Inhibition of nitrosamine formation by ascorbic acid

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ABSTRACT Nitrosation occurs under a wide variety of conditions by reaction of most types of amines with any of a large number of nitrosating species. Nitrite can be formed in vivo via bacterial reduction of nitrate and by activated macrophages and endothelial cells. The mechanism of nitrite formation by mammalian cells is via enzymatic oxidation of arginine to NO followed by oxidation to N_2O_3 and N_2O_4. Nitrosatable amines are found in many foods and some, eg, dimethylamine, are synthesized in the body. Precursors of *N*-nitroso compounds are thus almost constantly present together under favorable reaction conditions in vivo and there is, consequently, considerable interest concerning possible human health risks arising from endogenous formation of this class of compounds. Among many nitrosation inhibitors, most attention has focused on ascorbic acid, which reacts with many nitrosating agents and which is virtually nontoxic. This presentation discusses the chemistry of ascorbic acid inhibition of nitrosation reactions. *Am J Clin Nutr* 1991;53:247S–50S.

KEY WORDS Nitrite, macrophages, nitroso compounds, ascorbic acid

Introduction

Much has been written on the endogenous synthesis of nitrate, nitrite, and *N*-nitroso compounds, and the many papers dealing with this subject are a tribute to the current vigor of this area of research. This paper is not intended as a review of the field, but as the personal comment of one long-time observer and worker in the field.

The exposure of people to nitrosating agents occurs through multiple pathways ranging from NO_2 reactions in the lung to acid catalyzed nitrosation in the stomach to nitrosation mediated by mammalian cells or bacteria. The use of *N*-nitrosoproline (NPRO) as an index of endogenous nitrosation (1) has proven to be especially valuable for some of these pathways but may not be universally indicative.

What is increasingly apparent is that a nitrate-nitrite balance sheet does not give an accurate picture of the potential for *N*-nitrosation in various tissues and body compartments. Large quantities of nitrite may have only a small contribution to *N*-nitrosation if conditions are unfavorable for a reaction. Conversely small quantities may play an important role in carcinogenesis in specific tissues. This paper examines some of the issues related to endogenous nitrosation and the role that ascorbic acid may play in modulating the effects of various nitrosating agents in the body.

Chemistry of nitrosation

Sander and Buerkle (2) first demonstrated that the reaction between ingested secondary amines and nitrite could occur in vivo and could produce carcinogenic nitrosamines in laboratory animals. Many attempts to demonstrate endogenous nitrosation in humans were inconclusive, primarily because of inadequate analytical techniques (3) and lack of proper controls to ensure against the formation of artifacts during collection procedures and during analysis (4). Ohshima and Bartsch (1), however, designed an effective and relatively simple method for demonstrating the endogenous formation of *N*-nitrosoproline in humans. In a typical example of this technique, sequential oral doses of nitrate and proline are administered to a subject consuming a low nitrate diet and the resulting NPRO is measured in a 24-h urine collection. This method is effective because NPRO is not metabolized, is not carcinogenic, and can be quantitatively measured in urine.

The Ohshima and Bartsch method (1) has been used by other researchers who have confirmed that nitrosamines are formed endogenously (2, 5–7). All studies have demonstrated that urinary NPRO levels increase when nitrate and L-proline doses (typically 5 and 4 mmol, respectively) are given and that NPRO excretion returns to baseline levels of 14–30 nmol/d when a large dose (1 g) of ascorbic acid is administered along with the proline dose. A molar ratio of ascorbic acid to nitrite (2:1) is sufficient to completely inhibit NPRO formation in vitro, yet, ascorbic acid doses 20-fold larger than the estimated gastric nitrite [assuming 5% of the nitrate dose is reduced to nitrite (8)] do not eliminate urinary NPRO. Dietary sources of preformed NPRO do not account for the excess urinary NPRO. In studies where $^{15}N\text{-}NO_3^-$ was given to the subjects, ^{15}N-NPRO formation was completely inhibited by ascorbic acid, whereas baseline excretion of ^{14}N-NPRO was unaffected. Similar evidence has been obtained from studies in the ferret where background NPRO excretion is 2–4 nmol/d (9). Consequently, NPRO may be formed at some site other than the stomach, most likely one that is inaccessible to ascorbic acid, and probably via a mechanism other than that of acid-catalyzed nitrosation.

Two possible sources of endogenous nitrosating agents, in addition to dietary nitrate, are atmospheric nitrogen oxides and

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nitric oxide produced endogenously by cells. Nitric oxide is a labile species that can react rapidly with oxygen yielding NO_2, which exists in equilibrium with the potent nitrosating agents N_2O_3 and N_2O_4. Subsequent reaction of these compounds with secondary amines would yield nitrosamines; the competing reaction with water would yield nitrite and nitrate.

The reaction of atmospheric nitrogen oxides, particularly nitrogen dioxide (NO_2), with endogenous amines represents another possible nitrosation pathway. Cigarettes, eg, are one significant source of exposure; cigarette smoke contains as much as 1000 ppm nitrogen oxides (10). After administration of dimethylamine to mice followed by exposure to NO_2 at levels 20–50 times higher than normal atmospheric concentrations, Mirvish et al (11) found increased levels of *N*-nitrosodimethylamine (NDMA) in the urine. Garland et al (12) reported a positive correlation between atmospheric NO_2 levels and NDMA excretion in human subjects, although it could not be determined whether the increase was due to additional nitrosation of dimethylamine in vivo or nitrosation in the air and subsequent inhalation of NDMA. There was no correlation, however, between urinary NPRO and atmospheric NO_2 concentration. This could indicate that the urinary NDMA increase was a result of inhalation of higher levels of preformed NDMA in the air or that proline is nitrosated via a different mechanism or at a different site than dimethylamine.

Another pathway for endogenous nitrosation may be linked to the endogenous synthesis of nitrate, a mammalian process (13–15) estimated to produce ~1 mmol nitrate/d in man under normal conditions (7, 16). Immunostimulation increases endogenous nitrate synthesis; eg, a human subject on a nitrate balance study had a ninefold increase in urinary nitrate excretion while experiencing nonspecific intestinal diarrhea and fever (17). Rats treated with *Escherichia coli* Lipopolysaccharide endotoxin (LPS) had similarly augmented urinary nitrate levels. Two other immunostimulants, carrageenan and turpentine, caused smaller, although still significant, increases in urinary nitrate in rats (17).

Stuehr and Marletta (18) determined that the cell primarily responsible for immunostimulated nitrate synthesis is the macrophage. Macrophages and several established macrophage cell lines, when stimulated with LPS and/or lymphokines, produce nitrate and nitrite in vitro via intermediate production of nitric oxide (19, 20). When suitable secondary amines are added to growth media containing stimulated macrophages, the corresponding nitrosamines can be detected in the media (21). The major nitrogen source for these compounds is the terminal guanido-nitrogen of L-arginine (22). Endothelial cells (23), neutrophils (24), and brain cells (25) also produce nitric oxide from L-arginine in vitro. This is a general phenomenon occurring across species lines and in cells from many sources.

We have demonstrated that L-arginine is the precursor of endogenously synthesized nitrate in rats, ferrets, and humans (26). Following administration of an intraperitoneal dose of $^{15}N_2$-L-arginine, rats and ferrets excreted ^{15}N-NO_3^-. Nitrite is not measurable in mammalian urine because it reacts rapidly with oxyhemoglobin forming methemoglobin and nitrate (27). In the rat, LPS-induced immunostimulation was accompanied by increased excreted nitrate along with a parallel increase in incorporation of ^{15}N from $^{15}N_2$-L-arginine into nitrate. The wide range in the LPS-induced increases in excreted nitrate in rats is most likely attributable to individual variations in response to LPS. In a similar study, Wagner et al (15) treated Sprague-Dawley rats with LPS and observed a ninefold increase in urinary nitrate levels and large variations among individuals. They found that the enhancement of excreted nitrate correlated with the magnitude of fever induction. Macrophages are directly activated by LPS, although other cell types which will similarly convert L-arginine to nitric oxide may also be stimulated directly or indirectly by LPS. Endothelial cells, eg, play a role in vasodilation as a consequence of fever and could, therefore, make a contribution to the increase in excreted nitrate.

The effects of a prolonged period of exercise on excreted nitrate have been examined (28). In this study, two healthy males consumed a defined low-nitrate diet for 8 consecutive days and did not exercise nor physically exert themselves except on the fifth day, when they ran or bicycled almost continuously for 6 h. An increase in excreted nitrate was observed in the 12-h urine collection period during which the subjects exercised. This is the first time excreted nitrate has been intentionally increased in humans by a means other than dosing with nitrate. Again, elucidating the cell types involved remains for further investigation.

Bacteria are another cell type capable of mediating nitrosation and their role has been considered in hypotheses regarding the etiology of several cancers. It has been proposed, eg, that the association between a higher risk of gastric cancer and gastric achlorhydria may be due to increased levels of endogenous nitrosation via the higher populations of gastric bacteria which accompany elevated gastric pH. It was first thought that bacteria facilitated nitrosation primarily by reducing nitrate to nitrite (29, 30). Subsequent studies have shown that bacteria act directly in amine nitrosation (31–33) and that this activity is linked to nitrate reductase genes (34–36). *E. coli* grown anaerobically in the presence of nitrate form nitrosation products when nitrite and a suitable amine are added to the media (34). The reaction mechanism was further elucidated by Ji and Hollocher (37), who demonstrated that NO is produced from nitrite by *E. coli* under anaerobic conditions. Nitrosation occurs only after air is added to the system, indicating that the nitrosating agents are most likely N_2O_3 and N_2O_4.

In summary, studies in humans and animals have clearly demonstrated that endogenous nitrosation occurs intragastrically (38) and in at least one other extragastric site. Several types of cells including macrophages, endothelial cells, neutrophils, brain cells, and bacteria produce nitric oxide in vitro under certain conditions which may be duplicated in the whole animal. Nitrosation by macrophages and bacteria has been demonstrated in vitro. All of these cells, with the exception of bacteria, utilize nitrogen from L-arginine in the production of nitric oxide. We have demonstrated that L-arginine is a nitrogen source for biosynthesized nitrate in rats, ferrets, and humans. These same nitric oxide–producing cells may be responsible for this novel oxidation of L-arginine to nitrate in humans. Based on known chemistry for nitrosation reactions, it is possible that NO produced endogenously could react with oxygen and, subsequently, nitrosate secondary amines to produce carcinogenic *N*-nitrosamines in vivo.

Chemistry of ascorbic acid

Some relevant aspects of this topic have been discussed in greater detail in earlier reports but a summary is appropriate here. Ascorbic acid, under anaerobic conditions, can usually react with N_2O_3, $H_2NO_2^+$, and NOX with rates higher in each

case than the corresponding nitrosation rates for amides or dialkyl amines. Ascorbic acid can therefore generally inhibit the in vitro nitrosation of these classes of compounds. This property has been exploited to prevent nitrosamine formation in foods, and to some extent, to inhibit in vivo nitrosation.

Ascorbic acid thus appears to have potential importance as an in vivo nitrite scavenger. This potential, however, has yet to be completely realized; not only are the reactions that occur in live organisms more complex than might have been expected, but the in vitro model systems themselves are not straightforward. The equilibria, eg, involve several nitrosating species that can react with ascorbic acid to form NO, which does not nitrosate amines. Under anaerobic conditions these reactions can then exhaust the nitrosating capacity of the system. Oxygen, however, can react with NO to form N_2O_3 and N_2O_4, both of which are capable of nitrosation.

Under these circumstances, the ascorbic acid may be effectively removed from the system without significantly affecting the concentration of nitrosating species. In addition to these processes, ascorbic acid itself can also react directly with oxygen, undergoing conversion to dehydroascorbate (39, 40).

Evidence now exists that ascorbic acid is a limiting factor in nitrosation reactions in people. This has recently been demonstrated for gastrectomy patients (41) and for patients with chronic atrophic gastritis (42). The role that ascorbic acid may play in inhibiting processes of endogenous carcinogenesis has not yet been fully evaluated but future studies should pay close attention to the availability of reduced ascorbic acid in various compartments of the human body in populations at high risk for cancer.

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