Increased nitric oxide in exhaled air after intake of a nitrate-rich meal


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Exhaled and nasal NO (ENO, NNO) have been suggested as markers for inflammation in lower and upper respiratory tract respectively. It is still unknown how a number of factors, apart from airway inflammation, can influence NO levels. The aim of this study was to determine the effect of a nitrate-rich meal on ENO and NNO. Sixteen healthy subjects were observed during 1 week on normal diet before a nitrate-restricted diet was introduced in the next. On day 3 of the second week they were made to ingest a nitrate rich meal. ENO, NNO, plasma nitrate and plasma L-arginine were followed before the meal and afterwards for 3 h.

ENO and NNO as well as plasma nitrate and plasma L-arginine were significantly elevated after the nitrate-rich meal. The median maximal increase of ENO and NNO was 47% and 13% respectively. We found a moderate but significant correlation between the rise in plasma nitrate and ENO (r_s = 0.57, P = 0.027) but none between plasma nitrate and NNO (r_s = 0.02, P = 0.95).

As nitrate in the diet seems to substantially influence the levels of ENO it is important either to restrict or register the intake of nitrate-rich food prior to measuring ENO.

Key words: nitric oxide; nasal nitric oxide; nitrate; nitrite; L-arginine; breath analysis.
too, be formed non-enzymatically during oxidative stress, as proposed by Nagase et al. (13).

Although it is well known that the intake of food with a high nitrate (\(\text{NO}_3^-\)) content to a large extent influences blood nitrate levels, it is not clear how variations in plasma nitrate influence the exhaled NO levels.

In the gut, the nitrate in the diet is absorbed into the blood and actively transported to the saliva by the salivary glands (14). In the oral cavity it is converted to nitrite by bacteria, mainly in the deep clefts on the rear part of the tongue. The balance that exist between NO and nitrite is regulated by the pH, i.e. the more acidic environment the more NO will be formed. Salivary nitrate and nitrite have been shown to increase with increasing doses of ingested nitrate (15), although there has been shown to be a wide interindividual variability of conversion of nitrate to nitrite (16). Zetterquist et al. (17) have investigated the effect of an intake of 400 mg potassium nitrate solution on ENO levels, which they found to increase by a maximum of 150% 2 h after the ingestion. Concomitantly, they found an increase in nitrite in saliva following the pattern of its increase in plasma. Mouthwash with an anti-bacterial agent reduced NO release by almost 50%. They did not find any increase in nasal NO.

Studies have not been made of the way l-arginine in the diet can influence exhaled or nasal NO levels. After intake of l-arginine capsules in a dose of 0.05 g kg\(^{-1}\), no increase of exhaled NO was found among asthmatic patients (18), but Kharitonov et al. (19) found an increase in healthy subjects after ingestion of 0.1 g l-arginine kg\(^{-1}\). Nasal NO has been shown to increase after infusion of l-arginine (20).

The aim of the present study was to investigate whether the intake of a nitrate-rich meal would influence ENO and NNO levels. We analysed ENO and NNO after a normal diet, a nitrate-restricted diet and after intake of a nitrate-rich meal in 16 healthy volunteers. The levels of plasma nitrate and plasma l-arginine levels were simultaneously recorded.

### Subjects and methods

#### SUBJECTS

Twenty healthy (10 males/10 females), non-smoking volunteers with no history of obstructive lung disease or respiratory tract infection during the previous 3 weeks were recruited for the study. Three females had to be excluded because of an infection of the respiratory tract caught during the study period as did one male subject who developed symptoms of allergic rhinitis. ENO and NNO as well as plasma nitrate and plasma l-arginine levels were measured in 16 subjects. All subjects passed an examination including spirometry (21) and a Phadiatop\(^\text{TM}\) test (22). Basic data on the subjects are presented in Table 1.

<table>
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<tr>
<th>Table 1. Basic data on the subjects</th>
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<tr>
<td>Females ((n = 7))</td>
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<td>Males ((n = 9))</td>
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<td>Age (years)</td>
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<td>Phadiatop*</td>
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*0 = negative, 1 = positive.

The local committee of ethics approved the study and informed consent was obtained from all volunteers prior to their inclusion in the study.

#### EXHALED AND NASAL NO

Exhaled NO was measured during a slow single-exhalation for 20 sec against an oral pressure of 5 cm H\(_2\)O at a constant flow rate of 50 ml sec\(^{-1}\), in accordance with ATS recommendations (10). The measurements were performed in triplicate at each occasion and the mean NO concentration (ppb) and mean output (nl min\(^{-1}\)) during the plateau phase (12–18 sec of the exhalation) were registered. Nasal NO was measured by letting the analyser sample nasal air directly from one nostril at a constant sample rate of 8 ml sec\(^{-1}\), while the subject held their breath for 20 sec and closed the velum voluntarily. The nasal NO concentration during the plateau phase was registered. One measurement was performed in each nostril and the mean NO concentration was calculated.

A chemiluminescence analyser was used to measure nitric oxide, (CLD 700 AL; Ecophysics, Dürnten, Switzerland). A special computerized biofeedback system was used to regulate exhalation flow rate (Aerocrine NO System\(^\text{TM}\); Aerocrine AB, Stockholm, Sweden). A two-point calibration of the analyser was performed daily with a certified NO calibration gas. The exhaled NO output, i.e. ENO elimination rate \([\text{NO concentration (ppb) = nl l}^{-1} \times \text{flow rate (1 min}^{-1}\text{)}] \) is presented because these values are more accurate, the NO concentration being strongly influenced by small changes in the exhalation flow. To make comparisons possible with other studies where only NO concentrations are presented, the individual concentrations of exhaled NO are also presented. The intra-individual variability expressed as coefficient of variation (CV) for ENO, NNO and plasma nitrate were estimated through measurements taken on 3 days at a normal diet, with an interval of at least 2 days between each, on all included subjects.

#### NITRATE

Nitrate in plasma was analysed by the gas chromatography/mass spectrometry (GC/MS) method described by Tesch et al. (23) and further developed by Wennmalm et al. (24).
L-ARGININE

L-arginine was measured with a fully automatic ion-exchange-chromatography system, as previously described (25).

DIET

During the first week the subjects were kept on normal diet without any restrictions. During week 2 a low-nitrate diet was introduced (26), but with a normal L-arginine content. After 48 h of a low-nitrate diet the subjects were given a meal rich in nitrate: 100 g lettuce, 75 g spinach, 100 g ham, 75 g cucumber, three to five potatoes and a tomato. The nitrate content was equivalent to 1 g nitrate, and the L-arginine content to 1-4 g. ENO and NNO and plasma nitrate were then analysed every 15 min for 3 h and thereafter daily for 2 days. Plasma L-arginine was analysed before the meal and hourly during the first 3 h afterwards.

The nitrate rich meal was served at 09.00 hours for half of the subjects and for the other half at 12.00 hours in a randomized way. The ENO and NNO measurements taken on days of normal diet, and on the days following the nitrate-rich meal, were performed at the same time of the day as on the meal was served.

LEAN BODY MASS (LBM)

The increase in plasma nitrate and plasma L-arginine was considered to be dependent on plasma volume. A lean body mass index has been calculated for each individual as this is the best available index for plasma volume (27). The lean body mass index has been used in analysing the correlation between the changes in ENO, NNO, plasma nitrate and plasma L-arginine.

STATISTICS

Statistical analyses were carried out with SAS, version 6.12 (SAS Statistical package, Cary, NC, U.S.A.). The results have been given as medians of change in ENO output (nl min\(^{-1}\)), NNO concentration (ppb), nitrate concentration (\(\mu\text{mol}\) l\(^{-1}\)) and L-arginine concentration (\(\mu\text{mol}\) l\(^{-1}\)) in plasma, unless otherwise stated. The individual changes from baseline (i.e. the value before the meal was served) of exhaled and nasal NO, plasma nitrate and plasma L-arginine after the nitrate-rich meal were calculated as [value at respectively time point] – [baseline value]. For each individual the maximal change from baseline in ENO concentration was also noted. Significance testing was based on Student’s \(t\)-test using logarithm-transformed values. The area under the curve (AUC), representing the total increase from baseline during the follow-up period, was calculated for each parameter. Since the observations were equally spaced in time, the mean change for each parameter can be used, representing the area under the curve (28). As this method does not allow for any missing values, the mean of the two adjacent values has been used to represent the missing value.

Spearman’s rank correlation coefficient \((r_s)\) have been calculated to analyse any correlation between both maximal and total change in ENO/NNO and nitrate/L-arginine.

Results

We found significant increases of ENO and NNO, plasma nitrate and plasma L-arginine after intake of the nitrate-rich meal [see Fig. 1(A–D) (mean ± SEM)]. The individual NO concentrations before the nitrate-rich meal, the maximal increase of exhaled NO, irrespective of time-point (range 15 min–2 h 45 min), and the levels 3 h after the meal are presented in Fig. 2. The median of maximal ENO increase after the meal was 47% (range 7–89%) and the median of maximal NNO increase was 13% (range 0-1–36%).

The maximal increase in ENO and plasma nitrate occurred 2 h after the meal and subsequently decreased, although both ENO and nitrate were found to be significantly elevated throughout the measurement period. L-arginine was only examined before the meal and 1, 2 and 3 h afterwards. Here, too, the maximal increase occurred 2 h after the meal.

![Fig. 1](https://example.com/fig1.png)

**Fig. 1.** (A) Change in exhaled NO, mean ± SEM. (B) Change in nasal NO, mean ± SEM. (C) Change in plasma nitrate, mean ± SEM. (D) Change in plasma L-arginine, mean ± SEM.
We presumed that the increase of plasma nitrate and plasma L-arginine would depend on the subjects' plasma volume. As all the subjects were given the same amount of food, the intake of nitrate and L-arginine in relation to plasma volume differed markedly. We had chosen to use lean body mass (LBM) as an index for plasma volume. We found an inverse correlation between maximal increase in ENO and LBM, \( r_s = -0.78, P = 0.0003 \) and between maximal increase in plasma nitrate and LBM, \( r_s = -0.78, P = 0.0006 \). The maximal rise in plasma L-arginine was not dependent on LBM \( (r_s = -0.0044, P = 0.98) \). The maximal increase in NNO did not correlate to LBM \( (r_s = 0.11, P = 0.68) \).

As a measure of the total change of ENO, NNO, plasma nitrate and plasma L-arginine the area under the curve (AUC) for each factor was calculated, and used in the subsequent analysis. We found a significant correlation between the total change of ENO and plasma nitrate \( (r_s = 0.57, P = 0.027) \), but no significant correlation between ENO and plasma L-arginine \( (r_s = 0.19, P = 0.47) \). NNO was not significantly correlated with change of either plasma nitrate \( (r_s = -0.018, P = 0.95) \) or plasma L-arginine \( (r_s = -0.094, P = 0.73) \).

We did not find any significant change of ENO or NNO when the subjects were kept on a nitrate-restricted diet as compared with a normal diet (median 35.1 vs. 34.6 \( \text{ml min}^{-1} \) and 587 vs. 534 ppb respectively). In contrast, the plasma nitrate levels were significantly lower with a nitrate-restricted diet (median 36.9 vs. 45.6 \( \mu \text{mol} l^{-1} \), \( P < 0.02 \)). For these analyses we compared the median of three measurements on different occasions during week 1 with the median value before the meal on day 3 of the second week.

The intra-individual coefficient of variation (CV), for repeated measurements on three occasions at least 2 days apart, when the subjects were on normal diet, was 19% for ENO, 9% for NNO and 22% for plasma nitrate.

**Discussion**

Both ENO and NNO were significantly increased after the intake of a meal rich in nitrate. The increase in ENO was dose-dependent, i.e. the subjects with the highest dose per LBM (an index for plasma volume) showed the greatest increase.

The median increase of ENO for all subjects was 43% occurring 2 h after the meal. These findings are in agreement with Zetterquist et al., reporting a maximal increase of ENO 2 h after ingestion of 400 mg potassium nitrate solution. However the ENO increase was very much lower (43%) after intake of nitrate-rich food intake compared with the intake of potassium nitrate solution (150%), even though the total nitrate content of the meal was higher than the nitrate content of the solution. One possible explanation may be the different bio-availability of the nitrate in food and in potassium nitrate solution, enabling the latter to be more rapidly and completely absorbed than the nitrate in the food.

Contrary to Zetterquist et al., we found an increase of NNO. One explanation for this might be that the meal also contained L-arginine. The production of NNO may be limited by the availability of the substrate L-arginine (20). The L-arginine content of the meal, due to its content in the ham, was equivalent to 1.4 g L-arginine; we also found a significant increase of plasma L-arginine after the meal. Lundberg et al. (20) have shown that an L-arginine infusion of 16 mg per kg body weight, increases NNO from 420 ppb to 475 ppb (13%) as against the ingestion of 15 mg L-arginine per kg body weight in this study, also resulting in increased NNO by 13%.

Nevertheless, we found no significant decreases of either ENO or NNO after 3 days with a nitrate-restricted diet. The plasma nitrate levels were, however, significantly lower with a nitrate-restricted diet.

This might indicate that the plasma nitrate levels are less important than the other factors determining ENO levels, at least when there are only minor changes of plasma nitrate. The median decrease in plasma nitrate, on a nitrate-restricted diet, was 5-9 \( \mu \text{mol} l^{-1} \) compared with the median maximal increase of 81-5 \( \mu \text{mol} l^{-1} \) after the nitrate-rich meal. Alternatively, since ENO is influenced by salivary nitrate/nitrite concentrations (18) the result may indicate that nitrate uptake in the salivary glands is biologically resulted to keep a minimum of nitrate in the saliva for host defence purposes (12).

One limitation of the study is that the diet also contained L-arginine, which makes it difficult to separate the effects of increased plasma nitrate from that of L-arginine, at the ENO and NNO levels. The time courses for the increases of nitrate and L-arginine were also similar. However, in previous investigations the ingestion of L-arginine in amounts equivalent to the contents in the nitrate-rich meal, has not been shown to induce any increase in ENO levels in normal subjects (19).
The area under the curve (AUC) was used as an index of the total change of ENO, NNO, plasma nitrate and l-arginine during the follow-up period of 3 h. Although one could speculate that changes of plasma nitrate and plasma l-arginine would precede the changes in ENO and NNO, the kinetics for these processes are not known, making for ambiguity in any analysis based on lag-time.

Another limitation of the study is that we did not compare the changes in ENO after a nitrate-rich meal with variations in ENO during a normal day. There has been contradicting data concerning diurnal variation of ENO. Ten Hacken et al. (29) have not been able to demonstrate any such variation in asthmatic patients with nocturnal asthma. On the other hand Massaro et al. (30) have presented an abstract indicating great diurnal variation, although with a maximal increase of ENO between 04.00 hours and 08.00 hours and a maximal decrease at 20.00 hours in healthy individuals. In our study half of the subjects had their meal at 09.00 hours and the other half at noon. If there were a diurnal variation of ENO in the pattern suggested by Massaro et al., the ENO values would tend to decrease during the morning hours when we followed half of the group, as well as among the subjects receiving their meal at noon.

The ENO levels in our study started to return to baseline within 3 h, indicating that the changes were related to the food intake and not to diurnal variation.

The finding that ENO could increase up to 89% after a nitrate-rich meal, indicates that it is important to register or cease the intake of nitrate-rich food the same day as ENO measurements take place. Although a normal diet rarely consists of, for example, 100 g of salad and 75 g spinach, the effect on ENO might be substantial even with lower nitrate contents in recently ingested food.

How long subjects should refrain from nitrate-rich food may vary according to the need for accuracy and this will also have to be considered in relation to other sources of measurement errors. We followed the ENO levels 3 h after the meal, and at that time-point half of the subjects still had higher ENO levels than before the meal.

It seems, however, of no value to introduce a nitrate restricted diet for a longer period of time in order to avoid dietary influences on ENO levels.

Another way of reducing the influence of exogenous nitrate on ENO levels might be to reduce the nitrate reducing bacteria in the oral cavity and/or increase the pH on the oral cavity before the measurements are performed. Before making any such recommendations further studies are called for.

In conclusion we found that ENO increases significantly after ingestion of a nitrate-rich meal. It seems therefore important to restrict the intake of food rich in nitrate prior to measuring nitric oxide.

Acknowledgments

The authors acknowledge the valuable technical assistance of G. Granung and K. Wass. The work has been supported by the Swedish Heart-Lung Foundation, the Swedish Council for Worklife Research, Torsten och Ragnar Söderberghs Foundation and the Gothenburg Medical Society.

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