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NO Synthesis in Human Saliva

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Human saliva contains nitrate that is converted into nitrite by the activity of facultative, anaerobic bacteria of the oral cavity. Nitrite can be reduced to NO in the acidic gastric milieu; some NO may also form in the mouth at acidic pH values. In this paper, we show that bacteria (*S. salivarius*, *S. mitis* and *S. bovis*) isolated from saliva, may contribute to NO production in human saliva. NO formation by bacteria occurs at neutral pH values and may contribute to the antibacterial activity of saliva.

Keywords: Amperometric sensor; Bacteria; Nitrite; NO; Saliva; *Streptococcus salivarius*

INTRODUCTION

Human saliva is a biological fluid whose many components may vary upon time and upon physiological status; it also contains nitrites and nitrates in varying amounts.[11] Nitrates abound in vegetables, especially in lettuce and spinaches. In humans, ingested nitrate is absorbed from the duodenum and upper ileum into the blood stream and concentrated in salivary glands by an active transport system, increasing concentration up to 10 times that found in plasma.[5] About 25% of dietary nitrate excreted into saliva and most of it is converted to nitrite by microbial reductase activity of the facultative anaerobic bacteria on the surface of the tongue.[11]

NO may form in the mouth in amounts proportional to salivary nitrites and to dietary nitrates.[1] The mechanisms of oral production of NO are still unknown, although local decrease of pH values has been claimed to be responsible for this transformation.[1] In addition, NO may form in the stomach from nitrites following non-enzymatic reduction, due to the acidic environment, as demonstrated by the high values of NO (800–6000 p.p.b.) in gastric air.[2,7,10]

In this paper, we show a possible alternative route for the production of NO in human saliva through the bacterial reduction of salivary nitrite.

MATERIALS AND METHODS

Saliva Samples

Samples of saliva were collected from female, non-smoking, healthy volunteer donors aged 25–30. At the time of the collection, volunteers were fasting and had not chewed chewing-gum for at least 4 h. Saliva was collected in a sterile container and the amount of nitrites and NO were determined as described below.

Bacterial Cultures

An aliquot of saliva samples was inoculated to media containing Agar-blood or Agar chocolate (Beecton & Dickinson, Microbiology Europe, 30240 Meylan-Cedex France). Samples were incubated at 37°C for 24–48 h in 95% O_2 + 5% CO_2. Colonies were then

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identified and counted. Biochemical identification was performed with the Sceptor semi-automatic method (Microbiology Systems, Beecton & Dickinson Italia s.p.a., Via Caldera 21, 20153, Milano. Streptococci (identified as reported above) were removed, transferred into 3 ml sterile 0.9% NaCl. An aliquot (1 ml) was transferred to 4 ml of trypticase soy broth (Biolife, Italia s.r.l., Via Monza 272, 20128 Milano) and incubated in the presence or in the absence of 0.1 mM nitrite for 12 h at 25°C.

Determination of NO and Nitrites

The procedure to determine free NO and nitrites was as follows: (a) NO: an aliquot (0.2 ml) of streptococci in saline was transferred in the reaction vessel and used to determine the NO formed after the addition of different amounts of nitrite at 37°C. Produced NO was carried to the measuring equipment through a nitrogen flux. (b) Nitrites were determined at the beginning of the incubation (both in 0.9% NaCl and in the broth) and after 12 h. In addition, nitrites were determined in saliva as the release of NO. NO was liberated by adding to the reaction vessel 0.1 M cysteine + 0.1 M $CuCl_2$ and it was then carried to the measuring equipment through a nitrogen flux. These measurements were performed as previously described.[8,9] Briefly, free NO, liberated under anaerobic conditions, was carried to an amperometric gas sensor that determined the amount of NO through an electric current. The system was previously calibrated with increasing nitrite concentrations, as described.[8]

Determination of Protein

Bacterial content of suspensions was expressed as protein concentration. This was determined by the Lowry's method[6] on an aliquot of NaCl suspension.

RESULTS AND DISCUSSION

It has been reported that alimentary nitrate is absorbed and preferentially secreted in saliva in humans.[5] It has also been reported that nitrate, in the mouth can be converted to nitrite by the action of facultative anaerobic bacteria.[1,11] NO can be formed from nitrite in the stomach and in the mouth provided that pH values are low enough.[2,7,10] As far as we know, no bacterial production of NO in the mouth has been described.

In the present study we observed that the standing of saliva is accompanied by a decrease of nitrites. The same happened with exogenous nitrite added to saliva. The denitrifying activity of human saliva was variable (among donors and in the same donor at different times) and we postulated that this was due to bacterial action. Therefore, we tried to isolate from saliva the microorganisms responsible for this phenomenon. We directed our attention to *Streptococcus salivarius* that was present in all samples, although in some samples also *Streptococcus mitis* and *Streptococcus bovis* were present. Isolated bacterial cultures were used to perform the experiments described in this paper.

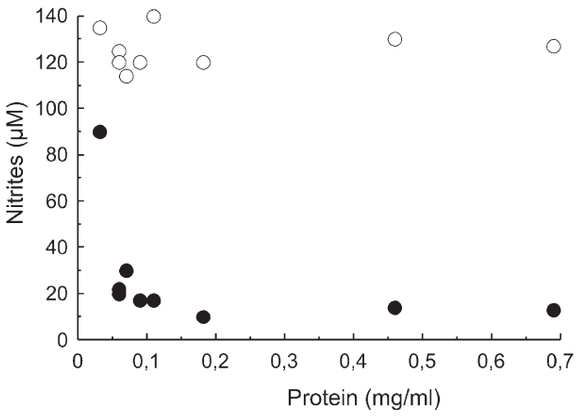

FIGURE 1 Denitrifying activity of *S. salivarius*. *S. salivarius* was isolated from human saliva and was incubated in blood-Agar for 24–48 h and the collected into 3 ml of sterile 0.9% NaCl. The amount of bacteria was expressed as protein ($mg.ml^{-1}$). An aliquot (1 ml) was then transferred into trypticase soy broth and incubated in the absence or in the presence of 0.1 mM nitrite for 12 h at 25°C. Nitrite content was determined immediately (○) after 12 h (●) on sample aliquots.

The production of NO and the consumption of nitrite was assessed by a method which permits the determination of nitrite and NO at nmolar concentrations; besides, it detects continuously the formed NO in bacterial cultures.[8]

Figure 1 shows the denitrifying activity of *S. salivarius* cultivated in the trypticase soy broth. The activity was proportional to the amount of bacteria in the suspension for low amounts of bacteria. At higher amounts the added nitrite became the limiting factor. *S. mitis* and *S. bovis* behaved similarly to *S. salivarius*.

The formation of NO was related to added nitrite (Fig. 2) for all tested *Streptococci* (*S. salivarius*, *S. bovis* and *S. mitis*). These experiments have been performed on bacteria suspended in saline. The results reported in Fig. 2 refer to 5 min of incubation at 37°C. The response versus nitrite was linear up to 1 µM nitrite, decreasing at higher nitrite concentrations, probably as the result of nitrite toxicity on bacteria.

The production of NO by salivary bacteria (*S. salivarius*, *S. mitis* and *S. bovis*) may be an important source of NO in the oral cavity. Oral bacterial flora may be responsible, through NO production for some beneficial antimicrobial effects.[3,4]

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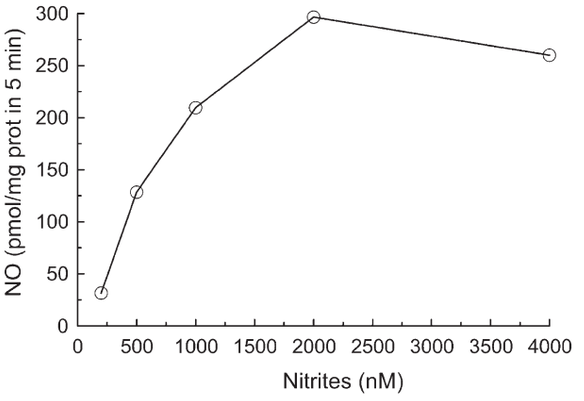

FIGURE 2 NO produced by *S. salivarius*. *S. salivarius* was isolated from human saliva and was incubated in blood-Agar for 24–48 h and the collected into 3 ml of sterile 0.9% NaCl. An aliquot (0.2 ml) was transferred to the reaction vessel connected to the amperometric sensor and nitrite added. Free NO, released under anaerobic conditions, was collected for 5 min at 37°C. Each point is the average of seven experiments. Vertical bars represent the S.E.M.

In conclusion, we report that human saliva contains bacteria able to form NO from nitrite (*S. salivarius*, *S. mitis* and *S. bovis*) at neutral pH values. As NO inhibits the growth of several bacteria and fungi, even at low concentration (<1 p.p.b.),[4] we propose that the production of NO through bacteria may play a very important role in the antimicrobial activity of human saliva.

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