Arteriovenous Differences in NO_2^- Kinetics in Anesthetized Rabbits

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Despite the importance of plasma NO_2^- as an index of endothelial nitric oxide (NO) formation and a substrate for NO production, only a limited kinetic knowledge is available *in vivo*. To address this issue, we intravenously injected NaNO_2 into anesthetized rabbits and quantified changes in arterial and venous plasma NO_2^- levels. Plasma NO_2^- levels in arterial blood ($956 \pm 220 \text{ nM}$) were slightly, but significantly, higher than those in venous blood ($889 \pm 214 \text{ nM}$) under control conditions. Although similar half-lives of the NO_2^- were observed in arterial and venous plasma, significant arteriovenous differences were observed in the volume of distribution of the central compartment ($0.158 \pm 0.007 \text{ l/kg}$ in arterial blood and $0.295 \pm 0.015 \text{ l/kg}$ in venous blood) and the peripheral compartment ($0.259 \pm 0.035 \text{ l/kg}$ in arterial blood and $0.135 \pm 0.034 \text{ l/kg}$ in venous blood). When NOR1 (authentic NO) was injected, increases in NO_2^- levels were greater in arterial plasma, and decreases in blood pressure significantly correlated with arterial NO_2^- levels only ($r = 0.90$, $p < 0.01$). These results indicate that changes in NO_2^- are larger and more easily detectable as an index of NO formation in arterial plasma.

Key words plasma nitrite; kinetic parameter; arteriovenous difference; anesthetized rabbit; authentic nitric oxide

The critical role of nitric oxide (NO) in regulating vascular tone is now widely recognized, and the search for reliable and practical indices of endothelial NO formation have been received much attention.¹⁾ Early studies that attempted to use plasma nitrate (NO_3^-) or NO_x (nitrite [NO_2^-] plus NO_3^-) as stable metabolites of $\text{NO}^{2,3)}$ encountered too high a degree of interference, resulting from numerous confounding factors (including contamination), for their results to be applied practically.^{4–6)} In addition, as-yet unexplained and paradoxical decreases in NO_x following increases in NO formation have been reported.^{7,8)} In contrast, plasma NO_2^- has been the source of much interest lately as a promising indicator of NO production following reports that approximately 70–90% of circulating plasma NO_2^- is derived from endothelial nitric oxide synthase (eNOS) activity in humans and animals.^{9,10)} Indeed, several studies have shown a close relationship between changes in endothelium-dependent blood flow and plasma NO_2^- levels.^{9,11,12)}

Furthermore, plasma NO_2^- is now believed to be a storage site for NO activity. Plasma NO_2^- is catalyzed by the nitrite reductase activity of deoxygenated hemoglobin (deoxyHb)¹³⁾; evidence in support of this includes the enhancement of vasodilatory activity of NO_2^- or production of NO in the presence of deoxyHb.^{14–18)} To further examine and evaluate these roles for plasma NO_2^- , accurate and highly sensitive methods for quantifying NO_2^- and precise information regarding kinetics *in vivo* are essential. Although there have been some preliminary and incomplete reports regarding NO_2^- kinetics *in vivo*,^{19,20)} to date, there are no systematic data based on standardized methods.

Therefore, the goal of the present study was to clarify the kinetic features of plasma NO_2^- *in vivo* using a canonical method (NO_2^- loading study) with an established highly sensitive quantifying technique,²¹⁾ taking possible arteriovenous (A–V) differences into consideration. That steady-state NO_2^-

levels might differ between veins and arteries has been subject to debate for many years.^{7,12,21–23)}

MATERIALS AND METHODS

Measurement of NO_2^- and NO_3^- We determined NO_x levels using a high-performance liquid chromatography (HPLC)-Griess system (ENO10 and ENO20; Eicom, Kyoto, Japan) consisting of a separation column, a flow reactor (with Griess reagent), a reduction column, and a detector at 540 nm, as described elsewhere.²⁴⁾ Operating under default conditions, the detection limit and sensitivity was $0.1 \mu\text{M}$ for both NO_2^- and NO_3^- with a loading volume of $10 \mu\text{l}$.

To determine nanomolar concentrations of NO_2^- , we removed the reduction column and increased the loading volume to $100 \mu\text{l}$, which improved the sensitivity and detection limits for NO_2^- to 1 nM and 2 nM, respectively.²¹⁾ In addition, a modified aqueous mobile phase was applied to improve the recovery time of the system.

Special attention was paid to excluding possible sources of NO_2^- contamination during the entire procedure, as is described elsewhere.²¹⁾ Unless otherwise specified, all laboratory wares were washed five times with pure water (resistance above $18.3 \text{ M}\Omega$ and almost completely free of NO_x contamination using MILLI-Q SP; Millipore, Bedford, MA, U.S.A.) to minimize both NO_2^- and NO_3^- contamination.⁵⁾

Animal Experiments and Sample Treatment Japanese white rabbits weighing around $2.4 \pm 3.5 \text{ kg}$ were anesthetized with intravenous sodium pentobarbital (30 mg/kg). Cannulae were inserted into the jugular vein (for drug administration), carotid artery (to monitor blood pressure) and abdominal aorta and caudal vena cava *via* femoral artery and femoral vein, respectively (for blood sampling). A tachometer to measure pulse rate was triggered by pulse waves of arterial pressure. After a stabilization period of for approximately

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20–60 min, we performed the experimental procedures. Several minutes were allowed to elapse for the recovery of hemodynamic parameters after the initial control blood sampling.

For NO_2^- kinetic analysis, 500 nmol/kg NO_2^- was injected (0.1 ml/kg of 5 mM NaNO_2 in saline), and arterial and venous blood samples were taken at the desired time points. During sampling, the initial blood sample (about 0.5 ml) was used to wash the sampling route (including syringe), and was discarded to avoid possible contamination of subsequent blood specimens. We then took samples of both arterial and venous blood (1.5 ml each from the artery and the vein). After each sampling procedure, an equivalent volume of saline was infused to compensate for blood loss, unless otherwise stated. The saline NO_2^- level was below 50 nM in every experiment, and the plasma NO_2^- concentration was not significantly affected by the saline supplementation in our preliminary study. Blood samples were immediately transferred to pre-washed microcentrifuge tubes (1.5 ml; QSP, Porex Bio Products Group, Petaluma, CA, U.S.A.) containing a heparin sheet (one-quarter of a sheet taken from a vacuum blood sampling tube; Venoject II/VP-H050K, Terumo, Tokyo, Japan). After centrifugation ($1600 \times g$ for 5 min at 4°C), plasma was mixed with methanol (1:1) and centrifuged ($10000 \times g$ for 10 min at 4°C) to remove proteins. The supernatant was immediately used for NO_2^- (and NO_3^-) analysis in every experiment.

The Ethics Committee of Kanazawa Medical University approved all animal procedures, and the animals were treated under conditions set by the “Guiding Principles for the Care and Use of Laboratory Animals” (Japanese Pharmacological Society).

Kinetic Analysis The data obtained from the NO_2^- loading study were analyzed according to the two-compartment open model as follows:

$$Y = A \cdot \exp(-\alpha \cdot t) + B \cdot \exp(-\beta \cdot t)$$

Where Y is NO_2^- concentration above steady state (Δ), A is the estimated concentration of the distribution phase at time 0, and B is the estimated concentration of the elimination phase at time 0. α and β are apparent elimination constants of the elimination phase and distribution phase, respectively, and t is time after administration. Data were fitted to the theoretical equation and hybrid parameters such as A , B , α , and β were estimated with the aid of SigmaPlot (version 8.02; SPSS, Chicago, IL, U.S.A.), which uses a least-squares principle based on an established algorithm.

Agents NaNO_2 (Wako Pure Chemical Industries, Osaka, Japan) was dissolved in saline (Otsuka, Tokyo, Japan). NOR1 was purchased from Dojindo (Kumamoto, Japan) and was dissolved in and diluted with DMSO (Wako Pure Chemical Industries, Osaka, Japan). All of the other chemicals were of analytical grade or of the highest grade commercially available.

Statistical Analysis All of the data were expressed as mean $\pm$ S.E.M. Comparisons within a group were made by paired t -test and comparisons between two groups were made by unpaired t -test. For more than three groups, comparisons were made by one-way analysis of variance followed by Tukey's method. Differences were considered significant at $p < 0.05$.

Fig. 1. Effect of NO_2^- Load on Hemodynamics and Plasma NO_2^- and NO_3^- Concentrations

NaNO_2 (500 nmol/kg) was intravenously injected into anesthetized rabbits ($n=8$, 2.75 ± 0.03 kg in body weight) and changes in hemodynamics (left upper and lower), plasma NO_2^- and NO_3^- concentrations (right upper and lower) were plotted against time. Results of the arterial blood gas analysis at time 0 were as follows: pH 7.44 ± 0.01 , PO_2 105.6 ± 9.5 mmHg, and PCO_2 32.6 ± 1.9 mmHg. HR: heart rate, mBP: mean blood pressure. * Significantly ($p < 0.05$, by paired t -test) different from venous plasma. Although plasma NO_2^- levels in both arterial and venous blood were significantly higher ($p < 0.05$, by paired t -test) than respective control (time 0) levels even after 30 min, asterisk denoting the significant differences are omitted to avoid complication.

RESULTS

To elucidate the kinetics of plasma NO_2^- *in vivo*, we intravenously injected eight rabbits with NaNO_2 solution (500 nmol/kg; 5 mM solution, 0.1 ml/kg) and sampled arterial and venous blood over the course of 1 h. The mean blood pressure (107.3 ± 3.8 mmHg at time 0) and heart rate (294 ± 13 beats/min at time 0) were relatively stable throughout the entire course of the experiment (Fig. 1). Arterial plasma NO_2^- levels (956 ± 220 nM) were slightly—but significantly ($p < 0.05$, by paired t -test)—higher than the NO_2^- levels in venous plasma (889 ± 214 nM) at the control state (time 0). After the injection, both arterial and venous NO_2^- concentrations increased markedly, followed by exponential decreases. A significant arteriovenous difference ($p < 0.05$, by paired t -test) was recognized at 0.5 min. The NO_2^- levels stayed significantly ($p < 0.05$, by paired t -test) higher than the respective control levels (time 0) even after 30 min had elapsed. There was no significant difference in plasma NO_3^- concentrations in arterial (64.3 ± 15.1 μM) and venous blood (64.6 ± 14.1 μM) at time 0. Although we observed that both arterial and venous NO_3^- concentrations after the NO_2^- injection tended to decrease slightly, there were no significant differences from the respective control values.

When differences in relation to the control values were plotted against time, two phases, the distribution phase and the elimination phase, were clearly distinguished (Fig. 2), and a two-compartment model was applied for further analysis. The hybrid parameters obtained by curve fitting were: 2097 ± 117 and 575 ± 89 ($p < 0.05$ by unpaired t -test) for arterial and venous A (nM), respectively; 965 ± 76 and 1152 ± 121 for arterial and venous B (nM), respectively; 0.54 ± 0.07 and 0.43 ± 0.06 for arterial and venous α (per min), respectively; and 0.028 ± 0.006 and 0.034 ± 0.006 for arterial and venous β (per min), respectively. There was no significant difference in the half-lives of the elimination phases of arterial and venous plasma NO_2^- (Table 1). Microscopic parameters were calcu-

Fig. 2. Changes in NO_2^- Concentrations in Arterial and Venous Plasma

Chronological changes in NO_2^- concentrations were plotted and lines were drawn using the hybrid parameters obtained by curve-fit analysis (see text for details). Data were obtained from the experiment shown in the Fig. 1. Solid and dashed lines indicate arterial plasma NO_2^- and venous plasma NO_2^- levels, respectively. * Significantly ($p < 0.05$, by paired t -test) different from venous plasma.

Table 1. Kinetic Parameters of NO_2^- in Anesthetized Rabbits

Parameters	Arterial blood	Venous blood
$t_{1/2}(\beta)$ (min)	34.8 ± 8.3	26.1 ± 5.6
k_{12} (/min)	0.360 ± 0.023	$0.107 \pm 0.012^{**}$
k_{21} (/min)	0.219 ± 0.044	0.310 ± 0.058
k_{el} (/min)	0.083 ± 0.013	$0.048 \pm 0.006^{**}$
Vd1 (l/kg)	0.158 ± 0.007	$0.295 \pm 0.015^{**}$
Vd2 (l/kg)	0.259 ± 0.035	$0.135 \pm 0.034^{**}$
Vdss (l/kg)	0.416 ± 0.040	0.431 ± 0.040

$t_{1/2}(\beta)$ (min): half-life of β slope ($=0.693/\beta$). k_{12} ($=A \cdot B \cdot (\alpha - \beta)^2 / (A+B) \cdot (A \cdot \beta + B \cdot \alpha)$) and k_{21} ($=A \cdot \beta + B \cdot \alpha / (A+B)$) are rate constants from central compartment to peripheral compartment. k_{el} : elimination constant ($=\alpha \cdot \beta \cdot (A+B) / (A \cdot \beta + B \cdot \alpha)$). Vd1 ($=D/(A+B)$, where D is a dose of nitrite): volume of distribution of central compartment. Vd2 ($Vd1 \cdot k_{12}/k_{21}$): volume of distribution of peripheral compartment. Vdss ($=Vd1 + Vd2$): volume of distribution under steady state. Data are presented as mean $\pm$ S.E.M. of 8 rabbits. ** Significantly ($p < 0.01$, unpaired t -test) different from arterial blood.

lated based on the hybrid parameters above. As shown in Table 1 and Fig. 3, the volume of distribution of the arterial central compartment (Vd1) was significantly smaller than that of the venous Vd1, and the reverse was found in peripheral compartments (Vd2). However, the volume of distribution at steady state (Vdss = Vd1 + Vd2) in the arterial system was very similar to that found in the venous system. The rate constant from the central compartment to the peripheral compartment (k_{12}), as well as the elimination constant (k_{el}) in the arterial system, was significantly larger than what was found in the venous system. Total clearance ($CL_{total} = D / (A/\alpha + B/\beta)$; see Table 1 for details) was estimated in both arterial (0.767 ± 0.109 l/kg/h) and venous systems (0.836 ± 0.104 l/kg/h), and were similar to one another. We performed further estimations of steady-state NO_2^- levels ($C_{ss} = R_{prod} / CL_{total}$) based on the assumption that the rate of NO_2^- production (R_{prod} : nmol/kg/h) is the same in both the arterial and the venous system. Arterial C_{ss} ($=R_{prod} / (93.3 \pm 16.3)$ nmol/l) tended to be higher than venous C_{ss} ($=R_{prod} / (79.9 \pm 9.9)$ nmol/l). However, the difference was not statistically significant.

To observe the effect of authentic NO on plasma NO_2^- levels, we intravenously administered the NO-releasing agent NOR1 (500 nmol/kg) in a separate series of experiments. As shown in Fig. 4, when compared with the vehicle control (DMSO) group, NOR1 decreased mean blood pressure transiently; a small decrease in heart rate was also observed.

Fig. 3. A Simplified Diagram of the Relationship among Kinetic Properties

Central compartments (plasma, Vd1) and peripheral compartments (organ tissues, extracellular fluid, RBCs, etc.; Vd2) are expressed as relative areas in relation to their calculated values (Table 1). Differences among rate constants (k_{12} and k_{21}), as well as elimination constants (k_{el}), are expressed as relative arrow sizes according to their values (Table 1). The continuity between arterial and venous systems via pulmonary (top) and peripheral (bottom) circulation is indicated. ** Significantly ($p < 0.01$, unpaired t -test) different from venous blood.

Concomitantly, plasma NO_2^- levels (173 ± 26 nM in arterial blood and 168 ± 19 nM in venous blood at time 0; $p = 0.13$ by paired t -test) increased transiently; the increases were significantly different from those in the vehicle control group (plasma NO_2^- levels at time 0 were 393 ± 101 nM in arterial blood and 340 ± 87 nM in venous blood; $p < 0.05$, by paired t -test). The increases in arterial plasma NO_2^- at 0.5 and 3 min were significantly larger ($p < 0.01$, by ANOVA followed by Tukey's method) than those in venous plasma NO_2^- . As shown in Fig. 5, changes in blood pressure significantly correlate ($p < 0.01$) with changes in arterial plasma NO_2^- levels ($r = 0.90$), but not with changes in venous plasma NO_2^- levels ($r = 0.37$).

Plasma NO_3^- levels in the vehicle control group (32.1 ± 9.0 μM in arterial blood and 28.8 ± 7.6 μM in venous blood at time 0) showed small and insignificant changes in arterial and venous blood. However, plasma NO_3^- levels after NOR1 injections (32.2 ± 5.9 μM in arterial blood and 31.5 ± 5.6 μM in venous blood at time 0) tended to decrease with time, and the decreases in venous plasma were significantly greater than those in the vehicle control group at 15 and 30 min (Fig. 4).

A-V differences in NO_2^- levels in control conditions were evaluated in 54 rabbits, including those in the series of experiments described above. As shown in Fig. 6, arterial NO_2^- concentrations (751 ± 107 nM) were slightly—but significantly ($p < 0.05$, paired t -test)—higher than venous NO_2^- concentrations (624 ± 78 nM), while no such A-V relationship was observed for either NO_3^- or NO_x concentrations.

DISCUSSION

We performed a standard kinetic study and provided fundamental kinetic parameters for NO_2^- *in vivo*. This knowledge will be very useful for future studies. The major finding of our study was that A-V differences exist in NO_2^- kinetic parameters, as well as in steady state NO_2^- levels.

The half-life for plasma NO_2^- during the elimination phase (β phase) was 34.8 min for arterial plasma and 26.1 min for venous plasma, and no significant A-V difference was observed. These half-lives are 2—3 times longer than those (11, 12 min) reported in *ex vivo* experiments,^{25,26} as well as those

Fig. 4. Effect of NOR1 on Hemodynamics and Plasma NO₂⁻ and NO₃⁻ Concentrations

NOR1 (500 nmol/kg) was intravenously injected into anesthetized rabbits ($n=6$, 2.92 ± 0.06 kg in body weight) and changes in hemodynamics (left upper and lower), plasma NO₂⁻ and NO₃⁻ concentrations (right upper and lower) were plotted against time. The arterial blood gas analysis at time 0 was as follows: pH 7.44 ± 0.01 , PO₂ 84.7 ± 8.5 mmHg, and PCO₂ 32.2 ± 1.6 mmHg. The vehicle control group received 0.1 ml/kg intravenous DMSO ($n=6$, body weight 2.95 ± 0.04 kg). The arterial blood gas analysis for the control group at time 0 was as follows: pH 7.45 ± 0.1 , PO₂ 88.4 ± 5.4 mmHg, and PCO₂ 30.1 ± 1.7 mmHg. HR: heart rate, mBP: mean blood pressure. *, ** Significantly different from the vehicle control group ($p < 0.05$ and $p < 0.01$, respectively, by unpaired *t*-test in changes in HR and mBP, and by ANOVA followed by Tukey's method to detect differences in ΔNO₃⁻ and ΔNO₂⁻). ## Significantly different from venous blood receiving the same treatment ($p < 0.01$, by ANOVA followed by Tukey's method to detect differences in ΔNO₂⁻).

Fig. 5. Relationship between Changes in Mean Blood Pressure (mBP) and NO₂⁻ Levels after NOR1 Administration

Changes in NO₂⁻ (ΔNO₂⁻) were plotted against changes in mean blood pressure (ΔmBP) (percent change from the control) 0.5, 3, and 15 min after NOR1 was administered. Data (three points from each rabbit) were taken from the results depicted in Fig. 4. A significant linear relationship ($r=0.90$, $p < 0.01$) was found in arterial blood only.

found in normoxic and hypoxic newborn lambs (8–9 min).¹⁹ However, they are similar to the 42.1-min half-life found in a study of healthy humans.²⁰

Information regarding the volume of distribution for NO₂⁻ *in vivo* is very limited. In newborn lambs, steady-state volumes of distribution (Vdss) are reported to be 0.209 l/kg and 0.215 l/kg in normoxic and hypoxic arterial blood, respectively, after central venous bolus injections of NO₂⁻, with no significant differences recognized in spite of the different oxygenation states.¹⁹ Arterially injecting NO₂⁻ into healthy human volunteers was reported to reveal the volume of distribution of the central compartment to be about 0.073 l/kg, and that during steady state to be about 0.355 l/kg in venous blood.²⁰ The results of our study are not markedly different from these studies, and add new and detailed knowledge to this area, especially with regard to simultaneous examinations of arterial and venous blood. Significant A-V differences were found in Vd1 and Vd2; Vd1 was smaller, and Vd2 was larger in the arterial system. Deducing from the results, changes in NO₂⁻ levels may be larger in arterial blood

Fig. 6. A-V Differences in Plasma NO₂⁻ Concentrations

NO₂⁻ levels evaluated during the control condition (before administration of agent) in arterial and venous blood are depicted. Data were obtained from 54 rabbits, including those used in preliminary and other studies. Body weight was 3.3 ± 0.1 kg, and mean blood pressure and heart rate were 100.9 ± 2.5 mmHg and 282 ± 5 beats/min, respectively. NO₂⁻ and NO₃⁻ concentrations in arterial blood (60.5 ± 5.5 , 61.3 ± 5.6 μM, respectively) were not significantly different from those in venous blood (60.3 ± 5.5 , 60.8 ± 5.6 μM, respectively). Data obtained from individual rabbits are linked by bars and large open circles with error bars indicate mean $\pm$ S.E.M. ** $p < 0.01$ by paired *t*-test.

(smaller Vd1) than in venous blood when changes in NO formation (and consequently changes in NO₂⁻) occur *in vivo*, because rapid change is reflected more in the central compartment and the concentration is expressed as the dose divided by the volume.

The apparent volumes of distribution of Vd1 in both arterial (about 0.158 l/kg) and venous blood (about 0.295 l/kg) exceeded the physical volume, which was estimated to be 0.048 l/kg (assuming that circulating blood volume is 8% of body weight and hematocrit is 40%). Therefore, we strongly suggest that NO₂⁻ is being distributed into spaces other than plasma. It should be noted that our estimated values are not far from the extracellular fluid volume (20% of body weight), and the rapid distribution of NO_x into RBCs,²⁷ and corre-

spondingly higher NO_2^- concentrations in RBCs,²⁸⁾ may contribute to the apparent volume of distribution. In addition, a substantial fraction of Vd_2 may be ascribed to tissue. Indeed, much higher (approximately two orders of magnitude) concentrations of NO_2^- have been reported in cardiac muscle,²⁹⁾ lung tissue,^{30,31)} and kidneys,³¹⁾ where uptake^{25,32)} and consumption of NO_2^- for further metabolic processes^{14–16)} takes place rather slowly.

The elimination constant (k_{el}) in arterial blood is significantly larger (approximately double) than it is in venous blood. However, no such A-V difference was noted in total clearance (from the entire body: the sum of the arterial and venous systems), as estimated by AUC (area under curve calculated as $A/\alpha + B/\beta$). Therefore, A-V differences in the respective k_{els} may not reflect actual elimination. A-V differences in Vd_1 , where blood sampling took place, should be taken into account. As arterial and venous systems are not independent, but continuous, an apparent decrease in NO_2^- based on an increase in apparent Vd_1 from peripheral tissue into venous deoxygenated blood may contribute to the apparent A-V difference in k_{els} , and *vice versa*. Therefore, it is difficult to properly elucidate the respective differences in arterial and venous k_{els} . Although A-V differences were recognized in both Vd_1 and Vd_2 , the sum of these compartments (Vd_1 and Vd_2), the volumes of distribution in steady state, are very similar in arterial and venous systems. This fact may support the above idea that apparent difference between arterial and venous k_{el} does not reflect real elimination in respective systems. To confirm this possibility, based on AUC, we estimated that the total clearance was similar in the arterial and venous systems. This strongly suggests that the A-V difference in k_{els} might be a reflection of the intrasystemic movements (or shifts) of NO_2^- through continuous arterial and venous systems with different Vd_1 values. The causes of this phenomenon are unknown at this point. However, as we mentioned previously, RBCs are a major site of NO_2^- storage in the blood,²⁸⁾ and the oxidoreductase activity of hemoglobin (oxygenation state-dependent) reduces NO_2^- to NO ,¹⁴⁾ which may be incorporated into other NO-related compounds,^{28,33,34)} contributing to the shift.

We performed a rough estimate of the plasma NO_2^- levels that we could expect using these clearance values and assuming that the constant production of NO from endothelial cells and the constant conversion to NO_2^- at a constant ratio occurs in both arterial and venous systems. The calculated constant for arterial NO_2^- levels (93.3) tended to be higher than that for venous levels (79.9), but without statistical significance. Although biological systems are too complex to allow for simple estimations, this may provide indirect support for A-V differences (higher arterial NO_2^- levels) in plasma NO_2^- in control conditions. The above considerations explain, at least in part, the differences in arterial and venous NO_2^- levels at steady state; further, they predict larger and more easily detectable changes in NO_2^- levels in arterial blood than in venous blood. To examine whether this latter speculation was operative *in vivo*, we injected NOR1 as a source of authentic NO. This compound decreased blood pressure for a short time, as was expected by its short half-life (1.8 min) at pH 7.4.³⁵⁾ This was accompanied by an increase in plasma NO_2^- levels, in which arterial concentrations were significantly higher than venous concentrations. In addition, there was a

significant linear correlation between changes in blood pressure and changes in arterial NO_2^- levels. This supports the idea that circulating NO_2^- can be used as an indicator of NO produced by endothelial cells.^{11,12)} However, with regard to our results, this is true only for arterial blood.

As NOR1 is known to release 1–1.5 mol of NO per mol administered (according to the technical note in the Dojindo catalogue), a 500 nmol/kg is approximately comparable to 500 nmol/kg NO_2^- (the dose applied in the kinetic study) once all of the NO is converted to NO_2^- . However, the actual changes in NO_2^- levels following NOR1 administration was unexpectedly low: the maximum increase in arterial NO_2^- level was 114 nM (about 2.5 μM by NO_2^- loading study in Fig. 1), which was obtained after 0.5 min of NOR1 (Fig. 4). The extreme complexity of NOR1 and NO chemical reactions in a biological system could contribute to the difference, and interpretations and explanations are difficult at present.

Although the significant decrease in NO_3^- after NO production seems paradoxical, we have experienced similar phenomena in humans^{7,8)}; however, the underlying mechanisms remain unclear.

In the first NO_2^- loading study, NO_2^- in a dose of 500 nmol/kg did not reduce blood pressure. In this case, conditions such as NOS inhibition may help development of vasodilation by the smaller doses of NO_2^- ,³⁶⁾ because small effect of exogenous NO_2^- could not overcome the regulation by intrinsic NO. In addition, the small amount of NO from such lower NO_2^- levels would not necessarily decrease blood pressure when the balance between NO generation and scavenging capacity of oxyHb may be responsible for NO availability for vasodilation.³⁷⁾

Indeed, extremely high doses of NO_2^- (10 mg/kg body weight of NaNO_2 , corresponding to 145 $\mu\text{mol/kg}$,¹⁹⁾ and 150 $\mu\text{mol/kg}$ in our preliminary study) did decrease blood pressure in anesthetized animals.

In conclusion, A-V differences in plasma NO_2^- levels exist not only in the NO_2^- loading condition, but also at steady state. The estimated kinetic parameters highlighted distinct A-V differences, and predicted that changes in NO_2^- would be more easily detectable in arterial blood than in venous blood. This was supported by our finding that after administration of authentic NO *in vivo*, arterial NO_2^- levels correlated with changes in blood pressure, while venous NO_2^- levels did not. Therefore, samples of arterial blood, on the basis of A-V differences in kinetic features, should be suitable for quantifying NO_2^- as an indicator of NO formation *in vivo*.

Limitation of this study: Both arterial and venous NO_2^- concentrations at the control state in NO_2^- loading study (Figs. 1, 2) were significantly ($p < 0.05$) higher than those in NOR1 loading study (Figs. 4, 5). It is statistically indicated that these groups may not belong to the same population, though these values are within reported ranges in rabbits (0.3–2.0 μM)²¹⁾ and in humans (0.5–3.0 μM).³⁸⁾ Therefore, attention may be required to interpret these data on the same basis.

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