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The increase in plasma nitrite after a dietary nitrate load is markedly attenuated by an antibacterial mouthwash

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ABSTRACT

Recent studies surprisingly show that dietary inorganic nitrate, abundant in vegetables, can be metabolized in vivo to form nitrite and then bioactive nitric oxide. A reduction in blood pressure was recently noted in healthy volunteers after dietary supplementation with nitrate; an effect consistent with formation of vasodilatory nitric oxide. Oral bacteria have been suggested to play a role in bioactivation of nitrate by first reducing it to the more reactive anion nitrite. In a cross-over designed study in seven healthy volunteers we examined the effects of a commercially available chlorhexidine-containing antibacterial mouthwash on salivary and plasma levels of nitrite measured after an oral intake of sodium nitrate (10 mg/kg dissolved in water). In the control situation the salivary and plasma levels of nitrate and nitrite increased greatly after the nitrate load. Rinsing the mouth with the antibacterial mouthwash prior to the nitrate load had no effect on nitrate accumulation in saliva or plasma but abolished its conversion to nitrite in saliva and markedly attenuated the rise in plasma nitrite. We conclude that the acute increase in plasma nitrite seen after a nitrate load is critically dependent on nitrate reduction in the oral cavity by commensal bacteria. The removal of these bacteria with an antibacterial mouthwash will very likely attenuate the NO-dependent biological effects of dietary nitrate.

From intense research performed over the past decade it now seems clear that the inorganic anion nitrite can be metabolized in vivo to form bioactive nitric oxide (NO)[1] [1–5]. This pathway complements the classical NO synthase/L-arginine pathway and is especially enhanced during hypoxia when NO formation by NOS's may be altered. A picture is now emerging suggesting important physiological functions of the nitrite-NO pathway in regulation of blood flow [6,7], gastric integrity [8–11] and in tissue protection against ischemic injury [12,13]. Several pathways exist for the reduction of nitrite to NO involving deoxyhemoglobin in blood [6] and xanthine oxidase [14], deoxymyoglobin [15,16], and mitochondrial enzymes [17] in the tissues. In addition to these enzymatic and protein-catalysed nitrite reductions, NO is also formed non-enzymatically from nitrite during acidic conditions [8,9] in a process that is greatly enhanced by vitamin C [18] and other antioxidants [19,20].

Recent studies now show that the much more stable anion nitrate can also be bioactivated in vivo to form NO. Larsen et al. showed that dietary supplementation with inorganic nitrate for three days resulted in a reduction in blood pressure in healthy volunteers [21]. The reduction in blood pressure was accompanied by an increase in plasma nitrite. The nutritional aspect of this finding is intriguing as the major dietary source of nitrate comes from vegetables. This has made us suggest that the well-known cardioprotective effect of a high vegetable intake, such as in the traditional mediterranean diet, is partly explained by its high nitrate content [22,23]. For nitrate to become vasodilatory NO however, it first needs to be reduced to nitrite, and it is believed that this reaction cannot be effectively performed by mammalian cells. Yet, the systemic levels of nitrite increased greatly after a dietary nitrate intake in the cited study by Larsen et al. [21] and in an earlier study by Lundberg and Govoni [24]. The likely explanation is that oral commensal bacteria are involved; they express highly effective nitrate reductase enzymes which can generate much nitrite from the dietary nitrate that accumulates in saliva after ingestion [25]. If oral bacteria are involved in regulation of systemic nitrite levels one would expect changes in plasma nitrite when the oral microflora is disturbed for example by the use of antibiotics.

In this study we have studied the effects of a commercially available antibacterial mouthwash solution on salivary and plasma levels of nitrate and nitrite after a dietary nitrate load.

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[1] *Abbreviations used:* NO, nitric oxide; NOS, nitric oxide synthase; HPLC, high performance liquid chromatography; C_{max}, maximum concentration.

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Experimental procedures

Chemicals

All chemicals were purchased from Sigma–Aldrich Sweden AB (Stockholm).

Study subjects and experimental protocol

The study was approved by the ethics committee at the Karolinska Institute and all subjects gave their informed consent. Seven healthy non-smoking volunteers aged 24–51 years, took part in the study. All subjects remained under low dietary nitrate intake the day before the experiment (no vegetables, no processed meat) and fasted overnight. Food or drinks were not allowed during the experiments. A catheter was inserted into the antecubital vein of the left arm for repeated blood sampling. The cross-over design consisted of two separate experiments (without or with mouthwash) conducted on separate days. In a first experiment (control experiment) samples of blood (5 ml) were drawn 30 min before and again immediately before ingestion of sodium nitrate (10 mg/kg in 100 ml water). In a second experiment (mouthwash experiment) the same protocol was followed with the addition of mouth washing (10 ml of Corsodyl®, gargled twice for 1 min) 15 min before ingestion of sodium nitrate. An additional blood sampling immediately before mouth wash was performed during this second experiment. After nitrate ingestion, blood was again sampled at 15, 30, 60, 90, 120, and 180 min, collected in tubes containing EDTA (final concentration 2 mM) and *N*-ethylmaleimide (final concentration 5 mM) and centrifuged at 1300*g* for 10 min at 4 °C within 30 s of sampling. Saliva (1 ml) was collected at the same time points. Samples of plasma and saliva were kept at 80 °C until measurements which were done later the same day (nitrite) or within 3 weeks (nitrate).

In a separate experiment in four of the healthy volunteers the middle and posterior tongue surface was scraped with a sterile inoculation loop before and 10 min after gargling with a chlorhexidine mouthwash solution exactly as described above. The collected material was placed in 1 ml Mueller Hinton broth containing 1 mM sodium nitrate. From this solution 100 µl was serially diluted and used for determination of viable counts on blood agar plates. Plates were incubated anaerobically for 20 h at 37 °C. The rest of the solution was left in the closed tube, incubated for 2–4 h at 37 °C to determine nitrite formation. Nitrite levels in the broth were below 1 µM at the start of the experiment.

Gas phase chemiluminescence assay for nitrite and nitrate

Salivary nitrite and nitrate and plasma nitrite were determined by gas phase chemiluminescence with a nitric oxide analyzer (Eco Physics AL 77, Switzwerland) after reductive cleavage and subsequent determination of the NO released into the gas phase. The method and apparatus has been described in details [24,26]. NO signals were displayed and reported as area under the curve using a chromatographic software (Azur v 3.0, Datalys, Saint-Martin d'Héres, France). Nitrite in plasma and saliva samples was reduced to NO with a solution consisting of 45 mmol/L potassium iodide (KI) and 10 mmol/L iodine (I_2) in glacial acetic acid at 60 °C and measurement was performed by direct sample injection (100 µl). The calibration curve was obtained with freshly prepared sodium nitrite solutions in ultrapure water. Nitrate in saliva was reduced to NO with a solution of vanadium (III) chloride in 1 N hydrochloric acid (saturated solution) at 95 °C. As vanadium (III)/HCl will also convert nitrite to NO, the amount of salivary nitrate was quantified by subtraction of the nitrite concentration in saliva. The calibration curve was obtained with freshly prepared nitrate standard solutions in ultrapure water.

High performance liquid chromatography (HPLC) assay for nitrate

Nitrate in plasma was measured after methanol precipitation of proteins (1:1 v/v) by using a dedicated HPLC system (ENO-20; Eicom, Kyoto, Japan). The method is based on the separation of nitrate by reverse-phase/ion exchange chromatography, followed by on-line reduction of nitrate to nitrite with cadmium and reduced copper. Reduced nitrite is then derivatized with the Griess reagent and the level of diazo compounds is measured by a visible detector at 540 nm. The retentions time for nitrate was 8.6 min. Parallel measurements with plasma samples showed excellent agreement between the HPLC and chemiluminescence methods for nitrate mesurements (data not shown).

Data analysis

Nitrite and nitrate values are reported as means ± SEM after subtracting the basal value. The differences in the mean values among the groups within the same experiment were analyzed by one way repeated measures ANOVA and to isolate the groups that differ from the others we used a post hoc Holm-Sidak multiple comparison procedure. Differences between groups were considered significant at $p \leqslant 0.05$. Statistics was performed using SigmaPlot 9.01 (Systat Software, Inc., Point Richmond, CA). Graphs were plotted using Origin for Windows, Version 7.0 (Microcal, Northampton, MA, USA).

Results

Saliva

The basal mean salivary nitrate levels (values taken from both experiments prior to mouth wash) were 0.72 ± 0.19 mM. The temporal changes in salivary levels of nitrate and nitrite are shown in Fig. 1.

Nitrate (Fig. 1a)

In the control experiment (nitrate load without antibacterial mouth wash), salivary nitrate reached its maximum concentration (C_{max}) already 30 min after nitrate ingestion with an increase of 12.7 ± 2.2 mM from basal levels. The levels then declined slightly and 3 h after nitrate ingestion the increase from basal levels was 5.8 ± 0.9 mM.

In the mouthwash experiment (nitrate load preceded by the antibacterial mouth wash), the maximum salivary nitrate concentration (C_{max}) was, as in the control situation, at 30-min after nitrate ingestion with an increase of 17.1 ± 2.7 mM from basal levels. After 30 min no significative variations from C_{max} were observed for the entire observation period.

Nitrite (Fig. 1b)

Basal mean salivary nitrite levels (values taken from both experiments prior to mouth wash) were 0. 26 ± 0.04 mM.

In the control experiment, salivary nitrite concentration increased by 2.11 ± 0.56 mM already 15 min after nitrate load and remained at this level along the entire observation time.

In the mouthwash experiment salivary nitrite was virtually abolished immediately after antibacterial wash and remained below basal levels during the entire observation period after nitrate ingestion.

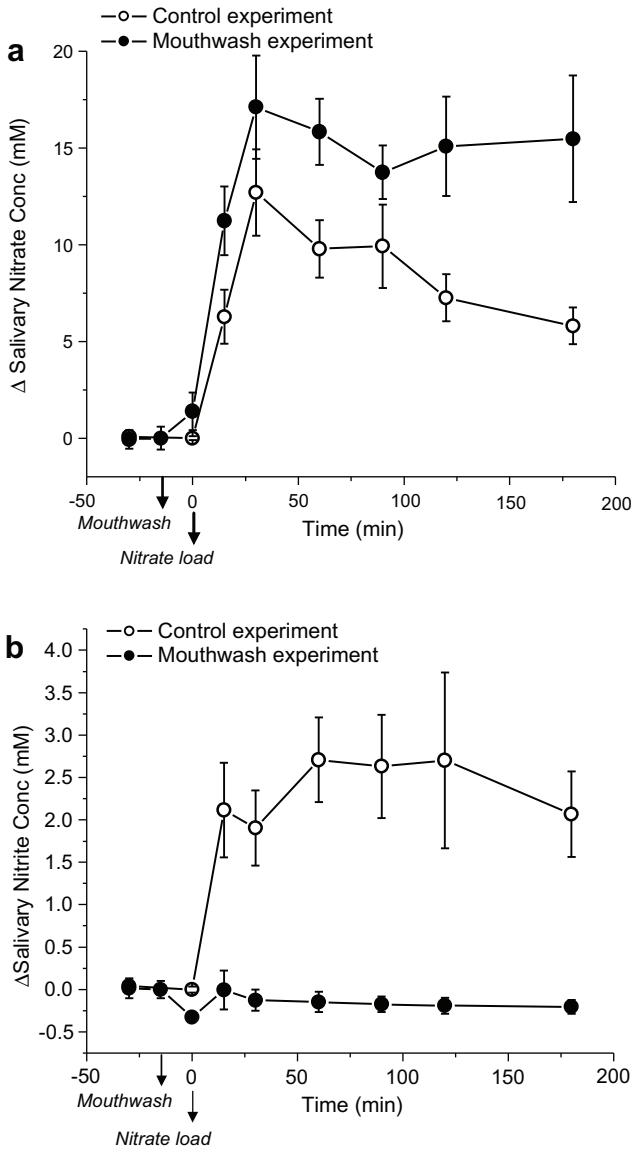

Fig. 1. Temporal changes in salivary levels of nitrate (a) and nitrite (b) after ingestion of sodium nitrate (10 mg/kg). Filled circles represent occasions when subjects rinsed the mouth with the antibacterial mouthwash 15 min prior to the nitrate load (mouthwash experiment). Open circles represent the control experiment without mouthwash. Data are expressed as means ± SEM, $n = 7$

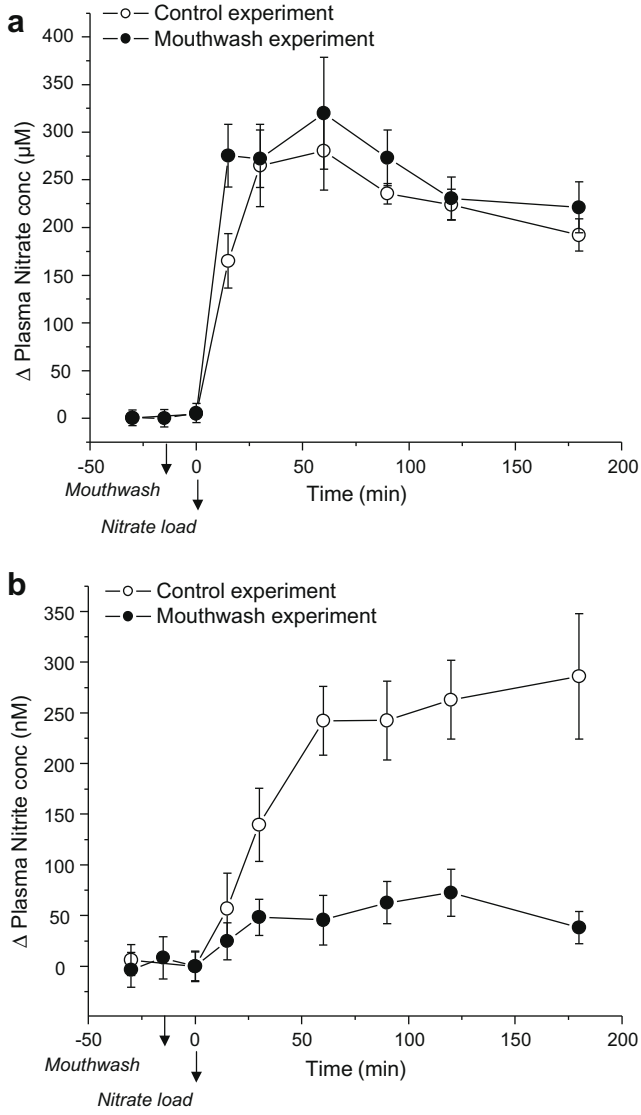

Fig. 2. Temporal changes in plasma levels of nitrate (a) and nitrite (b) after ingestion of sodium nitrate (10 mg/kg). Filled circles represent occasions when subjects rinsed the mouth with the antibacterial mouthwash 15 min prior to the nitrate load (mouthwash experiment). Open circles represent the control experiment without mouthwash. Data are expressed as means ± SEM, $n = 7$. The increase in plasma nitrate and nitrite over time was significant in both experiments ($p < 0.05$).

Plasma

Basal mean plasma nitrate levels (values taken from both experiments prior to mouth wash) were 12.52 ± 3.31 μM. The temporal changes in plasma levels of nitrate and nitrite are shown in Fig. 2.

Nitrate (Fig. 2a)

Plasma nitrate kinetic was very similar for both experiments. In the control experiment plasma nitrate increased by 265 ± 43 μM from basal levels 30 min after nitrate load reaching its C_{max} one hour after load (280 ± 41 μM above basal levels). Similarly, in the mouthwash experiment plasma nitrate increased by 272 ± 30 μM 30 min after the nitrate load, and reaching its C_{max} one hour later (320 ± 59 μM from basal levels). Three hours after the nitrate load, plasma nitrate was 192 ± 17 μM and 221 ± 27 μM in the control and mouthwash experiment, respectively.

Nitrite (Fig. 2b)

Plasma nitrite basal mean level (values taken from both experiments prior to mouth wash) was 103 ± 9 nM. In the control experiment plasma nitrite started to increase already 15 min after nitrate ingestion and continued to increase during the entire observation period. One hour after nitrate ingestion plasma nitrite had increased by 242 ± 34 nM and 3 h later the increase was 286 ± 62 nM.

In the mouthwash experiment nitrite levels remained unchanged early after the wash but then gradually increased following nitrate ingestion and reaching its C_{max} 2 h after the load with a 72 ± 23 nM increase from basal levels. However, the levels remained far below those seen in the control situation without the mouth wash.

Effects of the mouthwash on oral nitrate reducing bacteria

The bacterial counts were reduced by a mean of 80% after the mouth wash. The overall nitrate reducing capacity was virtually

abolished by the mouthwash procedure (Fig. 3). In the control situation nitrite levels were 30 μM after 2 h of incubation and these levels increased further to 950 μM at 4 h. Thus, essentially all nitrate had then been converted to nitrite. After the mouthwash, nitrite levels were virtually unchanged during the entire incubation period.

Discussion

We show here that the acute rise in plasma nitrite after a dietary nitrate load is predominantly a consequence of nitrate accumulation in saliva and reduction to nitrite by oral bacteria. Thus, when the nitrate reductase activity of oral bacteria is stopped by the action of an antibacterial mouthwash, nitrite formation in saliva is abolished and the acute rise in plasma nitrite is markedly attenuated. In a previous report we showed that the increase in plasma nitrite after nitrate ingestion was virtually absent if the test subject avoided swallowing for 1 h after the nitrate intake, but as soon as the test person started to swallow saliva, the plasma nitrite levels increased [24]. Together these two studies clearly illustrate that entero-salivary circulation of nitrate and reduction to nitrite by oral bacteria are central events in systemic nitrite formation after nitrate intake.

Interestingly, although the response was markedly attenuated, there was in fact a significant rise in plasma nitrite also after the mouthwash (Fig. 2b). This occurred despite the fact that this procedure completely abolished nitrite formation in the mouth, thereby excluding a salivary origin. At present we can see two possible explanations for this, the first being that the microflora in the stomach and small intestine contributes to some nitrate reduction. A more intriguing explanation would be that mammalian cells in the gut wall, the liver or elsewhere in fact are capable of some nitrate reduction. Although it is generally believed that nitrate cannot be metabolized by mammalian enzymes there are anecdotic reports of nitrate reductase activity in the gut wall of rats [27]. In two more recent studies, nitrate reductase activity was detected in rat liver homogenates [14,28]. However, these studies were done in vitro under strictly anaerobic conditions. In experiments using rat, mouse and human liver homogenates as well as germ-free animals in vivo, we can now confirm the existence of a mammalian nitrate reductase activity that is active also during normoxia [29].

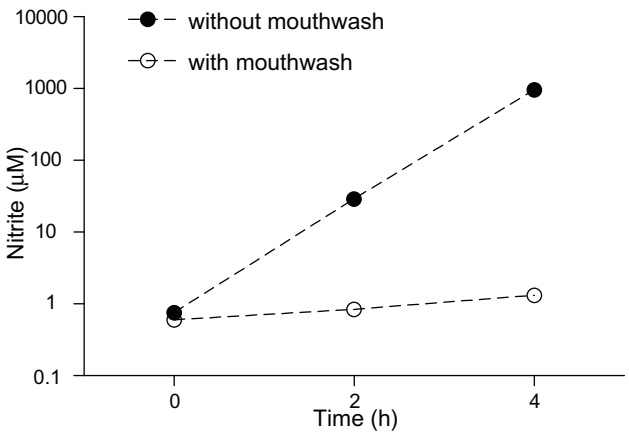

Fig. 3. The effect of an antibacterial mouthwash solution on nitrite formation by oral bacteria. A sample was taken from the tongue surface of four healthy volunteers before and after rinsing the mouth with a chlorhexidine solution. The sample was placed in growth medium containing 1 mM sodium nitrate and incubated for 2–4 h at 37 °C.

In the present study plasma nitrite increased as much as 70% even in the mouthwash experiments which is likely sufficient for biological effects. Indeed, a similar or even smaller increase is associated with reduction in blood pressure in healthy humans [21] and cardioprotective effects in a murine model of myocardial infarction [13]. However, despite this intriguing possibility, the fact still remains that the acute rise in plasma nitrite after nitrate ingestion is predominantly originating from bacterial nitrate reduction in the oral cavity.

Once salivary nitrate has been reduced to nitrite and reaches the systemic circulation, there are several pathways to generate NO. Nitrite can be converted to NO via reactions with deoxyhemoglobin, deoxymyoglobin, xanthine oxidoreductase, enzymes of the mitochondrial repiratory chain, and by acidic reduction which is enhanced by antioxidant conditions [1].

In this way dietary nitrate may increase or complement the intravascular and tissue storage pools of nitrite, which may serve to modulate important signal transduction pathways via NO formation.

The amount of nitrate ingested in the present study is equivalent to about 300 g of a nitrate-rich vegetable such as spinach, beetroot or lettuce [30]. A diet rich in vegetables is known to reduce the risk of developing hypertension and other cardiovascular diseases [31–33]. Although a direct link between nitrate and cardiovascular protection remains to be demonstrated there are several indications that nitrate could contribute to these effects trough prolonged low dose generation of NO. Indeed, recent studies show that dietary supplementation with inorganic nitrate reduces blood pressure in healthy volunteers [20] and it also inhibits platelet function [34]. Moreover, in a study by Bryan, dietary nitrate protected against myocardial ischemia–reperfusion injury in rats [35]. Webb et al. showed very recently that nitrate from a natural source (beetroot juice) acutely reduces blood pressure and platelet aggregation in healthy volunteers [36]. Interestingly, the effect was lost if the test subjects avoided swallowing after the nitrate intake, again demonstrating the importance of the entero-salivary circulation of nitrate for its bioactivation. Taken together, the present results provocatively suggest that the nitrate reductase action of oral bacteria plays a central role in the cardiovascular effects of nitrate.

It is important to note that nitrate and nitrite will accumulate in saliva also in the absence of a dietary intake. Endogenous NO is oxidized in blood to form nitrate and together with the nitrate coming from dietary sources it is taken up by the salivary glands and secreted in saliva. In this study the fasting salivary nitrite levels were as high as 0.3 mM and much of this is likely derived from endogenous NO. In this way the oral bacteria serve to "reactivate" oxidized endogenous NO so that it can become bioactive NO again. How important is then saliva as a source of systemic nitrite? The question is difficult to answer because we still do not know how much of the nitrite that survives metabolism in the gut and liver and enters the systemic circulation. Nevertheless, we allowed ourselves to make a rough calculation based on the data that are available today. A reasonable estimate of average nitrite concentration in whole blood is 200 nM, corresponding to a total of 1 μmol nitrite in the circulation. Remarkably, every three minutes, the same amount of nitrite enters the stomach, considering a daily production of saliva of 1–2 L (1 ml/min) with a concentration of 300 μM. Thus, even if only a fraction this nitrite would survive systemically, saliva would still be a major source of circulating nitrite.

In recent years there has been a widespread commercialization and use of antibacterial mouthwash solutions in treatment and prevention of dental plaque or gum disorders [37] but these products are also frequently used for less serious conditions such as oral malodor [38]. An important implication of the present study is that disturbances of the bacterial flora in the mouth could attenuate the NO-dependent biological effects of nitrate. It remains to be studied

if a sustained depression of oral nitrate reduction by the prolonged use of local or systemic antibiotics will have any pathological consequences in the gastrointestinal tract [10,11,39] or in the cardiovascular system [21,36].

In conclusion, dietary nitrate acutely increases the circulating levels of nitrite in a process that is heavily dependent on oral bacteria. Removal of oral bacteria with an antibacterial mouthwash markedly attenuates systemic nitrite formation thereby disturbing the nitrate–nitrite–NO pathway.

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MG, EÅJ, EW, and JOL designed and performed the study and wrote the report.

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