Quantitative Estimation of Endogenous Nitrosation in Humans by Monitoring \(N\)-Nitrosoproline Excreted in the Urine\(^1\)

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ABSTRACT

Endogenous formation of \(N\)-nitrosoproline (NPRO) was demonstrated by monitoring its excretion in the urine of a male volunteer who had ingested vegetable juice, as a source of nitrate, and proline. The resulting NPRO was analyzed after derivatization by combined gas-liquid chromatography thermal energy analysis. The amount of total NPRO excreted in the urine was found to be proportional to the proline dose and increased exponentially with the nitrate dose ingested. Neither nitrate nor proline, when taken alone, led to a detectable increase in NPRO in urine. The amounts of NPRO formed (as estimated from the amounts excreted within 24 hr) after dosing 325 mg nitrate (\(\text{NO}_3^-\)) followed by 500 mg proline, ranged from 16.6 to 30.0 (mean, 23.3) \(\mu\)g per person. The simultaneous intake of ascorbic acid or \(\alpha\)-tocopherol inhibited nitrosation of proline in vivo. Monitoring of NPRO or other \(N\)-nitroso compounds excreted in the urine thus appears to be a suitable procedure for estimating daily human exposure to endogenously formed \(N\)-nitroso compounds.

INTRODUCTION

Human exposure to carcinogenic \(N\)-nitroso compounds may result from ingestion or inhalation of preformed compounds in the environment or from nitrosation of amino precursors in the body. Although very few data exist to evaluate human exposure in quantitative terms, it has been suggested that the endogenous formation of \(N\)-nitroso compounds from ingested precursors is probably the largest single source of exposure to these compounds for the general population (6).

The formation of \(N\)-nitroso compounds in experimental animals in vivo has been demonstrated by identifying nitrosated products in the stomach contents (4, 16, 23) or in the whole animal (22) after feeding relatively high doses of precursors. Formation in vivo has also been demonstrated in human subjects who ingested diphenylamine and nitrate, by detection of \(N\)-nitrosodiphenylamine in their stomachs (24). Fine et al. (9) reported the formation of volatile nitrosamines in vivo as measured in the blood of a human subject who ingested a lunch consisting of spinach, cooked bacon, and beer. Although the endogenous formation of \(N\)-nitroso compounds from precursor amines and nitrosating agents has thus been proven experimentally, the extent to which nitrosation reactions occur in humans ingesting typical levels of nitrate, nitrite, and nitrosatable compounds has not yet been determined.

Recently, on the basis of animal experiments, we concluded that the monitoring of urinary levels of \(N\)-nitrosamine acids such as NPRO\(^3\) and \(N\)-nitrosodihydroxyproline could be a useful procedure for the quantitative estimation of nitrosation in vivo. Thus, more than 80% of a dose of NPRO administered p.o. to rats was excreted unchanged into urine within 24 hr; in a typical experiment, the simultaneous administration of 10 \(\mu\)mol of each proline and nitrite to rats resulted in a significant increase in urinary excretion of NPRO (24 nmol/24 hr/rat) (20). During the course of this study, Chu and Magee (5) reported independently that negligible \(^1^4\)CO\(_2\) production and DNA alkylation occurred in rats given \([^\text{\(\text{\(^{14}\text{C}\)}}\text{NPRO}\)]\); previously, it has been reported that NPRO is recovered almost quantitatively in the urine (8). NPRO has been reported to be noncarcinogenic and nonmutagenic (10, 17), perhaps because it appears that NPRO is not metabolized in vivo.

In the absence of adverse biological effects of NPRO, we conducted a number of kinetic studies on formation of NPRO in vivo in a human volunteer, who ingested vegetable juice (as a source of nitrate) and proline, and monitored urinary excretion of NPRO. Inhibitory effects of ascorbic acid and \(\alpha\)-tocopherol on formation of NPRO in vivo were also studied.

MATERIALS AND METHODS

Chemicals. NPRO and NPIC were synthesized according to the method of Liisinsky et al. (15); the purity and identity of these compounds were ascertained by thin-layer chromatography, gas-liquid chromatography, and mass spectrometry. All other reagents (purchased from Merck, Darmstadt, West Germany) were of analytical grade and were used without further purification. Diazomethane was prepared from \(N\)-methyl-\(N\)-nitroso-\(p\)-toluenesulfonamide.

Red beetroot juice was purchased in a local natural-food store in Lyon; 5 bottles were analyzed for nitrite and nitrate by Dr. C. L. Walters, British Food Manufacturing Industries Research Association, according to a published procedure (28), and they contained an average of 1300 ± 30 (±S.D.) mg nitrate (\(\text{NO}_3^-\)) per liter. \(L\)-Proline, beetroot juice, and other reagents used in this study contained no detectable levels of preformed NPRO or nitrite.

Ingestion of Beetroot Juice and Proline and Collection of Urine Samples. On the morning of the experimental day, the volunteer (70 kg body weight) consumed beetroot juice and then, 30 min later, 10 ml of an aqueous solution of proline; the amounts of nitrate (0 to 325 mg) and proline (0 to 500 mg) varied according to the different protocols (see "Results"). Ascorbic acid was dissolved in water, and \(\alpha\)-tocopherol was placed inside a small wafer capsule; when they were taken.

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\(^1\) In compliance with regulations for experimentation on human subjects, the Senior Scientific Committee of this Institute has judged that no carcinogenic risk to the human volunteer (a coauthor) was involved, when conducting the experiments described in this manuscript, because: (a) the experiments involved only an increased intake of commonly occurring food ingredients; (b) of the absence of carcinogenic and mutagenic effects of NPRO; (c) of the natural occurrence of low levels of NPRO in the urine of humans. The data were in part presented at the Vitamin C Symposium held in Warwick, United Kingdom, in April 1981 (20).

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\(^3\) The abbreviations used are: NPRO, \(N\)-nitrosoproline; NPIC, \(N\)-nitrosopiperidine; AS solution, 20% ammonium sulfamate solution in 3.6 N \(\text{H}_2\text{SO}_4\).
they were consumed with the proline solution. No foods or beverages
were taken for 2.5 hr after the ingestion of proline; water was taken ad
libitum. Meals, other foods, and beverages taken on the experimental
day were not standardized, but cured meat products and beer, which
were presumed to contain preformed NPRO, were avoided. No cigarettes
were smoked during the course of the experiment.

Urine samples were collected after the ingestion of nitrate and
proline during the time periods indicated in Chart 1. To prevent artificial
formation of NPRO during the collection and storage of the sample was negligible;
no increase in the amounts of NPRO was observed when the urine
sample, supplemented with 5 ppm nitrite and 100 ppm proline, was
stored as described above.

Analysis of NPRO. A 30-ml sample of urine, to which 600 ng NPIC
had been added as an internal standard, was extracted 3 times with 50
ml ethyl acetate in the presence of 10 g NaCl and 3 ml AS solution.
The combined ethyl acetate extracts were dried over anhydrous
Na2SO4 and concentrated to dryness by a rotary evaporator. The
residue was dissolved in 3 ml ethyl ether and derivatized with diazo-
methane for 5 min (14). The derivatized ethereal extract was concen-
trated to 0.5 ml under a stream of nitrogen; a 10-fil aliquot was used to
determine the amount of NPRO methyl ester in a gas-liquid chromatograph
equipped with a thermal energy analyzer (TEA 502; Thermo Electron). An au-
thentic sample of the methyl ester of NPRO was used as the reference
compound for quantitation.

A Tracor 550 gas chromatograph was equipped with a 3-mm (inside
diameter) x 2-m glass column packed with 10% Carbowax 20 M on
Chromosorb W, 80 to 100 mesh. The carrier gas, argon, was passed
at a flow rate of 20 ml/min. The temperatures of the column oven and
injection port were 190 and 220°, respectively.

Under these conditions, the peaks of the methyl esters of NPRO and
NPIC were well resolved (retention times, 11.2 and 9.3 min, respect-
ively), and no interfering peaks appeared on the chromatogram. When
urine samples were supplemented with either 10 or 20 ppb NPRO, the
average recovery was 85%; the minimum detectable level of NPRO
was 0.5 μg/liter.

RESULTS

Urinary Excretion of NPRO in a Human after Ingestion of
Nitrate and Proline. The typical pattern of excretion of NPRO
in the urine of the human volunteer who had ingested 200 ml
beetroot juice, containing 260 mg nitrate (NO3−), followed by
500 mg proline is shown in Chart 1. Since the urine analyzed
before intake of precursors contained only trace amounts of
NPRO (<3 μg/liter), ingestion of nitrate and proline resulted in
a dramatic increase in the level of NPRO. Under these condi-
tions, NPRO appeared to be excreted into the urine as such
(e.g., no formation of β-α-glucopyranosiduronic acid conjugate
occurred); incubation of the urine for 1 hr with 2 N HCl or 2 N
NaOH at 100° or with β-glucuronidase at 37° for 1 hr did not
increase the amount of NPRO recovered.

Urinary excretion of NPRO increased rapidly after ingestion of
precursors, reached a maximum after 6 to 8 hr, and
decreased over the following 12 hr (Chart 1). After 24 hr, the
concentration of NPRO had decreased to the level observed
before ingestion of precursors. When either beetroot juice or
proline was taken alone, no increase in urinary NPRO was
observed (Table 1). Intake of ascorbic acid (2 g) simul-
aneously with nitrate and proline reduced the amount of NPRO excreted
into the urine to the level observed when a low-nitrate diet was
taken, implying that the formation of NPRO in vivo was inhibited
by ascorbic acid (Chart 1; Table 1).

In order to determine the reproducibility and the rate of
elimination of NPRO from the human body, urine samples were
analyzed for NPRO after ingestion of variable amounts of
nitrate. In 5 experiments, 65, 130, 195, 260, and 325 mg
nitrate were ingested together with 500 mg proline. In each
case, a similar pattern of NPRO excretion was obtained, and
the amount of NPRO returned to the background level within
24 hr, indicating that NPRO was totally eliminated into the urine
within that period. Therefore, the NPRO analyzed in urine
collected over 24 hr after dosing was an indicator of daily
nitrosation in vivo.

Effects of Doses of Nitrate and Proline on Formation of
NPRO in a Human In Vivo. The effect of the dose of nitrate on
NPRO formation was examined by analyzing urine for NPRO
content 24 hr after ingestion of different volumes of beetroot
juice containing 0 to 325 mg nitrate, together with 500 mg
proline (Chart 2). The formation of NPRO in vivo was found to
be strongly dependent on nitrate intake; when less than 195
mg nitrate were ingested, a marginal or in most cases no
increase in urinary NPRO was observed, as compared with that
in a control experiment (no nitrate intake). However, when 260
and 325 mg nitrate were consumed, 5 and 15 times more
NPRO, respectively, were excreted into the urine than in the

Table 1
Effects of ascorbic acid and α-tocopherol on formation of NPRO in vivo in a
human

<table>
<thead>
<tr>
<th>Material ingested</th>
<th>μg of NPRO excreted in urine over 24 hr</th>
<th>α</th>
<th>b</th>
</tr>
</thead>
<tbody>
<tr>
<td>Beet juice (250 ml containing 375 mg nitrate)</td>
<td>1.69; 3.44; 3.86 (2.93)a</td>
<td>8.17; 7.45; 6.16 (7.26)</td>
<td></td>
</tr>
<tr>
<td>Proline (250 mg)</td>
<td>1.05; 1.40; 3.42 (1.96)</td>
<td>7.02; 8.39; 7.92 (8.13)</td>
<td></td>
</tr>
<tr>
<td>Beet juice (250 ml) + proline (250 mg)</td>
<td>14.0; 14.8; 15.9 (14.9)</td>
<td>14.0; 14.8; 15.9 (14.9)</td>
<td></td>
</tr>
<tr>
<td>Beet juice (250 ml) + proline (250 mg) + ascorbic acid (1 g)</td>
<td>2.39; 2.96; 3.13 (2.83)</td>
<td>2.39; 2.96; 3.13 (2.83)</td>
<td></td>
</tr>
<tr>
<td>Beet juice (250 ml) + proline (250 mg) + α-tocopherol (500 mg)</td>
<td>8.17; 7.45; 6.16 (7.26)</td>
<td>8.17; 7.45; 6.16 (7.26)</td>
<td></td>
</tr>
</tbody>
</table>

a Each value refers to an individual experiment.
b Numbers in parentheses, arithmetic means.

*Estimation of Endogenous Nitrosation in Humans*

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Inhibitory Effects of Ascorbic Acid and α-Tocopherol on Formation of NPRO in a Human in Vivo. The effect of these inhibitors was assessed quantitatively in another experiment (Table 1). The amount of NPRO excreted in the urine of a human subject who had ingested 325 mg nitrate and 250 mg proline ranged from 14.0 to 15.9 μg/24 hr, with a mean value of 14.9 μg; the excretion rate was 5 to 7.5 times higher than those in control experiments in which either nitrate or proline alone was ingested. Intake of ascorbic acid (1 g) simultaneously with the precursors was found to inhibit the nitrosation of proline in vivo, and the detected amounts of NPRO were the same as those in controls. α-Tocopherol (500 mg) was less effective and inhibited nitrosation in vivo by only about 50%.

DISCUSSION

Endogenous N-nitrosation was demonstrated in a male volunteer who ingested vegetable juice as a source of nitrate together with proline by quantitative monitoring of NPRO excreted into the urine. The rationale for use of this method is based on recent findings in this laboratory and by Chu and Magee (5): (a) NPRO, when administered p.o. to rats, was recovered in the urine unchanged within 24 hr at a yield of >80% (5, 8, 20); (b) simultaneous administration of proline and nitrite to rats resulted in a marked increase in urinary excretion of NPRO (20); (c) after gavage with [14C]NPRO, the [14C]CO₂ production and DNA alkylation were negligible, and [14C]NPRO was excreted quantitatively into the urine (5). NPRO has been reported to be noncarcinogenic and nonmutagenic (10, 17); this may be related to the fact that it appears to be neither absorbed nor metabolized in vivo and is excreted directly into the urine. Thus, a comparison of the levels of NPRO in the urine with those consumed may provide a quantitative estimate of nitrosation in vivo.

In our experiments, a significant increase in urinary excretion of NPRO occurred only after ingestion of nitrate and proline. The vegetable juice and proline used contained no detectable levels of preformed NPRO or nitrite. Ingestion of either of the precursors alone did not increase urinary excretion of NPRO; therefore, the NPRO excreted in the urine is most probably formed in the human body through the reaction of proline with nitrite, the latter being derived from nitrate (11, 25, 27). The simultaneous intake of ascorbic acid or α-tocopherol, both well-known inhibitors of nitrosation reactions in vitro (19, 21) or in animal experiments (12, 13), was found to suppress the increase in urinary NPRO excretion, strongly suggesting that nitrosation of proline in vivo in a human was also inhibited by these compounds.

NPRO formed in vivo was rapidly eliminated in the urine and may be cleared from the body within 24 hr (Chart 1). The rate of metabolism of NPRO ingested in food or formed in vivo has not yet been studied in humans; however, analyses of feces for NPRO and its decarboxylated product, N-nitrosopyrrolidine, and of urine for N-nitrosopyrrolidine (and other volatile nitrosamines, e.g., N-nitrosodimethylamine) after intake of nitrate and proline provided no evidence that the excretion rate of these compounds increased (data not shown). It therefore seems likely that in humans NPRO is excreted almost quantitatively into the urine, as has been demonstrated in rats (5, 8, 20). Thus, the monitoring of NPRO in the urine over 24 hr may be a new, suitable method for estimating daily endogenous nitrosation in humans.

Our experiments show that, with the highest doses of nitrate (325 mg) and of proline (500 mg), formation of NPRO ranged from 16.6 to 30.0 (mean, 23.3) μg/24 hr/person, corresponding to 0.002 and 0.004% of the ingested amounts of nitrate and proline, respectively. The formation of NPRO in vivo appeared to be proportional to the dose of proline and increased exponentially with the dose of nitrate ingested. Although we
did not determine the levels of nitrate and nitrite in the saliva after ingestion of the vegetable juice, the extent of nitrite formation in saliva in humans has been shown to be a function of the amount of nitrate ingested (25, 27). Kinetic studies in vitro have revealed that the rate of nitrosation of proline, as well as that of secondary amines, is proportional to proline concentration and to the square of nitrite concentration (18). It is therefore not surprising that nitrosation in vivo is markedly influenced by the amounts of precursors ingested, especially of nitrate which can be converted to nitrite in the oral cavity.

A possible mechanism for NPRO formation in humans may be as follows. Nitrate ingested in vegetable juice is resecreted in saliva, starting about 30 min after its ingestion and reaching a maximum after 1 to 2 hr (11, 25, 27). The resecreted nitrate is reduced to nitrite by the oral microflora. Proline ingested 30 min after intake of vegetable juice probably reacts with salivary nitrite to give NPRO under the acidic conditions prevailing in the stomach. When proline is taken simultaneously with ascorbic acid or α-tocopherol, the latter compounds compete with proline for nitrite, resulting in inhibition of NPRO formation in vivo.

The experiments reported here demonstrate that N-nitroso compounds can be formed endogenously in humans after ingestion of nitrate and precursor amines. Some epidemiological studies based on the geographical distribution of stomach cancer have associated an elevated exposure to nitrate with an increased incidence of this cancer (1, 26, 29); however, biological evidence of endogenous formation of N-nitroso compounds is at present insufficient, and the hypothesized conversion of nitrate to nitrite and subsequent nitrosation in vivo have not yet been unequivocally demonstrated (6). The method described for monitoring NPRO (or other N-nitroso compounds excreted in the urine) is simple and highly sensitive and may provide a valuable index for the extent of nitrosation in vivo and consequently of human exposure to endogenously formed N-nitroso compounds. It is therefore suitable for identifying high-risk groups or individuals in whom endogenous N-nitroso compounds may lead to an increased incidence of cancer at specific sites such as stomach, colon, and bladder. For example, a significant risk of the stomach cancer has been observed in patients with chronic atrophic gastritis or pernicious anemia or in patients who have undergone gastric surgery. The gastric achlorhydria observed in the stomach of these patients has been associated with an increased risk of this cancer (2, 3, 7). Following development of a model diet consisting of a nitrate source (vegetable juice) and a source for proline, the extent of daily in vivo nitrosation in such high-risk individuals is now being estimated by monitoring NPRO excreted in the urine. The method is currently also being used in this laboratory to study the endogenous formation of N-nitroso compounds in experimental animals, as related to various physiological and environmental conditions, e.g., stomach pH, catalysts and inhibitors in the diet, and bacterial flora.

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References


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