser) and response time (in this case 0–95% rise time) of a square wave for both analysers. Briefly, a three-way tap connected to an oscilloscope was turned, allowing test gas to flow down the apparatus at a rate comparable to the subjects expiratory flow rate (500 mL·min⁻¹), starting at the subject's mouthpiece. The output of the analysers was displayed on the same oscilloscope. The delay time was the time between throwing the switch and the onset of the analyser signal, and the rise time was the time from onset of the signal to 95% of the plateau value. The experiment was repeated 10 times in all. In our preliminary experiment [11], we demonstrated a good correlation, similar to that of other groups [12], between the peak NO and area under the curve (AUC). The peak NO was taken for these experiments. To see if different rates of flow actually generated different pressures, the calibration NO gas was applied to the analysers at a range of pressures from 4 to 40 mmHg. This resulted in an elevation of only 5.6 ppb with a 10 fold elevation in pressure.

Procedure

Each subject, wearing a noseclip, made 10 single consecutive exhalations from total lung capacity (TLC), each recording separated by 3 min. Five of the recordings, when the subject exhaled directly into the NO analyser, were made with measurements of NO, CO₂ and mouth pressure. Five recordings were made using a T-piece, to allow the additional measurement of flow. The subjects controlled their expiratory flow rates voluntarily using as feedback the signal observed from the pneumotachograph. The mouth pressure was standardized to 4 mmHg. In the direct method, the combined analysers sampled at 440 mL·min⁻¹ and, with the T-piece method, flow was standardized at an additional 225 mL·min⁻¹ (total 665 mL·min⁻¹). Subjects were randomized to carry out either direct or T-piece measurements first. The background level of NO was noted on each occasion, and studies were postponed if the background NO level was greater than 15 ppb.

The study was approved by the Brompton Hospital Ethics Committee and informed consent was obtained from each of the subjects.

Statistical analysis

Results were analysed using the Statistical Products and Service Solutions (SPSS) package, and employing Students t-test for matched pairs. A p-value of less than 0.05 was considered significant.

Results

Delay time for the NO analyser was 5.9 (SEM 0.8) s, and 95% response time was 1.1 (0.1) s. For the CO₂ analyser the delay time was 0.11 (0.003) s and the 95% response time was 41.5 (2.9) ms. Thus, the NO analyser responded much more slowly than the CO₂ analyser. Response time for the pressure transducer was 7.3 (0.2) ms and for the flow meter was 40.2 (0.8) ms, with no significant time delay. When comparing the NO and CO₂ recordings, allowance was made for the difference in time delay of the measurements from each machine and for the 2 mm offset of the pens on the recorder.

Ten measurements were performed successfully on each of the subjects. The coefficient of variation (CV) for individuals was 17% for the direct method and 11% for the T-piece method. The direct mean peak concentration of NO was 84.8 (SEM 14.0) ppb (range 25.1–189.0 ppb). There was greater variation in the female subjects, mean 92.4 (36.9) ppb (range 26.2–189.0 ppb), than male subjects, mean 71.7 (28.7) ppb (range 25.1–141.0 ppb). The peak NO levels measured through the T-piece system

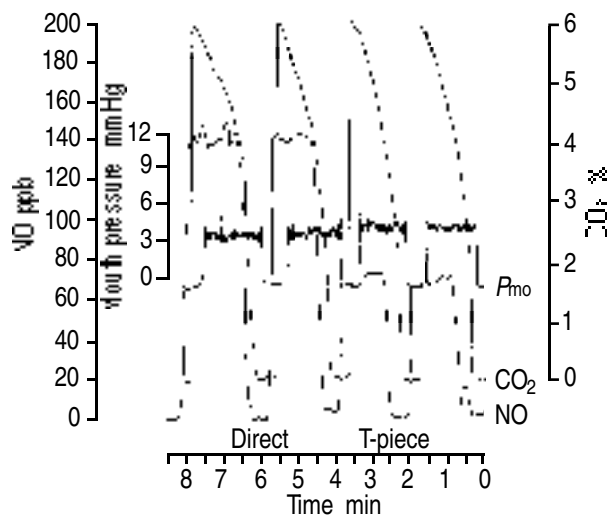


Fig. 1. — An example of raw data from one subject showing nitric oxide (NO), carbon dioxide (CO₂) and mouth pressure (P_{mo}). Note the difference in the exhaled NO concentration between the two methods of measurement.

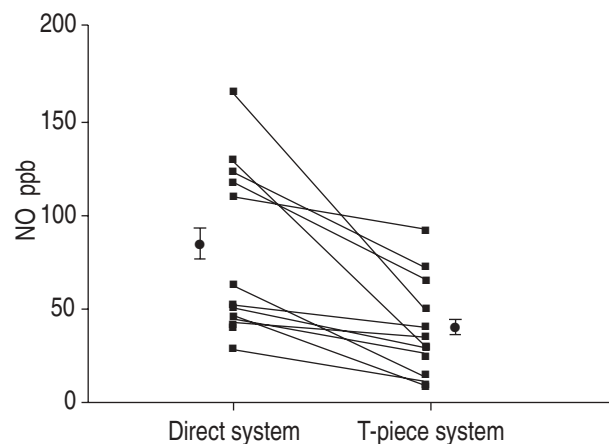


Fig. 2. — Comparison of mean peak exhaled nitric oxide (NO) levels measured through the direct and T-piece systems ($n=12$). Each point is the mean of five exhalations. Solid circles and vertical lines show mean and SEM, respectively.

were lower (figs. 1 and 2), with: total subject mean 41.2 (10.8) ppb (range 10.00–101.6 ppb); mean in males 33.2 (8.4) ppb; and mean in females 50.7 (12.3) ppb. There was no significant difference between peak CO₂ level with direct or T-piece measurements (mean 5.81 vs 6.19%; $p=0.66$), or between male and female subjects (5.84 vs 5.75% direct ($p=0.64$); and 6.41 vs 6.03% T-piece ($p=0.44$)).

In the direct measurements, the mean time to reach peak NO was 32.2 (SEM 4.3) s. This was significantly less ($p<0.001$) than the mean time to reach peak CO₂ levels at 50.5 (5.3) s, which was also less than the time to total exhalation of 56.2 (5.4) s ($p<0.001$) (fig. 3a). In the T-piece measurements, the mean time to reach peak NO was 23.1 (SEM 4.4) s. This was significantly less ($p<0.001$) than the mean time to reach peak CO₂ levels at 51.4 (4.6) s, which was again less than the total duration of exhalation 53.5 (4.7) s ($p=0.14$) (fig. 3b). There was a significant difference in the mean time to reach peak NO levels between the direct and T-piece method ($p<0.001$), whilst there was no difference between the time to reach CO₂ peaks between the direct and T-piece methods ($p=0.5$). The CO₂ level measured

at peak NO was 4.88 (SEM 0.14)%, which was significantly lower ($p<0.001$) when compared with peak CO₂ at 5.84 (0.21)%.

The background level of NO ranged 1–11 ppb, which did not alter the conclusions and so was not taken into account in the calculations.

Discussion

Previous studies have been unable to agree whether NO is of alveolar or airway origin [5–7]. If CO₂ and NO were both of alveolar origin, the time to peaks and the ratio of peak NO to CO₂ would remain the same even if measurement conditions were varied. We thus measured exhaled CO₂ and NO using two different conditions. In the direct experiments, the subjects exhaled directly into the NO analyser at 440 mL·min⁻¹, whilst in the indirect studies, exhalation was into a T-piece system at a faster rate of 665 mL·min⁻¹. The time to reach peak CO₂ level, and the level of the peak CO₂ were the same in both manoeuvres. However, the NO traces were very different. Firstly, the time to reach peak NO was shorter than the time to peak CO₂ in both manoeuvres (32.2 versus 50.5 s in direct measurements, and 23.1 versus 51.4 s in T-piece measurements). Secondly, altering the rate of exhalation alters the time to peak NO independent of the time to peak CO₂. Thirdly, at peak NO levels, the CO₂ levels were below their maximal peak. Fourthly, the ratio of peak NO to peak CO₂ differed between the two methods of measurement. Thus, although it is possible that a small amount of NO is of alveolar origin, the bulk of NO must be produced proximal to the alveolar membrane.

In any physiological study, it is important to consider whether the choice of apparatus or the manoeuvre selected could have significantly affected the conclusions. Ideally, we would have used fast-response time analysers, with identical delay times. Fast-response time analysers for NO have been used by others. PERSSON *et al.* [6] used a NO analyser with an integration time of 0.01 s for a study of changes in NO at rest and on exercise, and came to similar conclusions to ourselves despite using a very different analyser and manoeuvre. Our analyser would have been unable to detect rapid transient changes in NO. However, had these occurred, and they have not been described by other workers, they would have strengthened rather than detracted from our conclusions. Rapid transients have not been described for CO₂, and their presence in the NO signal would have implied a different source, which is the main conclusion of the present paper. Our method gives individual CVs similar to those of other groups [12].

We considered the possibility that differing pressures exerted on the NO analyser may have accounted for the difference in readings. In a series of experiments [13], we have shown that changes in pressure between 4 and 16 mmHg did not affect the analyser reading. Furthermore, we were careful to standardize to a mouth pressure of 4 mmHg during the expiratory manoeuvre, to eliminate the possibility of this artefact. The pressure drop from mouth to analyser was measured both for direct and T-piece apparatus, and found to be essentially the same. The expiratory flow rates between the two manoeuvres were deliberately different, to try to

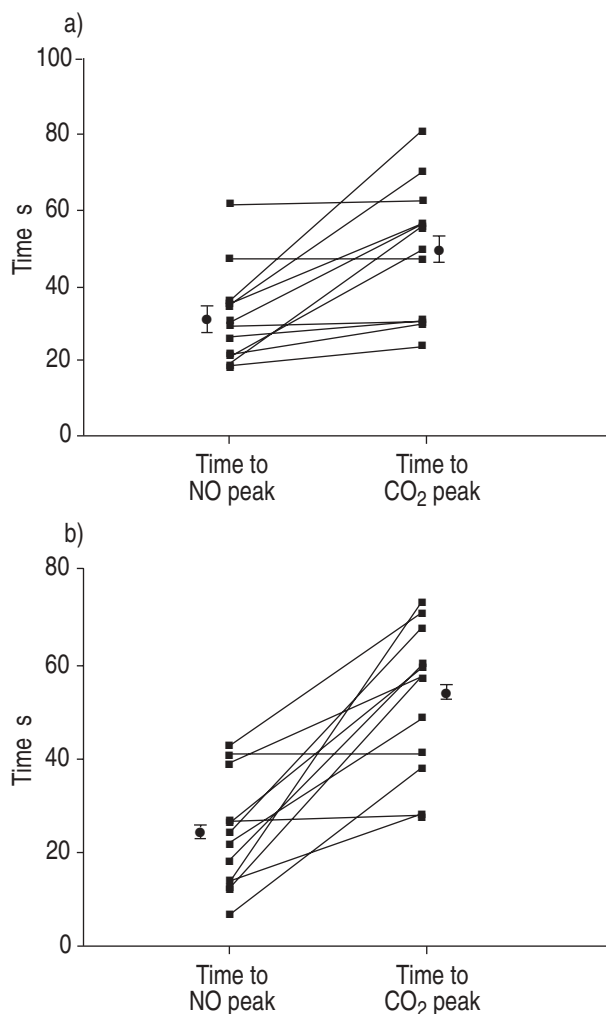


Fig. 3. – Mean time taken to reach peak nitric oxide (NO) and peak carbon dioxide (CO₂) concentrations: a) in the direct system (n=12); and b) in the T-piece system (n=12). Each point is the mean of five exhalations. Solid circles and vertical lines show mean and SEM, respectively.

separate the CO₂ and NO signals. However, the flow to the analyser remained constant throughout the study.

We also considered how the different response times of the analysers could have affected our conclusions. Ideally we would have used a mass spectrophotometer and equal analyser response times. The NO response was slower, but the NO peaked first. Had the NO response time been the same as that of the CO₂ analyser, the NO peak would have been even earlier. Thus, the analyser differences would serve to obscure, not enhance our conclusions.

We elected to study a single slow exhalation for the purposes of these studies, accepting that it may not be physiological or necessarily the best method for clinical practice. However, it was designed to supply an answer to the question posed. The possibility exists that the results were affected by back-diffusion of alveolar NO into the alveolar capillary blood, and by nasal contamination of the exhalate. During a prolonged expiration, if alveolar epithelium were the source of NO, then back-diffusion would allow some to be carried away combined with haemoglobin. However, although this could affect the height of the NO peak, it is inconceivable that it could affect the time to reach peak, and thus our conclusions are unaffected. Furthermore, the total time of expiration was very similar in the two different manoeuvres, suggesting that, were this mechanism to be operative, it would apply equally between the two tests.

Higher levels of NO are produced by the paranasal [10, 14] and nasal spaces, when compared to oral [7–9], sites, and these contribute a major proportion of the NO concentration measured by oral exhalation. Indeed, the paranasal sinuses also contribute a major proportion of the NO concentration measured by nasal exhalation [10, 14]. However, if all of the NO came from the nasopharyngeal region then the pattern of the exhaled NO trace would be expected both to peak and fall off early, rather than the continued plateau seen in our experiments. NO can be measured in exhaled air of tracheostomized rabbits (measured by chemiluminescence), guinea-pigs (measured by Quattro mass spectrometry) [15], and in humans (measured by chemiluminescence) [8]. We attempted to exclude nasal contamination by asking the subject to wear a noseclip, and to exhale against a moderate resistance (4 mmHg). One group has used nasal suction combined with an (amplitude unstated) expiratory resistance [16], and another has excluded nasal NO contamination by voluntary closure of the soft palate [9], but these techniques are by no means universally used. Whereas we cannot exclude a degree of nasal contamination, we believe that our technique will have excluded most contamination, and that, in any case, the degree of contamination will have been identical because of the very careful standardization of experimental conditions, including expiratory mouth pressure. This study was not designed to differentiate nasal and bronchial sources of NO. We elected to analyse the peak NO rather than AUC. The two are, in any case, closely correlated [11, 12]. We aimed to determine the sources of the two gases, not the total amounts produced, which we would expect to be independent of the experimental conditions. Thus, the difference in behaviour of the peak signals as the experimental conditions change gives more useful information than the AUC.

It might be argued that if airways (of volume 200 mL) contribute a peak NO at 85 ppb, a total of 10⁻¹⁰ mol, and the alveolar expirate (4 L) a plateau of 60 ppb, a total of 10⁻⁸ mol, then most NO comes from the alveoli. This assumes that subjects exhaled to residual volume, which is not the case. Furthermore, if this model was correct, peak CO₂ (definitely alveolar) and peak NO should move in the same direction as experimental conditions vary; in fact, the reverse was seen (fig. 2). In eight subjects, peak NO fell using the T-piece whilst CO₂ rose; in the other four subjects, peak NO fell and peak CO₂ remained the same. It is impossible to account for this on a model of major alveolar production of NO. The model that best accounts for the present data is continuous airway production of NO, diluted variably in a flow-dependent manner by NO-free (or low concentration NO) alveolar gas, analogous with a gaseous phase dye dilution method. The present data cannot exclude that alveolar gas contains a small amount of NO.

We have also demonstrated that the conditions of measurement critically affect the NO levels obtained. Thus, both the time to reach the NO peak and the peak level of NO reached were quite different in the two methods used in this study, although the patterns of the traces were similar. We considered whether the difference in results could have been artefactual, due to minor differences in the apparatus used in the two techniques. Teflon tubing, which does not absorb NO, connected the mouthpiece to the NO analyser. In the T-piece system, there was a 2.5 cm extension of plastic tubing leading from the mouthpiece to the actual T-piece, with the Teflon tubing then leading to the NO analyser off one arm, and further tubing off the second arm passing directly to the pneumotachograph for flow analysis. This would seem unlikely to account for the magnitude of differences obtained. The other alteration was that in the indirect method the flow rate was increased from 440 to 665 mL·min⁻¹. This may have been enough to account for the different results.

Clearly, there are major and unexplained differences between different studies. Our results show that the levels of NO measured in exhalate are critically dependent on measurement conditions. This could be related to expired flow rates but other factors may be important. It has been suggested that orally exhaled NO concentrations correlate with the inhaled ambient concentrations of NO [17], although we found, in a subsequent experiment, that a high ambient NO level rendered NO levels difficult to interpret [18]. The present study was only undertaken if the ambient NO levels were low. Furthermore, the greater NO levels and wider variations seen in females in the present study may be due to the reported effect of the menstrual cycle [19] on exhaled peak NO concentrations. This was not controlled for in the present study. However, as the measurements were performed sequentially on each subject on the same day, the conclusions concerning the origin of NO are not affected by phases of the menstrual cycle.

Much research is being carried out on levels of nitric oxide in exhaled air in health and in different respiratory and pulmonary vascular diseases. It is important to understand where and at what level nitric oxide is being generated so that abnormal results can be interpreted correctly.

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