Forum Mini Review

Malfunction of Vascular Control in Lifestyle-Related Diseases: Formation of Systemic Hemoglobin-Nitric Oxide Complex (HbNO) From Dietary Nitrite

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Abstract. Nitric oxide (NO) has many physiological functions. It is believed to be produced from L-arginine by nitric oxide synthase (NOS), and nitrite and nitrate are waste forms of it. By the way, nitrate and nitrite are abundant in vegetables and fruits, especially leafy vegetables and pickled vegetables. Orally-ingested nitrate is changed to nitrite by micro-organelles living in the hypopharynx area, and nitrite is expected to change to NO in the stomach due to its low pH. Indeed, some researchers reported that NO is produced in the gastric cavity, although few reports mentioned the physiological meanings of this NO formation. Therefore, we investigated whether the nitrite-derived NO can shift to the circulation and acts like NOS-derived NO does in tissues.

We adopted a stable isotope of nitrite (15NO2/g45) in order to distinguish between the endogenous nitrite and the exogenously administered one and measured nitrosyl hemoglobin (HbNO) as an index of circulating NO using electron paramagnetic resonance spectroscopy. It appeared that the oral administration of 15N-nitrite formed the Hb15NO in rat blood and decreased the blood pressure of chronic L-NAME treated rats. Our findings suggest that the intake of nitrite (or nitrate)-rich foods such as vegetables and fruits would alter the systemic HbNO dynamism, resulting in the improvement of cardiovascular diseases.

Keywords: nitric oxide (NO), hemoglobin-NO adduct, electron paramagnetic resonance, nitrite

Introduction

Nitric oxide (NO), a free radical molecule, has numerous roles in various physiological functions, such as regulation of the blood pressure (1), immune response to bacterial infection (2), and nervous systems (3). It is believed that nitric oxide synthase (NOS) makes NO by catalyzing the oxygen-, tetrahydrobiopterin-, and NADPH-dependent oxidation of L-arginine, and nitrite and nitrate are recognized as a waste forms of NO. However, an alternative pathway for NO production in biological systems has been found in the last decade. Catalysis by xanthine oxidoreductase was found to serve as an alternative enzymatic NO production pathway from organic- and inorganic nitrates under hypoxic conditions (4). In addition to enzymatic production of NO, non-enzymatic nitrite-derived mechanisms for NO generation has been recognized to occur by the following reactions (5):

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\begin{align*}
\text{NO}_2^- + \text{H}^+ & \rightleftharpoons \text{HNO}_2 & (\text{pKa} = 3.3) & (i) \\
\text{HNO}_2 + \text{H}^+ & \rightleftharpoons \text{H}_2\text{NO}_3^- & \rightleftharpoons \text{NO}^- + \text{H}_2\text{O} & (ii) \\
\text{H}_2\text{NO}_3^- + \text{NO}_2^- & \rightleftharpoons \text{N}_2\text{O}_3 + \text{H}_2\text{O} & (iii) \\
\text{N}_2\text{O}_3 & \rightleftharpoons \text{NO} + \text{NO}_2 & (iv)
\end{align*}
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These reactions are favorable under the acidic condition due to the low pKa value of reaction (i) (6), and nitrite-derived NO formation seems to occur in acidic environments such as the stomach (7, 8), oral cavity (9), and acidic urine (10).

By the way, it has been believed that vegetarian diets...
have hypotensive effects (11–14). And recently, the DASH (Dietary Approaches to Stop Hypertension) diet, which is rich in fruits and vegetables and low in saturated and total fats, showed hypotensive effects in randomized controlled trials (15, 16). It has not been determined which components of the DASH diet are responsible for the hypotensive effect. Nitrate and nitrite are abundant in vegetables and fruits (17, 18), especially leafy vegetables and Japanese pickled vegetables.

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 (19). The remaining ingested nitrate (almost equal to 25%) undergoes entero-salivary recirculation, and it is concentrated in the salivary glands and then secreted in the saliva (20, 21). Orally-ingested and salivary gland-derived nitrate changes to nitrite by micro-organelles living in the hypopharynx area. The rate of microbial reduction of nitrate to nitrite in the oral cavity is reported to be around 10% to 20% of total ingested nitrate (22, 23), and the nitrite is moved into the stomach by swallowing.

Indeed, NO is formed from nitrite in the gastric cavity (19), and blood pressure was lowered in a dose-dependent manner by oral nitrite uptake in spontaneously hypertensive rats (24, 25) and normotensive rats (26), although few reports mentioned what the physiological meaning of this nitrite-derived NO was. Therefore, we investigated 1) whether the nitrite-derived NO distributes to the circulation, and 2) whether nitrite-derived NO acts like NOS-derived NO does in vivo. We employed a stable isotope of nitrite (15\textsuperscript{N}-nitrite) in order to distinguish between endogenous nitrite and the exogenously administered one and measured nitrosyl hemoglobin (HbNO) as an index of circulating NO in whole blood using electron paramagnetic resonance spectroscopy (27).

**HbNO measurement by EPR spectroscopy**

It was reported that endothelium-derived NO diffuses into blood and binds to hemoglobin to form the relatively stable HbNO in erythrocytes (28), which means the amount of HbNO may reflect the blood NO concentration. Systemic HbNO concentration is reported to be 0.8 \mu M in rats (29) and 0.3–3 \mu M in humans (30, 31). EPR spectroscopy can detect the HbNO because HbNO is a paramagnetic species, although there are still some difficulties in obtaining fine HbNO signals because of the existence of paramagnetic compounds other than HbNO, such as ceruloplasmin, other heme proteins, and molybdenum enzymes, which give a strong EPR signal overlapping the same region of HbNO (32–37). Therefore, we developed an improved method of detecting the HbNO signal in whole blood by EPR spectroscopy (EPR subtraction method) (27). In addition, we adopted a stable isotope of nitrite (15\textsuperscript{N}-nitrite) because the Hb15\textsuperscript{N}NO EPR signal is different from the Hb14\textsuperscript{N}NO signal (Fig. 1: A and B), and using 15\textsuperscript{N}-nitrite enabled us to clarify whether the source of NO is nitrite.

**Sample preparation and EPR measurements**

Male Sprague-Dawley rats (12 weeks of age, weighing 350–400 g) were used for the present study. Venous blood was taken from the vena cava with a 1-ml plastic syringe under anesthesia (pentobarbital sodium (40 mg /kg body weight)). The kidney tissue was transferred to a 5-cm length of EPR quartz tubing by puncturing. Obtained samples were stored in liquid nitrogen until the EPR measurement.

EPR measurements were carried out in liquid nitrogen. The frozen sample was directly transferred to a liquid nitrogen-filled quartz finger dewer, which was placed in the cavity of the EPR instrument. A JES TE-300 EPR spectrometer (JEOL Co., Ltd., Tokyo) with an ES-UCX2 cavity (JEOL Co.) was utilized to collect EPR spectra at the X-band (9.5 GHz). Typical EPR conditions were as follows: power, 20 mW; frequency, 9.045 GHz; magnetic field, 3200 ± 250 gauss; modulation width, 6.3 gauss; sweep time, 15 min; and time constant 0.3 s. Spectra were processed using software ESPRIT 432 (JEOL Co.). The EPR signal subtraction was accomplished as reported previously (27).
Appearance of HbNO by bolus treatment of nitrite

In the control animal, no Hb\textsuperscript{15}NO (\(A_2 = 23.4\) gauss, \(g_Z = 2.01\) (38))-derived EPR signal was observed because of its low abundancy (data not shown). When 1 mg Na\textsuperscript{15}NO\textsubscript{2}/kg body weight was orally administered to the rat, marked Hb\textsuperscript{15}NO-derived doublet EPR signals and methemoglobin-derived EPR signals were observed in the blood and it gradually augmented with time (Fig. 2: B and C). This means that nitrite can be a source of circulating NO, and it binds with systemic Hb, forming HbNO. It is still unclear how NO produced in the stomach gets directly into erythrocytes and then scavenged by hemoglobin to form HbNO. One explanation is that NO diffuses through the epithelial barrier (39) to reach hemoglobin since NO is a hydrophobic molecule. Another proposed pathway for HbNO formation from nitrite is that orally ingested nitrite is rapidly absorbed from stomach to the blood stream (40) and then interacts with deoxyhemoglobin to form methemoglobin and HbNO (41). It seems that the latter mechanism is in accord with the observed formation of both Hb\textsuperscript{15}NO and methemoglobin-derived EPR signals. However, further research will be required to clarify the mechanisms.

Physiological role of nitrite: hypotensive effects and ischemia-reperfusion-related NO formation in kidney

Nitrite is known as a vasodilator at high concentrations in vitro (42–47) and ex vivo (48). However, its hypotensive effects in vivo are still under debate (49, 50). Therefore, we used chronic L-NAME-treated rats as an animal model of hypertension and examined the effect of nitrite on the level of HbNO as an index of NO. The oral administration of L-NAME (1 g/liter, in drinking water for 3 weeks) induced hypertension (168 ± 11 mmHg) with reduction of blood HbNO concentration (43% of the control). However, co-administration of nitrite with L-NAME dose-dependently lowered the systemic blood pressure (0.1 g NaNO\textsubscript{2}/liter: 151 ± 10 mmHg, 1.0 g NaNO\textsubscript{2}/liter: 141 ± 17 mmHg) and restored the HbNO level (90% and 93%). Although the orally administered nitrite concentration was high, our results demonstrated that nitrite treatment attenuated the decrease of blood NO in L-NAME-treated rats.

Next, we demonstrated that the nitrite can be an alternative source of NO in ischemic kidney and that it binds with hemoglobin and then is spread by the circulation after reperfusion (51).

Renal ischemia-reperfusion injury is a critical pathologic condition occurring as a result of kidney transplantation (52), and NO may play an important pathologic role in ischemic renal injury. By the way, it has been believed that NO is synthesized from L-arginine, NADPH, tetrahydrobiopterin, and molecular oxygen catalyzed by NOS. In other words, molecular oxygen is obligatory component for NO production by NOS. If so, how does NOS form NO under the hypoxic condition? In 2000, Hirabayashi et al. reported that the NO production from renal cortex tissue increased sharply during ischemia and that it was independent of L-arginine administration in rats (53), which may imply that NO is produced in an NOS-independent manner under ischemic conditions. Furthermore, Zweier et al. proposed that nitrite could be a source of NO in the event of ischemic heart (54). Taken together, it seems that NO is produced in an NOS-independent manner under ischemic conditions and that nitrite can be a candidate for the source of NO. It seems likely that endogenous nitrite in the kidney can be a source of NO under ischemic conditions because the kidney has relatively

Fig. 2. Changes in EPR-active species with time after oral administration of \textsuperscript{15}N-nitrite in blood. Spectrum A: control rat (-nitrite). Spectra B and C: rats were subjected to \textsuperscript{15}N-nitrite (1 mg NaNO\textsubscript{2}/kg body weight, p.o.) and then blood was collected from the vena cava 15 min (B) and 60 min (C) after treatment. EPR spectral conditions were same as Fig. 1 but the magnetic field was 2500 ± 2500 gauss (spectrum A – C) or 3200 ± 250 gauss (spectrum D); modulation width, 6.3 gauss; sweep time, 8 min; and time constant 0.1 s. The arrow in the figure indicates the position of high-spin iron (III) (methemoglobin).
high nitrite content (69 μM). Then we investigated whether nitrite can be a source of NO in rat kidney during and after in vivo renal ischemia induced by renal artery and vein occlusion using the EPR subtraction method.

We adopted a stable isotope of nitrite (15N-nitrite) because using 15N-nitrite enabled us to clarify whether the source of NO is nitrite. When 3 μmol/kg of 15N-nitrite was intravenously injected into rats and then followed by 40 min of ischemia, marked 15NO formation was observed with the appearance of a large doublet Hb15NO signal in kidney (Fig. 3B) in comparison to sham-operated rats (Fig. 3A). At 40-min ischemia and 1-min reperfusion in the kidney of the 15NO2−-administered rat, the Hb15NO signal in the kidney was decreased (Fig. 3C) compared to that of a 40-min-ischemia rat (Fig. 3B) and circulating Hb15NO was increased instead (data not shown). To investigate whether NOS contributes to NO generation from nitrite, the effects of L-NAME was examined in rats by 40-min occlusion. Rats were orally administered L-NAME (1 g/liter drinking water) for 1 week to induce NOS dysfunction (55, 56) and then received i.v. injection of 15N-nitrite (3 μmol/kg) followed by 40-min ischemia.

As shown in Fig. 3D, L-NAME treatment did not affect the Hb15NO formation in the ischemic kidney of 15N-nitrite-treated rats compared to the control (Fig. 3B). These results suggest that the nitrite can be an alternative source of NO in ischemic kidney.

Concluding remarks

In conclusion, in this review we described that 1) orally administered nitrite appears in the circulation as HbNO using a stable isotope of nitrogen and EPR spectroscopy, 2) nitrite treatment attenuates L-NAME induced hypertension in a dose-dependent manner, and 3) nitrite may be an alternative source of NO during renal ischemia. These results may explain, at least in part, the mechanism of the DASH diet-induced hypotensive and organ protective effects. Further research is needed to investigate the interaction between nitrite-nitrate intakes and human health.

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