Dietary doses of nitrite restore circulating nitric oxide level and improve renal injury in L-NAME-induced hypertensive rats

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Kanematsu Y, Yamaguchi K, Ohnishi H, Motobayashi Y, Ishizawa K, Izawa Y, Kawazoe K, Kondo S, Kagami S, Tomita S, Tsuchiya K, Tamaki T. Dietary doses of nitrite restore circulating nitric oxide level and improve renal injury in L-NAME-induced hypertensive rats. Am J Physiol Renal Physiol 295: F1457–F1462, 2008. First published August 27, 2008; doi:10.1152/ajprenal.00621.2007.—We have reported that pharmacological doses of oral nitrite increase circulating nitric oxide (NO) and exert hypotensive effects in Nω-nitro-l-arginine methyl ester (l-NAME)-induced hypertensive rats. In this study, we examined the effect of a chronic dietary dose of nitrite on the hypertension and renal damage induced by chronic l-NAME administration in rats. The animals were administered tap water containing l-NAME (1 g/l) or l-NAME + nitrite (low dose: 0.1 mg/l, medium dose: 1 mg/l, high dose: 10 mg/l) for 8 wk. We evaluated blood NO levels as hemoglobin-NO adducts (iron-nitrosyl-hemoglobin), using an electron paramagnetic resonance method. Chronic administration of l-NAME for 8 wk induced hypertension and renal injury and reduced the blood iron-nitrosyl-hemoglobin level (control 38.8 ± 8.9 vs. l-NAME 6.0 ± 3.1 arbitrary units). Coadministration of a low dose of nitrite with l-NAME did not change the reduced iron-nitrosyl-hemoglobin signal and did not improve the l-NAME-induced renal injury. The blood iron-nitrosyl-hemoglobin signals of the medium dose and high dose of nitrite were significantly higher than that of l-NAME alone. Chronic administration of a medium dose of nitrite attenuated l-NAME-induced renal histological changes and proteinuria. A high dose of nitrite also attenuated l-NAME-induced renal injury. These findings suggest that dietary doses of nitrite that protect the kidney are associated with a significant increase in iron-nitrosyl-hemoglobin levels. We conclude that dietary nitrite-derived NO generation may serve as a backup system when the nitric oxide synthase/l-arginine-dependent NO generation system is compromised.

Nitric oxide (NO) is a gaseous signaling molecule and exerts a variety of biological actions under physiological and pathophysiological conditions, such as regulation of the cardiovascular, inflammation, immune, and neuronal systems. NO also has numerous important functions in the kidney, such as the regulation of renal hemodynamics and glomerular microcirculation, the regulation of salt balance, the blunting of tubuloglomerular feedback, and the modulation of renal sympathetic nerve activity (18, 26). Chronic blockade of nitric oxide synthases (NOSs) with Nω-nitro-l-arginine methyl ester (l-NAME) in rats results in severe hypertension and progressive kidney damage (3, 11). On the other hand, NO production seems to be low in chronic kidney disease (CKD) patients, and NO deficiency may play a role in CKD progression (5, 32). Treatment strategies to maintain the circulating NO level in healthy young people may be useful in preventing or slowing the progression of renal diseases.

It is believed that NO is synthesized from l-arginine, NADPH, tetrahydrobiopterin, and the molecular oxygen catalyzed by NOSs in endothelial and other cells. The biological activity of NO is terminated by oxidation into nitrite and nitrate. Therefore, nitrite and nitrate in the blood are recognized as waste forms of NO. However, this dogma is now being challenged (20). Our previous studies (28, 35) indicate that nitrite should be considered as a storage form of NO in blood and tissues, and the oral administration of nitrite may be a useful treatment strategy to increase the circulating NO level.

It was first reported in 1926 that vegetarian diets have hypotensive effects (10), and recent studies have revealed that vegetarians tend to have lower blood pressures than nonvegetarians (23, 31). It is widely believed that a higher consumption of fruits and vegetables is protective against cardiovascular disease, and several nutrients in fruits and vegetables, such as dietary fibers, folate, potassium, flavonoids, and antioxidant vitamins, are considered candidate reducers of the incidence of cardiovascular diseases. Recent epidemiologic research has also shown that vegetarians have lower cardiovascular disease-related morbidity and mortality (14, 16). In addition, the Dietary Approaches to Stop Hypertension (DASH) diet, which is rich in fruits and vegetables and low in saturated and total fats, has exhibited significant hypotensive effects in randomized controlled trials (2, 24). However, it has not been determined which components of the DASH diet are primarily responsible for the hypotensive effect. Thöni and colleagues (4, 8) have suggested that a nitrate-derived NO formation pathway is a possible mechanism for the hypotensive effect of vegetable- and fruit-rich diets. Indeed, multiple dietary factors affect blood pressure (1), and blood pressure is a strong, consistent, continuous, independent, and etiologically relevant risk factor for cardiovascular and renal disease (7). In addition, vegetables and fruits contain large amounts of nitrate and nitrite (40), and we have clearly demonstrated that orally ingested nitrite can serve as an alternative to l-arginine as a source of NO in vivo (35). However, the physiological and pathophysiological role...
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MATERIALS AND METHODS

Materials. L-NAME was purchased from Nacalai Tesque (Kyoto, Japan). Sodium nitrite and other chemicals were from Wako Pure Chemical Industries (Tokyo, Japan).

Experimental design. Male Sprague-Dawley rats (7 wk old, weighing 240–280 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) and had access to tap water ad libitum until the day of the experiments. All animal care and treatments were conducted according to protocols reviewed and approved by the animal use and care committee of the University of Tokushima. All experiments were performed according to the Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health (NIH Pub. No. 85-23, revised 1985).

Rats received drinking water containing L-NAME alone or L-NAME with nitrite for 8 wk. Rats were divided into five groups (n = 5) at 8 wk of age: 1) control group, 2) L-NAME group (1 g of L-NAME in 1 liter of drinking water), 3) L-NAME plus low dose of nitrite (LDN: 0.1 mg of sodium nitrite in 1 liter of drinking water), 4) L-NAME plus medium dose of nitrite (MDN: 1 mg of sodium nitrite in 1 liter of drinking water), and 5) L-NAME plus high dose of nitrite (HDN: 10 mg of sodium nitrite in 1 liter of drinking water). The dose of sodium nitrite was selected on the basis of a vegetarian’s ingestion of nitrite (35). Rats were anesthetized with pentobarbital sodium (40 mg/kg ip), and venous blood was obtained from the vena cava for iron-nitrosyl-hemoglobin measurement at 8 wk. The blood sample was immediately immersed and stored in liquid nitrogen until electron paramagnetic resonance (EPR) measurement. The right kidney of each rat was removed and fixed in 10% buffered formalin and embedded in paraffin. Systolic blood pressure (SBP) was measured every week by the tail-cuff method with a BP-98A sphygmomanometer (Softron, Tokyo, Japan). Twenty-four-hour urine samples were collected at 8 wk in a metabolic cage. The amount of urinary protein excretion was measured by the Bradford method (Bio-Rad, Oakland, CA).

Morphological studies. The right kidney of each rat was removed immediately, fixed in 10% buffered formalin, and embedded in paraffin, and 4-μm sections were stained with periodic acid-Schiff (PAS) reagent. The severity of glomerular injury and tubulointerstitial injury was evaluated by light microscopy according to previously described methods with some modifications (30, 37). Briefly, 100 glomeruli were randomly selected and evaluated as follows. Protein droplets in glomerular visceral epithelial cells were assessed by calculating the percentage of affected glomeruli. The glomerular ischemic index was calculated by counting the affected glomeruli with ischemic change. The glomerular ischemic change was demonstrated as the wrinkling of glomerular basement membranes with apparent thickening and shrinkage and decrease in glomerular size in PAS-stained sections. Bowman’s capsular basement membranes were also multilaminated surrounding ischemic glomeruli. Tubulointerstitial damage was quantitatively assessed in the cortex and other medulla as mild (1), moderate (2), or severe (3), depending on the presence of infiltrated mononuclear cells and tubular damage in 20 microscopic fields with ×100 magnification, and average score per rat was calculated. The damage was evaluated by the appearance of tubular atrophy, dilatation, interstitial widening, and infiltration of inflammatory mononuclear cells. Mononuclear cells in the expanded interstitium were considered as inflammatory cells including lymphocytes and monocytes/macrophages in PAS-stained sections (6). A pathologist who was blinded to other findings semiquantitatively analyzed these lesions.

EPR measurement. All EPR measurements were carried out according to our previous report (17). 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 JES TE 300 ESR 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 normalized with ESPRIT 432 software (JEOL) to improve the signal-to-noise ratio. Typical instrument conditions were 20-mW microwave power, 6.3-G modulation amplitude, 1-s time constant, 60-min scan time, 3,200 ± 250-G range and, 9,045-GHz microwave frequency. Spectra were stored on an IBM personal computer for analysis. The iron-nitrosyl-hemoglobin signal was obtained by subtracting the EPR spectrum of iron-nitrosyl-hemoglobin-depleted whole blood from that of each sample as described previously (17). The relative iron-nitrosyl-hemoglobin concentration was obtained from the peak-to-peak amplitude of the first signal of the triplet signal of the z factor of iron-nitrosyl-hemoglobin at g = 2.01 (15).

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

RESULTS

Effects of L-NAME and nitrite on body weight, blood pressure, and iron-nitrosyl-hemoglobin concentration. Body weight continuously increased in all groups during the experimental period, and there was no difference in body weight among the groups during the 8-wk period (Fig. 1). Water consumption also was similar among all groups. Changes in SBP are shown in Fig. 2. SBP was identical among the five groups at the beginning of the protocol. The administration of L-NAME for 8 wk induced pronounced hypertension (SBP 163.0 ± 7.3 mmHg). Treatment with HDN significantly reduced SBP in the L-NAME-treated rats at 8 wk (l-NAME: 163.0 ± 7.3 mmHg,
l-NAME+HDN: 137.0 ± 5.0 mmHg). However, treatment with the dietary dose of nitrite tended to lower the SBP, but these changes were not statistically significant in other observation points and doses. As shown in Fig. 3, the EPR signal of iron-nitrosyl-hemoglobin was 38.8 ± 8.9 arbitrary units (AU) in the control group. Treatment with l-NAME alone significantly reduced the blood iron-nitrosyl-hemoglobin-derived EPR signal (6.0 ± 3.1 AU). The blood iron-nitrosyl-hemoglobin signal of LDN was similar to that of l-NAME alone. However, the blood iron-nitrosyl-hemoglobin signals of MDN and HDN were significantly higher than that of l-NAME alone (MDN: 29.2 ± 3.9 AU, HDN: 29.0 ± 6.2 AU).

Effects of a dietary dose of nitrite on l-NAME-induced proteinuria and renal histological changes. l-NAME-treated rats showed a higher 24-h urinary protein excretion (36.7 ± 1.7 mg/day) than control rats (20.5 ± 2.0 mg/day) at 8 wk (Fig. 4). LDN did not reduce the urinary protein excretion. The urinary protein excretion was significantly lower in the MDN group (24.2 ± 3.4 mg/day) and the HDN group (22.3 ± 2.9 mg/day) than in the l-NAME-alone group. Previously, it was demonstrated that rats chronically NO blocked as the result of L-NAME display protein droplets in glomerular epithelial cells, ischemic change in the glomerular tuft, and tubulointerstitial injury (30). In addition, this model also exhibits chronic fibrotic lesions such as glomerulosclerosis and interstitial fibrosis (3, 27). We examined whether l-NAME-treated rats without nitrite treatment would display these lesions in the present study. Accordingly, ischemic changes (Fig. 5B) and protein droplets in glomeruli (Fig. 5C) and inflammatory lesions in the tubulointerstitium (Fig. 5D) were observed in the present experiments, but chronic fibrotic lesions were not detected. MDN reduced the glomerular damages induced by l-NAME treatment (Fig. 5E). As shown in Fig. 6, there was no significant effect on the renal histological damages induced by l-NAME at a low dose of nitrite. However, MDN and HDN diminished the l-NAME-induced glomerular damages, as evaluated by glomerular ischemic index and the protein droplets. MDN and HDN also improved the tubulointerstitial damage. The beneficial effects of dietary nitrite on the renal histological damages induced by chronic l-NAME intake may be dependent on the circulating blood iron-nitrosyl-hemoglobin level.

DISCUSSION

We designed the present study to assess the effect of a dietary dose of nitrite on l-NAME-induced hypertension and renal damage in rats. We showed that chronic administration of nitrite in rats restored the proteinuria and renal histological changes induced by chronic oral intake of l-NAME. In fact, the oral administration of MDN (1 mg/l) and HDN (10 mg/l) increased the blood iron-nitrosyl-hemoglobin concentration to as much as the normal level and improved the l-NAME-induced proteinuria and renal histological damage. However, LDN (0.1 mg/l) did not improve the proteinuria or renal histological damage induced by chronic NOS inhibition, and LDN also did not increase the circulating blood iron-nitrosyl-hemoglobin concentration reduced by chronic l-NAME treatment. Chronic treatment with oral nitrite tended to lower SBP, but only HDN treatment lowered SBP at 8 wk. These findings suggest that dietary doses of nitrite that protect the kidney are associated with significant increase in iron-nitrosyl-hemoglobin levels. Our present data also suggest that the dietary nitrite-dependent NO production system works as an alternative to the l-arginine/NOS NO production system and helps to
prevent the progression of renal disease in a state of NO deficiency, as seen in CKD (5, 32).

In circulation, NO can be sequestered by hemoglobin in its α-hemes (41) and NO exists as a relatively stable iron-nitrosyl-hemoglobin (29), which means that the amount of iron-nitrosyl-hemoglobin may reflect the blood NO concentration. The physiological vasodilatory properties of circulating nitrite have been appreciated, and nitrite could be a stable endocrine carrier and transducer of NO-like bioactivity within the circulation (12, 21). In this study, we demonstrated that dietary doses of nitrite that protect the kidney are associated with significant increase in iron-nitrosyl-hemoglobin levels. We can speculate as to at least three mechanisms to increase iron-nitrosyl-hemoglobin levels after the dietary intake of nitrite: 1) Dietary intake of nitrite is decomposed to NO gas (NO) in the strong acidic environment of the gastric lumen. This NO penetrates the gastric wall and is trapped by the hemoglobin of red blood cells. 2) Absorbed and increased circulating nitrite is reduced to NO by xanthine oxidoreductase. Then, iron-nitrosyl-hemoglobin levels increase after dietary intake of nitrite. 3) Absorbed nitrite is reduced by the nitrite reductase activity of deoxyhemoglobin. We already have demonstrated that orally administered nitrite is detectable in the circulation as iron-nitrosyl-hemoglobin (17). We also found that iron-nitrosyl-hemoglobin levels increased after oral administration of nitrite were not influenced by pretreatment with xanthine oxidoreductase inhibitor in rat experiments (unpublished data).

Moreover, we examined the effects of iron-quercetin complex on orally administered nitrite-derived NO formation in rats (unpublished data). If nitrite were rapidly absorbed in the

Fig. 5. Photomicrographs of glomeruli in rats. A: control rat. B–D: ischemic change (B), protein droplets (C), and inflammatory lesions in tubulointerstitium (D) in L-NAME-treated rats. E: L-NAME + nitrite(1 mg/l)-treated rat.

![Fig. 5. Photomicrographs of glomeruli in rats. A: control rat. B–D: ischemic change (B), protein droplets (C), and inflammatory lesions in tubulointerstitium (D) in L-NAME-treated rats. E: L-NAME + nitrite(1 mg/l)-treated rat.](image)

![Fig. 6. Semiquantitative assessment of renal injury. A: glomerular ischemic index. B: % with protein droplets. C: tubulointerstitial damage score. *P < 0.01 compared with L-NAME group.](image)
gastrointestinal tract and circulating iron-nitrosyl-hemoglobin levels were derived from circulating nitrite, the time course and the concentration of circulating iron-nitrosyl-hemoglobin levels would depend on circulating nitrite concentration. However, our data contradict this hypothesis. Indeed, after orally administered nitrite plus iron(II) with quercetin, blood levels of iron-nitrosyl-hemoglobin increased rapidly and peaked at ~5 min. On the other hand, after oral administration of nitrite alone, blood levels of iron-nitrosyl-hemoglobin gradually increased and peaked at 15 min. Meanwhile, our experiments showed that circulating nitrite concentration was significantly lower in the group of nitrite plus iron(II) with quercetin than in the group of nitrite only. These data suggest that increased circulating iron-nitrosyl-hemoglobin comes from the acidic decomposition of nitrite in the gastric lumen. Our experimental findings did not properly exclude the possibility that the increased circulating iron-nitrosyl-hemoglobin levels come from the increased absorbed nitrite. Indeed, the intravenous infusion of sodium nitrite increased the iron-nitrosyl-hemoglobin EPR signal in rat, but the iron-nitrosyl-hemoglobin EPR signal intensity is not correlated with plasma concentration of nitrite because of the complicated disposal pathways of plasma nitrite (19, 28). There may be some factors to modify the nitrite-derived NO formation in blood. We need further study to clarify the mechanisms of this increase.

We already have reported that orally ingested nitrite can be an alternative to L-arginine as a source of NO in vivo, and that the coadministration of nitrite with L-NAME attenuates the hypertension induced by a NOS inhibitor. However, in a previous study, we used pharmacological doses of nitrite to clarify the nitrite-derived NO production in vivo. In this experiment, we evaluated the effect of a dietary dose of nitrite on L-NAME-induced hypertension and renal injury. We speculated that vegetarians ingest a maximum of ~14.2 mg of nitrite per day (0.24 mg/kg body wt for a 60-kg person) (35). If we estimate the body weight of the rat to be 300 g and the daily water consumption of the rat to be ~30 ml, 2.4 mg of sodium nitrite per liter of drinking water would provide the rat with a representative maximum vegetarian dose of nitrite (0.24 mg/kg body wt). According to this conjecture, we used three doses of nitrite (LDN 0.1 mg/l, MDN 1 mg/l, HDN 10 mg/l) to evaluate the effect of dietary dose of nitrite on L-NAME-induced hypertension and renal damage. MDN (1 mg/l), which is less than the maximum vegetarian dose, restored the circulating blood iron-nitrosyl-hemoglobin concentration to that of normal rats and improved the proteinuria and renal histological damages induced by chronic administration of L-NAME. However, a small dose of nitrite (0.1 mg/l) did not change the circulating iron-nitrosyl-hemoglobin level or renal injury in the L-NAME-treated rats. These findings suggest that a dietary dose of nitrite can, at least in part, play a role in compensating for the depletion of L-arginine/NOS-derived NO. The triply NOS−/− mice were viable, and the plasma NOX concentration and urinary NOX excretion in the triply NOS−/− mice were extremely decreased but still detectable (25). This study suggests that there is a NOS-independent NO production system in the mouse, and an NOS-independent NO production system might play an important role in maintaining homeostasis.

It is widely reported that vegetarian diets are associated with low blood pressure and that a higher consumption of fruits and vegetables is protective against cardiovascular disease (1, 14). The DASH diet, which is rich in fruits and vegetables, has exhibited hypotensive effects in randomized controlled trials (2, 24). Well-established dietary modifications that lower blood pressure include reduced salt intake, weight loss, and a moderation of alcohol consumption (among those who drink), but other dietary factors, such as increased intake of fibers, antioxidant vitamins, phytochemicals, calcium, and magnesium, may also affect blood pressure (1). Vegetables and fruits contain large amounts of nitrate and nitrite (40). Himeno et al. (13) reported that the NOx content was low in drinks such as cola but extremely high in vegetable juice, and that the intake of high-NOx water increased the plasma NOx concentration in healthy volunteers. In addition to the L-arginine and NOS pathways, formation of NO from nitrite is well known in the field of chemistry. However, nitrite has not attracted attention as a dietary factor for improving hypertension and cardiovascular diseases because nitrite has not only been considered to be a toxic compound (33) but also considered to be a risk factor for methemoglobinemia and cancer (39). Moreover, circulating nitrite has been considered to be an inert waste form of NO. On the other hand, Classen et al. (8) reported that a nitrate-derived NO formation pathway is a possible mechanism for the hypotensive effect of vegetable- and fruit-rich diets. It was also reported that intravenous infusion of sodium nitrite decreased the blood pressure of anesthetized rats in a dose-dependent manner (38). These observations have stimulated our interest in nitrite as a dietary factor with potential for lowering blood pressure and preventing organ damage.

In the kidney, NO has numerous important functions in the control of renal hemodynamics and excretory function (22, 26, 34). It is also well known that chronic blockade with the NOS inhibitor L-NAME results in hypertension and renal glomerular injury (3, 37). In this study, we confirmed that chronic treatment with a NOS inhibitor decreased the circulating iron-nitrosyl-hemoglobin level and induced proteinuria and renal damage in rats. It has been believed that NO is synthesized from L-arginine by NOSs, and that nitrite and nitrate are the inert end products of NO. However, we have already demonstrated that orally ingested nitrite can serve as an alternative source of circulating NO (35) and there is a nitrate-derived NO generation system during renal ischemia (28). In this study, we examined the effect of small comparative amounts of orally ingested nitrite to clarify the physiological role of a dietary dose of nitrite. The findings demonstrate that chronic administration of a medium-level dietary dose of nitrite restores the circulating iron-nitrosyl-hemoglobin levels reduced by L-NAME and maintenance of the circulating iron-nitrosyl-hemoglobin level in a controlled range protects L-NAME-induced renal injury. van Loon et al. (36) reported that there is no positive association between the intake of nitrate or nitrite and gastric cancer risk from a prospective cohort study. Moreover, Coss et al. (9) suggested that long-term exposure to drinking water nitrate at levels below the maximum contaminant level of nitrate nitrogen (10 mg/l) is not associated with pancreatic cancer. Taking these findings together, we propose that dietary supplementation of nitrite is one of the potentially important non-pharmacological strategies for maintaining circulating NO level so as to prevent or slow the progression of renal diseases.

In conclusion, our findings suggest that the beneficial effects of dietary nitrite on the renal damage induced by chronic L-NAME intake are dependent on circulating blood iron-
nitrosyl-hemoglobin levels and that dietary nitrite-derived NO generation serves as a backup system for NO production when the NOS/L-arginine-dependent NO generation system is compromised. These results explain, at least in part, the mechanism by which vegetable- and fruit-rich diets help prevent cardiovascular diseases (14).

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