Forum Minireview

New Methods to Evaluate Endothelial Function: A Search for a Marker of Nitric Oxide (NO) In Vivo: Re-evaluation of NOx in Plasma and Red Blood Cells and a Trial to Detect Nitrosothiols

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Abstract. Although plasma NOx (NO\textsubscript{2}\^- and NO\textsubscript{3}\^-) has been used as an index of nitric oxide (NO) formation in vivo, many unreasonable results appeared even after active elimination of NOx contamination from laboratory ware. For example, plasma NOx concentrations did not increase during vasodilation mediated by the NO/cGMP pathway or after organ perfusion. A possible shift of NOx from plasma to erythrocytes (RBCs) as a cause of these phenomena has been excluded, leaving the destination of NOx (after leaving plasma) unknown. Kinetic analyses have revealed that steady state NOx concentrations in plasma and whole blood did not correlate with the NOx formation rate, but rather with the NOx elimination rate. Therefore, the supposition that the NO status is directly reflected by plasma NOx concentrations appears untenable. As nitrosothiols (R-SNOs), possible carriers of NO bioactivity, have been flagged as alternative indices of NO status in vivo, efforts have been made to detect these substances. When interference by ultrafiltration was eliminated, low molecular weight R-SNOs such as nitrosocysteine and nitrosogluthathione were undetectable. However, a high-molecular weight R-SNO, nitrosoalbumin, was detected in human blood. Further research is required into the significance and practical use of nitrosoalbumin as a marker of NO in vivo.

Keywords: cardiovascular system, nitric oxide (NO), NOx (nitrite and nitrate), nitrosothiol, blood

Introduction

Nitric oxide (NO) from endothelial cells is widely recognized as an essential regulator of vascular tone. Endothelial dysfunction involves reduced production or bioavailability of NO (1, 2), and hence markers of NO in vivo representing its regulatory status by the endothelial function-NO-cGMP system in the whole body are subjects of intensive search (3 – 5). Stable metabolites of NO (NOx: NO\textsubscript{2}\^- and NO\textsubscript{3}\^-) have been frequently used as a tentative index of NO formation since the early days of NO research because of the established simple procedure (Griess reaction) for their measurement. However, many confounding factors that affect NOx levels in biological fluids or the NOx measurement procedure became apparent. Our studies to clarify the regulatory status of NO in vivo by eliminating these factors encountered several conflicting results. Thus, there was a need for critical re-evaluation of the blood NOx concentration for use as an index of NO formation in vivo. In this review, we will consider problems arising from the use of blood NOx concentration under many circumstances and examine whether or not NOx is feasible for this purpose.

Recent advances have focused on NO-derived molecules that stabilize the bioactivity of NO for remote delivery of NO and oxygen, and these substances are expected to reflect the regulatory status of NO (3, 5 – 8). Therefore, we will also discuss detection of high- and low-molecular weight nitrosothiols as alternative indices of NO in vivo.

NOx measurement

Sample processing and elimination of NOx contamination

For accurate determination of NOx, it is necessary to
eliminate NOx contamination from every step. As sterilized syringes and vacuum sampling tubes can be contaminated with NOx (9, 10), it is strongly recommended to use equipment with the lowest contamination possible. It is practically impossible to remove NOx contamination by washing (see below) under sterilized conditions. For blood sampling, a suitable anticoagulant should be selected, as it is well documented that heparin, but not ethylenediaminetetraacetic acid (EDTA), may interfere with the enzymatic reduction of NO$_3^-$ to NO$_2^-$ (11, 12). When heparin is used, other methods free from such interference (13) should be employed.

Without exception, laboratory ware for sample handling is contaminated with NOx (mainly NO$_3^-$) to various degrees (9). A model procedure for processing blood samples for NOx determination requires several pieces of laboratory ware (Fig. 1), and any contact of the sample with contaminated laboratory equipment causes an apparent (artificial) increase in NOx. Most NOx contamination originates from conical tubes and the disposable tips of a mechanical pipette in our model procedure (Fig. 2). It is not practical to seek out laboratory equipment with negligible NOx contamination, as the contamination level of individual pieces of laboratory ware cannot be predictable. Washing all laboratory ware with nominally NOx-free water is a practical and effective countermeasure to minimize the contamination (9). However, great caution has to be paid with respect to the type of water used. A reverse osmosis column is effective in reducing NOx levels in water and further treatment with an ion exchange column (for example, with MilliQ) reduced NOx to negligible levels (14, 15). Only fresh and confirmed pure water after passage through an ion exchange column is suitable for this purpose. However, the NOx concentration in fresh pure water is not always negligible (16) and careless use of pure water without confirmation of its purity, or use after a certain period (for example, overnight) without reconfirmation should be avoided. Use of ultrafiltration units to remove protein from plasma is a cause of heavy contamination (9, 16) that persists to a certain degree even after several washes with pure water (9). On the other hand, plasma NO$_2^-$ levels would fall after acidification for deproteinization, because reduced thiols in plasma could react with NO$_2^-$ to form S-nitroso compounds under acidic conditions (10).

**Fig. 1.** A model procedure for sample (blood) treatment. The sample was first collected in a sterilized disposable syringe and then transferred to a sterilized conical tube and mixed with anticoagulant before centrifugation. A portion of the supernatant was transferred to a small conical tube for preservation at $-80^\circ$C. The frozen sample was thawed and vortexed before transfer of a portion to another microcentrifuge tube for mixing with methanol for deproteinization. After centrifugation, the supernatant was obtained by decant and used for NOx determination. [based on our previous report (Ref. 9)]

**Fig. 2.** Origin of NOx contamination. In phosphate buffer subjected to the model procedure (Fig. 1), contamination by NO$_2^-$ (ranging from non-detectable to 0.02 $\mu$M) and NO$_3^-$ (0.35 to 1.349 $\mu$M) was confirmed. Another set of experiments revealed that each step of the procedure was subject to a varying degree of NOx contamination. NOx from each step was stacked from the bottom as per the procedure shown in Fig. 1. [based on our previous report (Ref. 9)]
Is NOx an indicator of NO formation?

Plasma NOx levels are widely considered to reflect NO formation in vivo. This is based on the fact that NOx is a stable metabolite of NO covering about 60–90% of the NO endogenously formed (17) or exogenously applied (18). However, despite special attention to eliminate NOx contamination throughout the procedure for NOx determination, our experience in utilizing plasma NOx as an index of NO formation has sometimes resulted in the unexpected results described below.

NOx gradient across the coronary circulation: We previously investigated whether levels of NOx in the coronary circulating blood reflect endothelial dysfunction due to coronary atherosclerosis (13). In that study, NOx levels in plasma obtained from the ostium of the left coronary artery were compared with that in plasma from the coronary sinus of patients with or without coronary atherosclerosis. It is generally recognized that perfusion of a certain vascular bed is expected to result in some increase in NOx concentration in the perfusate due to basal endothelial NO release (19). It may result in a higher concentration of NOx in venous blood than in arterial blood in the local (organ) circulation. A reduced basal activity of NO has been found in human atherosclerotic epicardial and microvascular coronary arteries (20). Based on the above background, we anticipated a net increase in NOx concentration across the normal coronary circulation, which could be attenuated in atherosclerotic coronary circulation. However, to our surprise, the mean arterio-venous NOx concentration gradient (NOx gradient) across the coronary circulation without atherosclerosis was not positive, but rather was zero. Furthermore, the difference in NOx concentration across atherosclerotic coronary circulation was negative. These results are in accordance with another report (21) and our successive observations (22–24) that the NOx gradient across the coronary circulation is around zero in the healthy condition and is significantly negative in diseased conditions. Although the absolute value of the NOx gradient across the coronary circulation is difficult to explain, the relative values of the NOx gradient indicate an interesting relationship between coronary endothelial dysfunction and NOx. Our subsequent study (24) revealed that the Gensini Score, an index of the extent and severity of atherosclerosis, in the left coronary artery negatively correlated with the NOx gradient and positively correlated with the arterio-venous difference of serum amyloid A protein (SAA), a marker of inflammation. Moreover, the NOx gradient showed a significant negative linear relationship with the arterio-venous difference of SAA. These results indicated that the severity and extent of coronary atherosclerosis was related to the degree of local inflammation, which has a possible association with coronary endothelial dysfunction as indicated by the relative decrease in the NOx gradient.

In patients with vasospastic angina, basal coronary artery tone at the spastic site shows a negative linear correlation with the NOx gradient; the greater the basal arterial tone, the more negative the NOx gradient becomes (22). A similar negative relationship was recognized between basal coronary artery tone and the dose of ergonovine required for spasm provocation, leading to a positive relationship between the dose of ergonovine and the NOx gradient (22). These analyses indicated that a negative NOx gradient would be a qualitative and quantitative representation of coronary endothelial dysfunction in vasospastic angina. Furthermore, we have shown that the arterio-venous difference of ADMA (asymmetric dimethylarginine) negatively correlates with the NOx gradient (arterio-venous difference) (23). Since ADMA is known to be an endogenous competitive inhibitor of NO synthase and since high concentrations of ADMA are thought to be a cause of endothelial dysfunction (25) in patients with vasospastic angina, elevated ADMA may interfere with arginine to reduce NO formation, leading to a higher susceptibility to spasm provocation with elevation of basal coronary artery tone and a (relatively) reduced NOx gradient.

The above interpretations of the NOx gradient across coronary circulation are based on the NOx gradient relative to zero in control (healthy) coronary circulation. Indeed, absolute values in diseased coronary circulation were negative and hard to reconcile with the notion that there should be a net increase in NOx concentration across normal coronary circulation (positive gradient), which could be attenuated in diseased coronary circulation (positive gradient was still anticipated). These discordant findings lead us to suspect that some basal (fixed) amount of NOx disappears across both healthy and diseased coronary circulation. However, a possible sink for the NOx from plasma does not appear to be RBCs, as has been suggested by Recchia et al. (26) (described below).

These observations led us to expect definitive positive findings of elevation of plasma NOx concentrations under conditions such as enhanced NO formation by endothelial cells or by exogenous administration of NO-releasing vasodilators. Changes in NOx induced by arginine infusion: Enhanced NO formation by endothelial cells was induced in patients with type 2 diabetes mellitus in whom endothelial dysfunction was suspected and in patients without diabetes mellitus (including healthy volunteers), presumably free from endothelial dysfunction (27).
Intravenous infusion of L-arginine (30 g/300 ml for 30 min), a substrate of NO formation, resulted in a fall in arterial blood pressure with a concomitant increase in plasma cGMP, a second messenger downstream to activation of nitric oxide synthase, in non-diabetic patients. Similar, but smaller changes in the depressor response and an elevation of plasma cGMP were observed in diabetic patients, indicating endothelial dysfunction in these patients. However, contrary to our expectation, plasma NOx concentration decreased in both diabetic and non-diabetic patients. This is not surprising given the findings of early reports; in human subjects, the hypotensive effect of L-arginine was not associated with a substantial increase in plasma NOx (28 – 30), although increased NO2− (only a few percent of total NOx) has been reported (31). Increased NOx has been reported mainly in urine after L-arginine (28, 30, 32, 33), but not in plasma. Therefore, part of the paradoxical decrease in plasma NOx may be explained by “NOx escape” to urine, although quantitative evaluation is required.

Changes in NOx induced by ISDN: Our second investigation in which we anticipated an increase in plasma NOx was performed by infusion of a NO-releasing vasodilator, isosorbide dinitrate (ISDN), and comparison of this with the effect of a calcium channel blocker, nicardipine, in human subjects (34). Since ISDN is known to be a more effective vasodilator in veins than arteries, special attention has been paid to whether the venodilating effect of ISDN is associated with a preferential increase in plasma concentrations of NOx in venous compared to arterial blood. As expected, nicardipine decreased blood pressure (mainly by arterial dilatation), but did not affect either arterial or venous plasma NOx concentrations at all. However, the ISDN-induced venodilatory response was accompanied by a decrease in the NOx concentration in both arterial and venous blood. Interestingly, the decrease in total NOx consisted of two contrary components: a small increase in NO2− compensated for by a large decrease in NO3−. Further analysis revealed that plasma NO2− increased in the pulmonary circulation; that this increase was preserved after nicardipine and ISDN; and that ISDN, but not nicardipine, increased plasma NO3− in the pulmonary circulation. At present, these relationships have not been comprehensively elucidated. Nonetheless, in response to ISDN, we failed to detect either an increase in plasma NOx or a higher concentration of NOx in venous blood than arterial blood as we had initially expected.

Possible accumulation of NOx in RBCs: The absence of an increase in plasma NOx was confusing; however, Recchia et al. (26) have suggested an explanation that may provide a theoretical solution to the derangement. They reported that NOx accumulates into RBCs at a higher concentration than that in plasma (RBCs/plasma = 4.4 – 14.6) and that a small change in the NOx distribution ratio between RBCs and plasma (mainly regulated by plasma HCO3−) might result in a large change in plasma NOx concentration. If such a mechanism is operative under our conditions, it might explain our inconsistent observations. However, our investigation subsequent to their report failed to detect such a high NOx concentration in RBCs (RBCs/plasma = 0.5 – 0.8) (35). This difference may result from different procedures employed in the removal of hemoglobin that interferes with the Griess reaction product at 540 nm from the hemolyate (for determination of whole blood NOx). Recchia et al. used ultrafiltration for this purpose (26). However, we did use an ODS column in line to remove hemoglobin, as the membrane of the ultrafiltration unit is known to be heavily contaminated with NOx (9, 16) as described above. NOx contamination in this step results in an over-estimation of whole blood NOx from which a much higher concentration of RBCs NOx concentration is derived. The possibility that plasma HCO3− or PCO2 might be a regulator of the NOx ratio (RBCs/plasma) was examined by hyperventilation. This maneuver in healthy volunteers resulted in increases in plasma pH and PCO2 with a concomitant decrease in PCO2 and HCO3−, denoting respiratory alkalosis. However, there was no change in the NOx ratio. In addition, it was clear that there was a linear relationship between the plasma NOx concentration and whole blood NOx concentration or RBCs NOx concentration (35). These observations indicated that NOx equilibrium is maintained between RBCs and plasma (no excessive accumulation into RBCs) and that the transporting mechanism modulated by HCO3− is not operative for the equilibrium.

The absence of an increase and possible attenuation in NOx concentration across hearts with healthy and atherosclerotic coronary circulation was examined further by determining NOx concentrations in both RBCs and plasma by blood gas analysis. However, there was no escape of NOx from plasma to the RBCs in either circumstance and the NOx distribution in both compartments had no relationship with HCO3− (our unpublished observation).

Therefore, in our experience, the destination of NOx escape would appear to be compartments other than RBCs and the nature of the changes in plasma NOx remains obscure. Thus, it would not be appropriate to use plasma or whole blood NOx concentration as a marker of acute changes in NO in vivo even after careful elimination of NOx contamination from all laboratory equipment.

NOx kinetics and steady state NOx concentration:
Another use of plasma NOx is for estimation of whole body basal endothelial NO formation by assessing the steady state NOx concentration. Plasma NOx concentration is confounded by many factors (36), and no informative data would be derived from random blood sampling due to the large variations reflecting different contributions of such factors at any point in time (Fig. 3). Thus, the steady state NOx concentration is required to eliminate the influence of exogenous NOx as much as possible. The largest factor influencing plasma NOx concentration is the NOx contained in food and beverages and as such, can be controlled. Although a hospital diet for several days or an overnight fast has sometimes been regarded as the “control” or “steady” state (37, 38), we should be aware that these conditions do not necessarily reflect the actual steady state (Fig. 4). Plasma NOx concentration after an overnight fast mostly depends on the amount of NOx taken before the fast and the renal excretion rate (described later). Since there are no published data concerning the NOx content of Japanese daily food and beverages, we have constructed such a list of NOx content for over 200 food and beverage items (14). No firm notion of how to achieve a NOx steady state has ever been suggested, and thus we had to determine how to establish a steady state with the aid of the list. We set two conditions for each subject: one commenced at a higher NOx concentration (NOx intake approximately 370 μmol/day) and decreased to the steady state, and the other commenced at a lower NOx concentration (NOx intake <100 μmol/day) and increased to the steady state. NOx concentrations under these two conditions converged to a similar level (steady state), and we confirmed that after a 13-h fast subsequent to a diet containing NOx content <100 μmol/day, the plasma NOx concentration reached the steady state (Fig. 5). This means that when the last meal was taken at 18:00, the steady state could be expected at 07:00.
the next morning. After the steady state was attained, a known amount of NOx was administered and chronological changes in blood NOx concentration were monitored and analyzed to elucidate whole body NOx kinetic parameters. Our preliminary kinetic data on plasma NOx, about 0.26 l/kg volume of distribution (Vd) and about 0.13/h elimination constant (Kel), were in good accordance with those determined by others: 0.28 – 0.33 l/kg Vd and 0.09 – 0.17/h Kel (4, 39, 40), and these parameters were further used for estimation of the NOx formation rate. A theoretical equation is used to obtain steady state drug concentrations by continuous drug infusion:

\[ \text{NOx}_{\text{formation}} = C_{\text{ss}} \times \text{Kel} \times \text{Vd} \]

where \( \text{NOx}_{\text{formation}} \) is a NOx formation rate that was the original drug infusion rate, and \( C_{\text{ss}} \) is a steady state NOx concentration.

According to the equation, we determined a NOx formation rate of about 0.5 \( \mu \text{mol/kg-h} \), in concordance with values of NO formation estimated in anesthetized and awake rats (0.33 – 0.85 \( \mu \text{mol/kg-h} \)) using isotope-labeled oxygen (41), in humans (0.38 – 0.96 \( \mu \text{mol/kg-h} \)) using isotope-labeled oxygen (42) or arginine (43), or by theoretical analysis without isotope-labeled substrate (44). It should be noted that the NOx formation rate does not necessarily reflect NO formation directly in vivo; however, it would be acceptable as an alternative measure when the real NO formation rate is hard to evaluate. Determination of the parameter in several volunteers enabled us to examine relationships among kinetic parameters such as NOx formation rate, elimination rate, and steady state. The results were interesting in that there was no significant correlation between NOx formation rate and the steady state NOx concentration, but there was a significant linear correlation between NOx elimination rate and the steady state NOx concentration. A similar case was observed when NOx kinetics were evaluated in whole blood and in RBCs. This analysis indicates that the steady state NOx concentration established after a NOx-restricted diet followed by a 13-h fast depends on NOx elimination, but not on its formation. Indeed, we have found a significant linear relationship between the steady state NOx concentration and serum creatinine concentration, a rough index of renal function. The same result was reported from another laboratory, indicating that studies in which the NOx concentration is used as an index of NO production can be interpreted only if renal function has been taken into consideration (45). From this perspective, it is understandable that plasma NOx levels in patients with diabetes mellitus, in whom endothelial dysfunction was suspected, were paradoxically higher than those in non-diabetic patients (37). It may be due to possible diabetic renal dysfunction.

The above considerations led us to the conclusion that in spite of active exclusion of many confounding factors, NOx concentration in plasma could not be used as an index of NO formation. We are not able to estimate how much NO is transformed to NO\(_3^-\) (and/or NO\(_2^-\)) and to what extent the NOx escape from blood occurs under various conditions. In addition, the blood NOx concentration largely depends on renal excretion rather than NOx formation. Plasma NOx concentration may therefore be of use if restricted to the monitoring of disease progression or response to therapy in individual patients or subgroups (46) at the steady state, after taking renal function into consideration (45).

**Detection of nitrosothiols**

Since blood NOx concentration does not seem to be a reliable index of NO formation, intrinsic NO carriers, nitrosothiols (R-SNOs) have been subjected to intensive investigation as an alternative measure of the regulatory status of NO in blood (6). They include S-nitrosocysteine (Cys-SNO) and S-nitrosoglutathione (G-SNO) as low-molecular weight nitrosothiols (LMWR-SNOs) and S-nitrosoalbumin (Alb-SNO) as a high-molecular weight nitrosothiol (HMWR-SNO). However, agreement has not been established yet about their basal levels, or the existence of some R-SNOs in human plasma under basal conditions. A major problem in assessing plasma R-SNOs is the separation and quantification of individual substances. We have employed a device combining HPLC for separation and Saville’s method for quantification to measure R-SNOs (47). Since two different sets of apparatus (mainly depends on the separation column) and different sample treatments are required for determination of LMWR-SNO and HMWR-SNO, our observations will be described separately.

For determination of LMWR-SNO, the sensitivity and discrimination ability of the apparatus equipped with a C\(_{18}\)-reverse phase column was 1 – 2 nM for both Cys-SNO and G-SNO. As we have previously reported in a preliminary observation, our attempts to detect LMWR-SNOs in human plasma encountered false positive results (3). After treatment of the plasma sample with a common ultrafiltration unit (with 5,000 \( \times \) g) to remove high molecular substances, a peak of unknown nature appeared at the position of G-SNO. This signal behaved as real nitrosoglutathione in that it was partially Hg\(^{2+}\)-sensitive and decayed with time. However, the use of pre-washed units or another ultrafiltration unit that required a lower g force (1,800 \( \times \) g) or direct application of plasma to the HPLC-Saville’s system did
not yield such a signal. Thus, we must be cognizant of the interference that originates from the ultrafiltration unit in the precise quantification of LMWR-SNOS, and there was no G-SNO detectable (or <1 nM) in human plasma, in accordance with recent reports (7, 48, 49).

Our next trial to detect Alb-SNO was performed using a HPLC-Saville’s system equipped with a gel filtration column. The detection limit was 5 nM and discrimination ability was 1 nM. This sensitivity would be expected to be sufficient to quantify reported levels (several tens of nM or more) of endogenous Alb-SNO (5, 8, 50). However, the signals obtained in our study from human plasma were too small to quantify. Thus, we devised a method to amplify the signal and summation of several sweeps of the chromatogram of a sample enabled us to quantitatively determine Alb-SNO levels. Values we obtained were 3 to 5 nM in arterial blood and lower in venous blood. Although the arterio-venous difference of Alb-SNO in this preliminary result was significant, a different value has been reported (7). Further examination is required in this regard. Because this trial to detect Alb-SNO did not take into account some considerations, such as use of EDTA that promotes degradation of R-SNO in plasma and non-use of MEN (N-ethylmaleimide) to protect –SH (5), and because improvement of sensitivity of the apparatus is needed, we are attempting various strategies to improve the results.

At present, Alb-SNO is the only remaining candidate as an index of NO status in vivo.

Appendix

*: During the editorial process of this manuscript, a report outlined here was accepted for publication (Tashimo O, Ishibashi T, Yoshida J, et al. Interference with Saville’s method in determination of low-molecular weight S-nitrosothiols by ultrafiltration. Nitric Oxide, In press).

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