Experimental Evidence for Inhibition of N-Nitroso Compound Formation as a Factor in the Negative Correlation between Vitamin C Consumption and the Incidence of Certain Cancers

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Abstract

Ascorbic acid (ASC) consumption is negatively correlated with the incidence of certain cancers. This is a review and update of the theory, which has recently been neglected, that this negative correlation is due to ASC inhibition of in vivo nitrosation. The review covers the older literature on ASC inhibition of carcinogenesis by nitrite administered with amines or amides and more recent studies on ASC inhibition of nitrosation by bacteria, nitrogen oxides, and activated macrophages; the role of oxygen in ASC inhibition of gastric nitrosation; ASC inhibition of N-nitrososoprine formation in subjects from areas with high incidences of certain cancers; dose and temporal relationships between ASC and in vivo nitrosation in humans; the role of substances other than ASC in the inhibition of nitrosation by vegetables and fruits; and the active secretion of ASC into the human stomach.

Introduction

Block recently reviewed the evidence that the intake of ASC in fresh vegetables and fruits is negatively correlated with cancer of the stomach, esophagus, oral cavity, and pancreas, and, somewhat less definitely, the cervix, rectum, breast, and lung (1, 2). The mechanisms of this action remain controversial. Block reviewed evidence that ASC is a radical scavenger and thereby spares vitamin E, protects lipid membranes from peroxidation, and protects the immune system (1, 2). Nitrite can react with secondary amines and N-substituted amides under the acidic conditions of the stomach to form N-nitrosamines and N-nitrosamides, collectively called NOC. (The reaction is called nitrosation.) Most NOC are carcinogenic. There is a firm experimental basis for the view that ASC and other components of edible plants can protect against cancer by inhibiting this in vivo nitrosation. However, this view was mentioned only briefly in Block's recent reviews (1, 2). ASC was required to reduce nitrosation in aqueous solutions contained in open flasks than in closed flasks under nitrogen (17). In the open flasks, the required amount of ASC depended on the rates of mass transfer of oxygen and nitrogen oxides between the air and water phases. ASC accelerated the nitrosation of weakly basic lipid-soluble amines in aqueous solution (18), perhaps because amine micelles accumulated lipid-soluble nitrogen oxides, which nitrosated the amines. [N₂O₃ and N₂O₄ are strong nitrosating agents in organic solvents (9).]

ASC inhibited nitrosation by bacteria at neutral pH (19). This may be important because bacterial nitrosation in the achlorhydric stomach could be involved in the etiology of gastric cancer. ASC also inhibited nitrosation of morpholine (a secondary amine) by the gas NO₂ bubbled into aqueous solutions of the amine at pH 7.4 and 13 (20). This may be significant because NO₂ can produce nitrosamines on the skin (21) and perhaps in the lungs.

Animal Tests

ASC strongly inhibited N-nitrosomorpholine formation from morpholine and nitrite in mice (22) and methylnitrosourea formation from methyurea and nitrite in the rat stomach (23). Other experiments on the in vivo inhibition by ASC of NOC formation have been reviewed (8, 9, 12). ASC reduced the acute liver toxicity which was produced by feeding dimethylamine and nitrite to rats, but did not reduce the toxicity of the preformed nitrosamine (24, 25). The authors attributed all these effects to reduction of gastric NOC formation by ASC.

Lung adenomas attributed to NOC formation were induced in mice by chronic feeding of NaNO₂ (1 g/liter drinking water) and the amines morpholine or piperazine, or the amide methyurea (2-7 g/kg diet). Feeding sodium ascorbate (23 g/kg diet) produced 89-98% inhibition of these tumors (26). ASC and the amines/amides were both given in the diet so that they would compete with each other for the nitrite. Lower ASC doses were less effective. ASC did not reduce tumor induction by the corresponding NOC. In recent tests in DBA mice using an unusually low amine/nitrite ratio, as little as 250 mg ASC/liter drinking water inhibited the induction of liver adenomas by morpholine (200 mg/liter drinking water) and NaNO₂ (4.4 g/kg diet) (27). Liver tumors were produced in rats by chronic administration of morpholine (10 g/kg diet) plus NaNO₂ (3 g/liter water). The tumor to NO by ASC, which is oxidized to dehydro-ASC and competes with the amine or amide for the nitrite. ASC is particularly effective at pH 2-4 because ascorbate anion, which predominates above pH 4.3 [the acidic dissociation constant (pKₐ) of ASC], is 230 times more effective than ASC at reducing nitrite (15, 16). Urea and other nitrite scavengers are as effective as ASC at pH 1, but are much less effective at pH 2-4 (14). ASC competes well with most amines and amides because it reacts with nitrite so rapidly that formation of the intermediate nitrosating agent, N₂O₃, is rate limiting (15). ASC is water soluble water and can only inhibit nitrosation in aqueous solution. It is now routinely added to nitrite-preserved meat products to inhibit nitrosamine formation during cooking.

In a gas-water system containing oxygen, nitrogen oxides that are formed in the gas phase by the oxidation of NO can reenter the solution and regenerate one-half the original nitrite (14). Hence, more ASC was required to reduce nitrite in aqueous solutions contained in open flasks than in closed flasks under nitrogen (17). In the open flasks, the required amount of ASC depended on the rates of mass transfer of oxygen and nitrogen oxides between the air and water phases. ASC accelerated the nitrosation of weakly basic lipid-soluble amines in aqueous solution (18), perhaps because amine micelles accumulated lipid-soluble nitrogen oxides, which nitrosated the amines.
VITAMIN C INHIBITION OF NITROSATION

Table 1. Effect on NPRO production of taking ASC before, with, or after a meal

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Nitrates</th>
<th>ASC</th>
<th>Time of ASC dose (h)</th>
<th>No. of urines</th>
<th>Mean ± SE NPRO (nmol/day)</th>
<th>Net NPRO (mean - basal)</th>
<th>Inhibition by ASC (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Proline only (basal)</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>20</td>
<td>10 ± 2</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Standard (nitrate and proline)</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>50</td>
<td>26 ± 4</td>
<td>16</td>
<td>-</td>
</tr>
<tr>
<td>ASC before meal</td>
<td>+</td>
<td>+</td>
<td>-2</td>
<td>10</td>
<td>11 ± 2</td>
<td>1</td>
<td>94</td>
</tr>
<tr>
<td>ASC with meal</td>
<td>+</td>
<td>+</td>
<td>0</td>
<td>9</td>
<td>9 ± 3</td>
<td>0</td>
<td>100</td>
</tr>
<tr>
<td>ASC after meal</td>
<td>+</td>
<td>+</td>
<td>+1</td>
<td>20</td>
<td>12 ± 2</td>
<td>2</td>
<td>87</td>
</tr>
<tr>
<td>ASC after meal</td>
<td>+</td>
<td>+</td>
<td>+2</td>
<td>20</td>
<td>22 ± 3</td>
<td>12</td>
<td>25</td>
</tr>
</tbody>
</table>

* Adult male volunteers followed a low-NPRO low-proline diet for 5 days. On days 4 and 5 they also avoided high-ASC foods, took 548 mg NaNO, (400 mg nitrate, except in the first group) in one-half a glass of apple juice and, 1 h later, ate a standard 650-calorie lunch (mainly a beef sandwich) with 500 mg l-proline in a glass of apple juice; and took 1 g ASC in one-half a glass of apple juice before, with, or after the test meal (or in the juice with proline when ASC was taken with the meal). The 24-h urines were collected and analyzed for NPRO by a standard method.

** Mean NPRO minus basal value when proline alone was taken.

The induction period rose 1.7-fold and tumor incidence fell slightly when sodium ascorbate (12 g/kg diet) was also given (28). ASC was estimated to reduce nitrosomorpholine production by 50%, not as much as in our study (26) on mice, and ASC appeared to increase the induction of forestomach tumors.

When single doses of ethylurea and NaNO, (200 and 100 mg/kg, respectively) were gavaged to pregnant hamsters on day 15 of gestation, 50% of the female offspring developed tumors of the peripheral nervous system after 27–69 weeks. These tumors were completely prevented when sodium ascorbate (200 mg/kg) was gavaged with the ethylurea and nitrite (29). Similarly, when pregnant Wistar rats were gavaged with ethylurea and NaNO, (150 and 100 mg/kg) 1 day before giving birth, 72% of the offspring developed tumors of the central and peripheral nervous system, compared to none of the offspring when the mothers also received ASC (200 mg/kg) (30). ASC also inhibited lung tumor induction in rats by aminopyrine and nitrite (31). In most of these studies, ASC was shown not to affect carcinogenesis by the preformed NOC.

ASC and a-tocopherol inhibited the carcinogenicity of many preformed carcinogens, including NOC, by 30–60% (10). This effect of ASC was probably due to its radical-scavenging property (1, 2) and was usually less effective than when nitrite and amines/amides were fed. In retrospect, some of the inhibition of carcinogenesis by nitrite and amines/amides might have been due to effects of ASC on carcinogenesis by the resulting NOC. Although ASC did not inhibit carcinogenesis by the preformed NOC, this inhibition was sometimes examined with such large doses of NOC that a small reduction of their effective dose would not have been detected.

The NPRO Test in Humans

In 1983, Ohshima and Bartsch demonstrated in vivo nitrosation in humans by showing that a fasting man who was fed 325 mg nitrate and 500 mg L-proline excreted 23 µg (160 nmol) of NPRO in his 24-h urine (32). In humans, 25% of ingested nitrate is actively secreted into the saliva and 5% of ingested nitrate is reduced to nitrite by oral bacteria and then mostly swallowed (33). This normally accounts for 90% of gastric nitrite, the normal level of which is 120 µg/liter. Rats and mice have cleaner mouths than people and do not reduce oral nitrate to nitrite. This explains why feeding sodium nitrate with piperasine did not induce tumors in mice (34). In humans, most endogenous NPRO is thought to be produced in the stomach by acid-catalyzed chemical nitrosation, absorbed into the blood, and excreted in the urine. Thus, NPRO excretion measures gastric nitrosation of proline. The test is considered safe because NPRO is noncarcinogenic and quantitatively excreted in rats. Proline is nitrosated with moderate facility relative to other amines (35), so that NPRO formation may be a reasonable indicator of the formation of carcinogenic nitrosamines. When 1 g ASC was taken with proline and nitrate, it inhibited NPRO formation by 81% (32). Feeding ASC also lowered the gastric levels in humans of total NOC and of thermal- and acid-labile compounds measured by thermal energy analysis (36).

Subjects in areas of China, Japan, and Poland with high incidences of esophageal or stomach cancer showed increased NPRO formation when they were fed proline (100 mg 3 times daily) but not nitrate, compared to nearby areas with low incidences of these cancers (12, 37–39). Urinary nitrate excretion was raised in the high-risk areas of China and Japan (but not that of Poland), indicating increased intakes of nitrate. ASC (100 mg with meals 3 times daily) strongly inhibited NPRO formation in all 3 high-risk areas. This proved that the increase in NPRO was endogenous and suggested that the ratio of ASC intake to nitrate intake (33) was depressed in the high-incidence areas.

ASC completely inhibited [15N]NPRO formation from [15N]nitrate in humans (40) and ferrets (13), but did not affect the excretion of unlabeled NPRO (26 nmol/day), which was probably due to dietary NOC or to in vivo nitrosation outside the stomach. NPRO formation was studied in the cannulated dog stomach after nitrite, proline, and ASC were administered (41). It was deduced that the ability of ASC to prevent NPRO formation was inhibited by oxygen diffusing into the stomach from the blood. Rate equations were developed that explained the observed NPRO yields (42). These equations indicated that proline was nitrosated in the human stomach for 2 h after it was fed (in agreement with results in Table 1) and that dimethylnitrosamine in human body fluids did not arise from dietary dimethylamine.

To test the effect of varying the ASC dose, volunteers ate a low-nitrate, low-ASC diet for 2 days before and during the test. On the test day, they took 325 mg nitrate in water 2–4 h after lunch and, 30 min later, took 550 mg proline and 1 of 6 doses of ASC in water (43). When 9 and 1000 mg (the lowest and highest doses) of ASC were taken, the NPRO excretion of 42 nmol/day in the absence of ASC was reduced by 15 and 44%, respectively. NPRO was significantly and linearly related to log(ASC dose). The 9-mg dose was surprisingly small compared to the recommended minimum daily allowance of 60 mg. In another study by the same group, the time of ASC administration was varied using a similar protocol and a dose of 466 mg ASC (44). When ASC was given (in separate experiments) 5 h before, with, 0.5–1.0 h after, and 2 h after the proline, the NPRO excretion of 42 nmol/day in the absence of ASC was reduced by 44, 77, 39, and 0%, respectively. These inhibitions were calculated after subtracting the "blank" NPRO of 12 nmol/day obtained when proline, but not nitrate, was fed. The results support the evidence (see below) that ASC is secreted into the stomach, because little of the ingested ASC would have remained in the stomach 5 h after it was fed.

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We confirmed some of these results under somewhat different conditions (45). Because most amines/amides (other than drugs) are taken as components of meals, volunteers ate a standard meal containing added proline 1 h after taking nitrate. When 1 g ASC was given 2 h before, with, 1 h after, or 2 h after the test meal, net NPRO excretion was inhibited by 94, 100, 87, and 25%, respectively (Table 1).

To test whether the inhibition of in vivo nitrosation by vegetables/fruits is due solely to their ASC content, Helsel et al., (46) conducted the NPRO test as before (43, 44), but instead of ASC they gave 100 ml of vegetable or fruit juice to which ASC had been added to give a total of 46 mg ASC. Green pepper, pineapple, strawberry, and carrot juices inhibited NPRO formation by 41–63%, compared to 24% when 46 mg ASC was given in 100 ml water. [ Juices are listed in decreasing order of effectiveness. I calculated these inhibitions after subtracting the blank NPRO in Ref. 44.] In a similar study, subjects from a high-stomach-cancer area of China were given proline and fresh juices of 4 Chinese fruits (including kiwi) or green tea, each containing 75 mg ASC, or orange peel powder with 3 mg ASC. NPRO yield was reduced significantly more than when 75 mg ASC was given in water (47). These results demonstrate that some vegetables/fruits contain compounds other than ASC that inhibit endogenous nitrosation. These compounds probably include polyphenols which reduce or are nitrosated by nitrite (8, 9). Hence, negative associations between vegetable/fruit consumption and cancer are probably not due solely to ASC, even if only the inhibition of NOC formation is involved.

Saliva was collected for 10 min while 20 mg proline with or without 20 mg ASC was kept in the mouth, and was analyzed for NPRO (48). Salivary NPRO levels were higher in subjects with poor oral hygiene than in those with good oral hygiene, with mean levels of 190 versus 24 μg NPRO/liter. ASC inhibited NPRO formation by >90% in both groups. Hence, chewers of betel nuts and tobacco may be exposed to nitrosamines produced in the mouth in addition to those already present in these products or formed in the stomach.

NOC can arise from NO generated during the oxidative burst of macrophages and related cells. Some of this NO is apparently converted to N₂O₃ and other nitrogen oxides, which can nitrosate amines. This may help explain the association of chronic infection, e.g., in ulcerative colitis, with the development of cancer. When cultures of murine macrophages containing added amines were activated by bacterial lipopolysaccharide, the formation of nitrate and nitrosamines was increased. Addition of ASC increased nitrate formation but inhibited that of nitrosamines (49). Rats of a strain that (unlike normal rats) cannot synthesize ASC were fed ASC-deficient or ASC-sufficient diets, and were given injections i.p. of thioproline and bacterial lipopolysaccharide. Urinary nitrite excretion increased 5 times whether or not ASC was given. Urinary N-nitrosodiethylamine (like NPRO, a measure of in vivo nitrosation) increased 8 times in the ASC-deficient group but only 1.8 times in the ASC-fed group (50). Hence, ASC can inhibit in vivo nitrosation due to NO.

Secretion of ASC into the Stomach

In humans, the normal median level of ASC plus dehydro-ASC, as measured by the 2,4-dinitrophenylhydrazine method, is 50 mg/liter gastric juice and 7 mg/liter plasma (51); see also Refs. 52 and 53. (In some of these studies, ASC without dehydro-ASC was also determined by high-performance liquid chromatography.) These results indicate an active secretion of ASC into the stomach. The estimated basal secretion into the stomach of 0.42 mg ASC/h increased to 1.65 mg/h after treatment with pentagastrin (52). The median level of ASC plus dehydro-ASC was only 3.4 mg/liter gastric juice in cases of chronic atrophic gastritis, a precursor lesion for stomach cancer (51). This low level could be due to a decreased secretion of ASC and/or to a greater instability of ASC in the high-pH gastric juice associated with the gastritis. Such an instability has been observed (52). These results suggest that high ASC levels in the stomach provide an evolutionary advantage, which could be the ability of ASC to inhibit nitrosation.

Conclusions

ASC reduced NPRO formation when it was given before proline, although not as efficiently as when it was given with proline (44); see Table 1. Presumably, ASC that was taken before proline was absorbed and later secreted into the stomach at concentrations sufficient to inhibit nitrosation. Therefore, in intervention trials of ASC, such as those performed in a high-esophageal-cancer area of China (54), ASC taken between meals should inhibit nitrosation, but not as efficiently as when it is taken with meals.

The striking results in the animal tests suggest that some of the negative correlations between ASC intake and cancer are due to ASC inhibition of in vivo N-nitroso compound formation, rather than to a direct inhibition of carcinogenesis. This may apply particularly to cancer of the stomach, esophagus, and nasopharynx, for which there is strong evidence for a role of in vivo nitrosation. Although the smaller direct effect of ASC on carcinogenesis has a firm experimental basis (1, 2, 10), we should also consider whether in vivo N-nitroso compound formation plays a role in the etiology of the remaining cancers which are negatively correlated with ASC consumption, including cancer of the oral cavity, pancreas, cervix, rectum, breast, and lung (1, 2).

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