Nitrite is an alternative source of NO in vivo

Koichiro Tsuchiya, Yasuhisa Kanematsu, Masanori Yoshizumi, Hideki Ohnishi, Kazuyoshi Kirima, Yuki Izawa, Michiyo Shikishima, Tatsuo Ishida, Shoji Kondo, Yoshiharu Takiguchi, and Toshiaki Tamaki

1Department of Pharmacology and 2Department of Pediatrics, The University of Tokushima School of Medicine, and 3Department of Clinical Pharmacology and 4Department of Pharmacokinetics and Biopharmaceutics, Institute of Health Biosciences, The University of Tokushima, Tokushima, Japan

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Nitrite is an alternative source of NO in vivo. Am J Physiol Heart Circ Physiol 288: H2163–H2170, 2005. First published December 30, 2004; doi:10.1152/ajpheart.00525.2004.—In this study, we investigated whether orally administered nitrite is a source of NO in vivo and whether nitrite attenuates hypertension in a dose-dependent manner. We utilized a stable isotope of [15NO2]nitrite (15NO2) as a source of nitrite to distinguish between endogenous nitrite and that exogenously administered and measured hemoglobin (Hb)-NO as an index of circulating NO in whole blood using electron paramagnetic resonance (EPR) spectroscopy. When 1 mg/kg Na15NO2 was orally administered to rats, an apparent EPR signal derived from Hb15NO (A2 = 23.4 gauss) appeared in the blood. The peak blood HbNO concentration occurred at the first measurement after intake (5 min) for treatment with 1 and 3 mg/kg (HbNO: 4.93 ± 0.52 and 10.58 ± 0.40 μM, respectively) and at 15 min with 10 mg/kg (HbNO: 38.27 ± 9.23 μM). In addition, coadministration of nitrite (100 mg/l drinking water) with Nω-nitro-l-arginine methyl ester (l-NAME; 1 g/l) for 3 wk significantly attenuated the l-NAME-induced hypertension (149 ± 10 mmHg) compared with l-NAME alone (170 ± 13 mmHg). Furthermore, this phenomenon was associated with an increase in circulating HbNO. Our findings clearly indicate that orally ingested nitrite can be an alternative to l-arginine as a source of NO in vivo and may explain, in at least part, the mechanism of the nitrite/nitrate-rich Dietary Approaches to Stop Hypertension diet-induced hypotensive effects.

nitric oxide; hypertension; electron paramagnetic resonance

NITRIC OXIDE (NO) is a free radical molecule that has numerous roles in various physiological functions, such as regulation of the cardiovascular, immune, and nervous systems. An imbalance of NO is implicated in many diseases, such as hypertension, arteriosclerosis, septic and hemorrhagic shock, ischemiareperfusion injury, diabetes, and neurodegenerative disorders (28, 65, 98). It has been believed that NO is synthesized from l-arginine, NADPH, tetrahydrobiopterin (BH4), and molecular oxygen catalyzed by NO synthases (NOSs). However, an alternative pathway for NO production in biological systems has been described in the last decade. In addition to NOSs, xanthine oxidoreductase can be an alternative enzymatic pathway for NO production through reduction of the therapeutic organic nitrate nitrateglycerin as well as inorganic nitrate and nitrite under hypoxic conditions in the presence of NADH (61). Besides enzymatic production of NO, nonenzymatic nitrite-derived mechanisms for generation of NO reported include the following reactions (20):

\[ \text{NO}_2^- + \text{H}^+ \rightleftharpoons \text{HNO}_2 \] (1)

\[ \text{HNO}_3 + \text{H}^+ \rightleftharpoons \text{H}_2\text{NO}_2^- + \text{NO}^+ + \text{H}_2\text{O} \] (2)

\[ \text{H}_2\text{NO}_2^- + \text{NO}_2^- \rightleftharpoons \text{N}_2\text{O}_3^- + \text{H}_2\text{O} \] (3)

\[ \text{N}_2\text{O}_3^- \rightleftharpoons \text{NO} + \text{NO}_2^- \] (4)

The low pKₐ value (3.3) (86) of reaction 1 makes it unlikely that nonenzymatic NO formation from nitrite (NO2) occurs at physiological pH of the blood (91). Hence, researchers reported that NO2-derived NO formation is found in acidic environments such as the stomach (57, 59), oral cavity (18), acidic urine (56), and ischemic rat heart (100).

It was reported that blood pressure was lowered in a dose-dependent manner by oral nitrite uptake in spontaneously hypertensive rats (4, 30). Furthermore, nitrite treatment decreased mean arterial blood pressure in normotensive rats (94). However, it was unclear whether the orally ingested nitrite-derived NO was responsible for this blood pressure-lowering effect.

In the circulation, NO exists as a relatively stable hemoglobin (Hb)-NO adduct (HbNO) (71). Because NO can be sequestered by Hb in its α-hemes (99) until its eventual release to maintain physiological concentrations (−10⁻⁷ M < [NO] < −10⁻⁹ M) in the blood, the amount of HbNO may reflect the blood NO concentration. In addition, it has been reported that the concentration of HbNO is relatively high [0.8 μM in rats (35) and 0.3–3 μM in humans (24, 44)] compared with the concentration of NO. HbNO has a characteristic electron paramagnetic resonance (EPR) spectrum, and we succeeded in measuring the blood HbNO level as an index of NO by using the EPR HbNO signal subtraction method in a previous study (38). Therefore, in the present study, we examined the formation of HbNO after nitrite treatment to clarify whether nitrite acts as a systemic source of NO.

MATERIALS AND METHODS

Materials. Nω-nitro-l-arginine methyl ester (l-NAME) was purchased from Nacalai Tesque (Kyoto, Japan). Sodium nitrite and other chemicals were obtained from Wako Pure Chemical Industries (Tokyo, Japan). The stable sodium nitrite isotope (Na15NO2) was obtained from Cambridge Isotope Laboratories (Andover, MA). NO gas was obtained commercially from Sumitomo Seika Chemicals (Osaka, Japan).

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Address for reprint requests and other correspondence: T. Tamaki, Dept. of Pharmacology, The Univ. of Tokushima School of Medicine, Tokushima 770-8503, Japan (E-mail: tamaki@basic.med.tokushima-u.ac.jp).
Japan), and higher oxides such as NO2 and N2O2 (NO2) were removed by passing NO gas through a trap containing 1 M KOH. A NO-saturated aqueous solution was prepared by bubbling NO gas for 15 min through water that had been previously deoxygenated by bubbling with purified argon gas for 15 min (45).

Animals. Male Sprague-Dawley rats (12 wk old, weighing 350–400 g) were obtained from Japan SLC (Shizuoka, Japan) and kept in plastic cages at a controlled temperature (25°C) under controlled lighting conditions (12:12-h light-dark cycle). The animals were fed a commercial diet (nitrite concentration was 5.2 mg/kg or less) and had access to tap water ad libitum until the day of the experiments. All animal care and treatments were conducted in accordance with the guidelines of the animal care committee of the University of Tokushima. All experiments were performed according to the Guide for the Care and Use of Laboratory Animals (NIH Pub. No. 85-23, Revised 1985).

Preparation of HbNO for calibration curves. Rats were anesthetized with pentobarbital sodium (40 mg/kg ip). Venous blood was collected from the vena cava and aliquots (1 ml) were mixed with various amounts of the NO-saturated solution at 0°C. The samples were immediately drawn up into disposable 1-ml plastic syringes and then immersed and stored in liquid nitrogen until EPR measurement.

Preparation of NOS-derived, NO-depleted whole blood. To prepare the NOS-derived, NO-depleted blood (38), 12 rats were administered tap water containing l-NAME (1 g/l) for 1 wk. The rats were euthanized, and venous blood was taken from the vena cava with a 1-ml plastic syringe and stored in liquid nitrogen until use.

Acute administration of nitrite. At the age of 13 wk, the rats were administered distilled water for 1 wk (Fig. 1). On the day after the completion of treatment, sodium nitrite (1, 3, or 10 mg/kg) was administered by oral gavage (1 ml/kg). The rats were euthanized, and venous blood was obtained from the vena cava at 5, 15, 30, or 60 min after nitrite administration (see Preparation of NOS-derived, NO-depleted whole blood). We used sodium nitrite instead of potassium nitrite to prevent possible effects of potassium on the cardiovascular system and blood pressure (73, 83).

1. Acute administration of nitrite

Day 0

Distilled water

Nitrile
1,3,10 mg/kg

Day 1

Day 2

Day 3

Day 4

Day 5

Day 6

Day 7

Day 8

2. Chronic administration of nitrite

Day 0

Group 1

Group 2

Group 3

Group 4

Nitrile (100 mg/liter)

L-NAME

L-NAME

L-NAME

L-NAME

Nitrite (1000 mg/liter)

Distilled water

Fig. 1. Experimental design. Down arrows indicate blood drawing points. l-NAME, Nω-nitro-l-arginine methyl ester.

Chronic administration of nitrite with simultaneous NOS inhibition. At 13 wk of age, the rats were divided into four groups (group 1, l-NAME; group 2, l-NAME + low-dose nitrite; group 3, l-NAME + high-dose nitrite; and group 4, control; Fig. 1). Each group had seven rats, and all animals except controls received l-NAME (1 g/l; days 0 to 21) without or with sodium nitrite (100 mg/l, group 2; 1,000 mg/l, group 3; days 0 to 23). Both l-NAME and sodium nitrite were dissolved in tap water. On the basis of the drug solution intake, the effective daily consumption of l-NAME was estimated to be ~65 mg·kg⁻¹·day⁻¹. To evaluate the endothelial function in maintenance of blood NO levels, we stopped l-NAME treatment 2 days before the experiments were conducted. Systolic blood pressure (SBP) was measured every week via the tail-cuff method using a BP-98A sphygmomanometer (Softron, Tokyo, Japan). On the 24th day, rats were anesthetized with pentobarbital sodium, and venous blood was collected (see Preparation of NOS-derived, NO-depleted whole blood) for HbNO measurement.

EPR measurement. All EPR measurements were carried out according to a previous report from our laboratory (38). Briefly, the frozen blood was directly transferred to a liquid nitrogen-filled quartz EPR finger dewar, which was placed in the cavity of the EPR measurement device. A JE Instruments ESC spectrometer (JEOL, Tokyo, Japan) with an ES-UCX2 cavity (JEOL) was utilized to collect EPR spectra at the X band (9.5 GHz). Each sample was measured four times, and values were normalized using EPRIT 432 software (JEOL) to improve the signal-to-noise ratio. Typical experimental conditions were as follows: microwave power, 20 mW; modulation amplitude, 6.3 gauss; time constant, 1 s; scan time, 60 min; scan range, 3,200 ± 250 gauss; and microwave frequency, 9,045 GHz. Spectra were stored on an IBM personal computer for analysis.

The HbNO signal was obtained by subtracting the EPR spectrum of HbNO-depleted whole blood from that of each sample as described previously (38). The relative HbNO concentration was obtained from the peak-to-peak amplitude of the first signal of the triplet signal of the Z factor of HbNO at g = 2.01, and the absolute HbNO concentration was calculated by double integration of the region of the HbNO signal (35) and then compared with that of NO-supplemented blood samples.

Statistical analysis. All data are expressed as means ± SD. Data were analyzed using two-way ANOVA, followed by the Tukey test for comparisons between groups. P < 0.05 was accepted as statistically significant.

RESULTS

Bolus nitrite treatment and HbNO formation. In the control animal, a small Hb15NO (A2 = 17.5 gauss, gZ = 2.01)-derived EPR signal was observed due to the basal HbNO in the blood (36) (Fig. 2A). When Na14NO2 was orally administered to rats (1 mg/kg), triplet EPR signals due to Hb14NO2 in the blood were increased (Fig. 2B). To further clarify the hypothesis that exogenously administered nitrite is responsible for the augmented HbNO signal, we adopted a stable isotope of sodium nitrite (Na15NO2) instead of naturally abundant (>99%) sodium nitrite (Na14NO2). The shape of the EPR signal of Hb15NO is different from that of Hb14NO, which enabled us to distinguish them from each other. As shown in Fig. 2C, when 1 mg/kg Na15NO2 was orally administered to rats, marked Hb15NO-derived doublet EPR signals [A2 = 23.4 gauss (43)] were observed in the blood, which indicated that orally administered nitrite can be a source of circulating NO as a form of HbNO.

Next, we investigated the time course of blood HbNO concentration changes after ingestion of nitrite. As shown in Fig. 3, oral administration of nitrite (1–10 mg/kg body wt) produced a pronounced increase in blood HbNO. The HbNO concentration remained considerably higher even at the end of
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DISCUSSION

We undertook the present study to assess the effect of orally administered nitrite on circulating HbNO formation by using the EPR technique. Using $^{15}$NO$_2^-$, we clearly showed that orally administered nitrite is an alternative source of NO.

It was first reported in 1926 that vegetarian diets have hypotensive effects (16), and recent studies revealed that vegetarians tend to have lower blood pressures than nonvegetarians (60, 74, 75). In addition, the Dietary Approaches to Stop Hypertension (DASH) diet, which is rich in fruits and vegetables and low in saturated and total fats, showed hypotensive effects in randomized controlled trials (1, 67), although these results may be due to increased intake of fiber, unrefined carbohydrates, polyunsaturated fatty acids, potassium, magnesium, calcium, ascorbate, and vitamin E or lower intake of energy, total and saturated fat, cholesterol, protein, and sodium (66). It has not been determined which components of the DASH diet are responsible for the hypotensive effect.

Thöni and colleagues (4, 11) suggested that a nitrate-derived NO formation pathway is a possible mechanism for the hypotensive effect of vegetable- and fruit-rich diets. Vegetables and fruits contain large amounts of nitrate and nitrite (97) and supply 86% of the daily nitrate intake in the general US population (an average of 75 mg of NO$_3^-$ in nonvegetarians and 268 mg of NO$_3^-$ in vegetarians) and 16% of the daily nitrite intake (an average of 0.77 mg of NO$_2^-$ in nonvegetarians and 1.7 mg of NO$_2^-$ in consumers of a diet in cured meat) (14). When nitrate is ingested, it is rapidly absorbed in the upper small intestine, and up to 75% is excreted in the urine within 24 h (70). The remaining ingested nitrate (~25%) undergoes...
enterosalivary recirculation, and it is concentrated in the salivary glands and then secreted in the saliva (3, 8). The rate of microbial reduction of nitrate to nitrite in the oral cavity is reported to be ∼10–20% of total ingested nitrate (19, 81), and the nitrite is moved into the stomach by swallowing. All combined, vegetarians ingest a maximum of 0.24 mg/kg body wt for a 60-kg person). In the case of rats, 0.156 mg of nitrite seems to be supplied by rat chow (the nitrite concentration of rat chow is 5.2 mg/kg on average, and a rat consumes ~30 g/day). It had been believed that ingested nitrite is absorbed by the gastrointestinal tract (51) or reacts in the stomach with secondary or tertiary amines and amides present in foods such as cheese or meat to form N-nitroso compounds (7). However, the rate of nitrosation of amines is dependent on the nitrite concentration to a power of greater than unity, which means that nitrite ingested at one time over a short period will be more active in the synthesis of N-nitroso compounds than a continuous supply at lower concentrations over long periods (95). In addition, findings of recent studies of nitrite metabolism suggest another reaction mechanism rather than nitrosamine formation pathways. Mc-Knight et al. (59) reported that a large and sustained increase in chemically derived gastric NO concentration after an oral nitrite load and very low gastric nitrite concentrations resulting from nitrate would suggest that nitrosamine formation is not likely in healthy volunteers. Once the nitrite is mixed with stomach acids, it interacts with hydrogen to form nitrous acid in accordance with reaction 1 (57). Nitrous acid forms NO by spontaneous decomposition through reactions 2–4; alternatively, several reducing species such as ascorbic acid are expected to be good candidates for NO production from nitrous acid or other nitrite metabolites according to reaction 5 (70, 84, 96). Nitrate and thiocyanate are secreted by the salivary glands; 25% of the nitrate is then reduced by bacteria on the dorsum of the tongue, and these compounds move to the stomach. Thioscyanate is a powerful catalyst of nitrosation of secondary amines by acidified nitrite, and this simultaneous delivery of nitrite and thioscyanate to the acidic environment of the stomach therefore has been regarded as a potentially important source of endogenous formation of carcinogenic N-nitroso compounds (6). Incidentally, ascorbic acid is actively secreted into gastric juice (77, 79) and plays a key role in preventing acid-catalyzed luminal nitrosation competitively with the secondary amines and amides (62, 63), which results in the formation of NO and dehydroascorbate in the stomach (2, 52), and then NO diffuses into the adjacent epithelium barrier of the stomach (84). These hypotheses are supported by the facts that 1) omeprazole, a proton pump inhibitor, suppresses the formation of NO in the stomach with a rise in intragastric nitrite (69); 2) thiocyanate is secreted from salivary glands, and it catalyzes the nitrosation of secondary amines by acidified nitrite (6), although ascorbic acid, which is secreted into the gastric juice, prevents acid-catalyzed luminal nitrosation competitively with the secondary amines and amides (62, 63); and 3) a large amount of NO is formed when lettuce and saliva are mixed under acidic conditions (57); hence, lettuce contains ascorbic acid and nitrite.

However, it is still unclear whether orally ingested nitrite is responsible for the formation of NO in the blood and whether the NO generated in the stomach contributes to systemic blood pressure. Therefore, we measured the increase in HbNO concentration after oral nitrite treatment to confirm the origin of the increased HbNO. In addition, we measured the HbNO formation in nitrite-treated rats with l-NAME-induced hypertension by using the EPR subtraction method (38) and compared HbNO concentrations and blood pressure among the rats.

The results of bolus nitrite treatment demonstrated that orally administered nitrite forms HbNO in vivo (Fig. 2B), and this phenomenon was further confirmed by use of $^{15}$NO$\text{O}_2$ instead of $^{13}$NO$\text{O}_2$ (Fig. 2C) (44). The formation of HbNO by NO donors has already been confirmed by investigators applying the EPR technique both in vivo (10, 41) and in vitro (41,
44). Kohno et al. (41) succeeded in detecting the EPR signal of HbNO in rats treated with an intravenous injection of a large amount of nitrite (6.9 mg NaNO2/rat), although they did not detect the HbNO signal when 0.69 mg NaNO2/rat was injected because of the decomposition of nitrite by oxyhemoglobin (42, 80). However, our results indicate that treatment with 0.4 mg NaNO2/rat (1 mg NaNO2/kg body wt po) produces an apparent HbNO EPR signal with a peak concentration of 4.93 μM at 5 min after treatment (Fig. 3). A possible explanation for the difference in the effects of oral administration and intravenous treatment on HbNO formation may be the existence of acid decomposition pathways of nitrite in the stomach (57), followed by the salvage of NO by Hb in erythrocytes (99).

Nitrite has been considered a toxic compound [the oral median lethal dose (LD50) in rats is 180 mg/kg (78)], although it is present throughout the body in various concentrations: 500 μM in rat lung tissue, 69 μM in the human kidney, 10 μM in aortic tissues (72), and 12 μM in the rat heart (100). In contrast, the nitrite concentration in the blood circulation is relatively low compared with tissues levels, and its values vary from undetectable (27) and submicromolar [0.2 μM (34), 0.45 μM (48) in healthy humans] to micromolar [6.6 μM in human serum (21)] and a few tens of micromoles [26 μM in patients with left-to-right shunt (25)]. Recently, Kleinbongard et al. (39) reviewed the physiologically relevant plasma nitrite concentration range in various mammals (100 – 600 nM), and they concluded that it is 0.3 μM in humans and 0.2 μM in rats.

Nitrite is known as a vasodilator at high concentrations in vitro (29, 32, 33, 47, 58, 68) and ex vivo (15). However, Lauer et al. (46) demonstrated that nitrite had no vasodilatory effects when it was infused intra-arterially into humans (the venous plasma nitrite concentration exceeded 130 μM when nitrite was infused at 36 μmol/min for 1 min), even at concentrations 200 times that achieved during maximal endothelial NOS stimulation with acetylcholine. In our experiments, when nitrite was chronically administered to L-NAME-treated hypertensive rats for 3 wk, SBP dropped [L-NAME control: 170 ± 13 mmHg; L-NAME + nitrite (1,000 mg/l): 149 ± 10 mmHg] (Fig. 4) as reported elsewhere (4, 11, 30, 94). In addition, the blood HbNO concentration was augmented with the decrease of SBP in nitrite-treated rats (Fig. 5). These results suggest the following reaction mechanisms.

First, acid-derived disproportionation of nitrite to NO occurs in the stomach (76). This process forms nitrous acid as the first step, as follows:

\[
\text{NO}_2^- + \text{H}^+ \rightarrow \text{HNO}_2 \quad (6)
\]

The nitrous acid can exist as a resonance form, i.e., a nitrosonium ion with a hydroxide-like structure as an intermediate (2):

\[
\text{HNO}_2 \leftrightarrow \{\text{HOON}\} \quad (7)
\]

This intermediate reacts with another nitrite ion to form dinitrogen trioxide (76), which results in the formation of NO by decomposition:

\[
\{\text{HOON}\} + \text{NO}_2^- \rightarrow \text{N}_2\text{O}_3^- + \text{OH}^- \quad (8)
\]

\[
\text{N}_2\text{O}_3^- \rightarrow \text{NO} + \text{NO}_2 \quad (9)
\]

Alternatively, the intermediate decomposes to nitrosium ion and hydroxyl ion in accordance with its pKₐ value (3.4) (5, 59):

\[
\{\text{HOON}\} \rightarrow \text{OH}^- + \text{NO}^+ \quad (10)
\]

Thereafter, the nitrosium ion interacts with reductive compounds (R), such as flavonoids (85) and ascorbate (87), to form NO:

\[
\text{NO}^+ + \text{R} \rightarrow \text{NO} + \text{R}' \quad (11)
\]

and then is effectively scavenged through reaction with Hb in erythrocytes to form HbNO (99).
It is still unclear how NO produced in the stomach gets directly into erythrocytes and is scavenged by Hb, because there are various compartments between the stomach lumen and erythrocytes. One possible explanation for this is that the NO produced diffuses through the epithelial barrier (84) to get to Hb, because NO is a hydrophobic molecule. However, it also has been reported that erythrocytes themselves and/or both erythrocytes and endothelium have diffusional barrier systems against NO (9, 12, 31, 50, 53, 54, 92, 93), and hence these barriers are estimated to decrease the rate of NO scavenging by Hb more than 6,000-fold (22). Therefore, the following pathway for HbNO formation from nitrite has been proposed. When nitrite is orally administered to animals, nitrite in the stomach is absorbed rapidly into the blood stream (95) and then interacts with deoxyhemoglobin to form HbNO (17). In 2003, Cosby et al. (13) demonstrated that nitrite infusion (up to 36 \( \mu \)mol/ml into the brachial arteries of human volunteers at 1 ml/min for 5 min to achieve an intravascular nitrite concentration of \( \sim 200 \) \( \mu \)M) under basal conditions and after exercise with or without regional forearm NOS inhibition caused a dose-dependent increase in blood flow, and this phenomenon was associated with the formation of HbNO. If this pathway were valid, the time course of changes in the blood concentration of HbNO following oral administration would depend on the nitrite concentration in the blood.

However, the peak blood concentration of HbNO was observed at the first measurement after intake (5 min) for 1 and 3 mg NaNO2/kg and at 15 min for 10 mg NaNO2/kg treatment (Fig. 3), and these values are shorter than the time (30 min) of peak plasma levels of nitrite following oral administration in rats (40). This discrepancy may result, in part, from the contribution of acids in the stomach to the rapid decomposition of nitrite (70).

Once HbNO has been produced, it acts as a pool of NO under physiological conditions. Luchinger et al. (55) and Stamler (82) proposed that HbNO transfers NO to cysteine 93 to form S-nitrosohemoglobin, which in turn interacts with physiologically relevant millimolar concentrations of GSH to transfer the NO from erythrocytes (23) or with cysteine thiols in the cytoplasmic domain of membrane-associated band 3 protein (AE1), thus exporting NO to extracellular areas (71).

There is a further candidate for nitrite-derived NO formation in the circulation other than an acid-degraded decomposition and deoxyhemoglobin pathways. Xanthine oxidoreductase catalyzes the reduction of nitrite to NO under both anaerobic (61) and aerobic conditions (49).

In conclusion, we have demonstrated that 1) orally administered nitrite is detectable in the circulation as HbNO by using a stable isotope of nitrogen and EPR spectroscopy, and 2) nitrite treatment attenuates \( l \)-NAME-induced hypertension in a dose-dependent manner. Although the metabolic pathways from nitrite to NO in rats (64) seem comparable to those in humans (84), species differences make further study necessary to extrapolate these conclusions directly to humans. In addition, the daily intake of nitrite in the present study (2.7 mg in 100 mg NaNO2/l drinking water, and 27 mg in 1,000 mg/l, Table 1) is considerably higher than that provided by normal rat chow (0.156 mg) or a vegetarian diet (0.1 mg). However, these results may explain, at least in part, the mechanism of the DASH diet-induced hypotensive effects. In this study we used a pharmacological dose of nitrite to clarify the nitrite-derived NO production in vivo; we next need to examine the physiological role of a dietary dose of nitrite. Further research is needed to investigate the interaction between nitrite-nitrate intake and human health.

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