Indexes of NO Bioavailability in Human Blood

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Disturbances of nitric oxide (NO) bioavailability may play a key role in vascular dysfunction and in the development of atherosclerotic lesions. Thus assessment of a reduced NO bioavailability in human circulation is of particular interest. Here we summarize potential biomarkers of NO availability in human blood and critically discuss their respective significance and application fields.

Previous experimental studies have demonstrated the crucial importance of the endothelium in the regulation of vascular homeostasis (9) by participating in different metabolic, synthetic, and regulatory pathways. Normal endothelial function includes control of antithrombotic and thrombolytic activity, vascular architecture and permeability, leukocyte interactions with the vessel wall, and regulation of vascular tone during rest and exercise. In this context, several studies have suggested the particular importance of endothelial-derived nitric oxide (NO) (9). Disturbances of NO bioavailability have been suggested to play a key role in vascular dysfunction and the development of atherosclerotic lesions. Thus assessment of a reduced NO bioavailability in human circulation is of particular interest. A state of reduced NO availability in humans was traditionally assumed if a pathological vasoconstriction occurred following administration of acetylcholine in the dependent vascular bed. The specificity of this test was improved by simultaneous infusion of NO synthase (NOS) inhibitors, which unmasked the NOS-independent fraction of the acetylcholine response. However, using this approach, it has not been feasible to discriminate between alterations of NO production, NO inactivation, or NO sensitivity, which requires an additional measurement of NO. A direct measurement of NO and its adducts in humans causes considerable analytic difficulties due to the short half life and the rapid metabolism, which is still poorly understood. Only recently could this dissatisfying condition be improved. Here we summarize the present understanding of NO metabolism in human blood and its relevance for the assessment of NO bioavailability.

Balance of synthesis and decomposition of NO

NO is a soluble gas synthesized in various mammalian cells. NOS are the enzymes responsible for NO generation. To date, three distinct isozymes have been identified: neuronal NOS (type I), inducible NOS (type II), and endothelial NOS (type III) [for further details, the reader is directed to recent reviews (2)]. NOS isoforms catalyze an overall five-electron oxidation of one $N^\alpha$ atom of the guanidino group of L-arginine to NO and L-citrulline with the intermediate $N^\alpha$-hydroxy-L-arginine (NOHA; Ref. 2; see Fig. 1). NO synthesis is critically influenced by various cofactors like tetrahydrobiopterin, flavin mononucleotide, and flavin adenine dinucleotide, the presence of reduced thiols, endogenous NOS inhibitor asymmetric dimethylarginine (ADMA), and substrate availability. Additionally, NOS I and III are dependent on calmodulin and Ca$^{2+}$. In biological systems, the mode and rate of NO elimination depends on its concentration, its diffusibility, and the concentration of other bioreactants (see Fig. 1). In principle, NO may react by electron gain to form the nitroxy anion NO$^{-}$ and the electron loss to form NO$^+$, the nitrosionium ion. Various metabolic routes and reactions contribute to the breakdown and conversion of NO, NO$^+$, and NO$^{-}$, e.g., heme proteins such as guanylate cyclase, catalase, xanthine oxidase, superoxide dismutase, and hemoglobin (Hb), or high-energy free radicals such as the hydroxyl radical or carbon-, oxygen-, and nitrogen-centered radicals (4). The charge neutrality of NO presumably facilitates its free diffusibility in aqueous solution and across cell membranes. This is a prerequisite for NO to travel significant distances and to enter the blood vessels. Matters are further complicated by the fact that, due to a variable plasma/blood cell ratio, the metabolic routes for NO in human blood are likely to vary along the vascular tree (1). Thus, to help guide the reader through these various reactions, it is useful to differentiate between the compartment of blood plasma and that of the erythrocytes (RBCs).

Metabolism of NO in plasma

The major immediate breakdown product of NO in human plasma is nitrite (NO$_2^{-}$; Fig. 2). In vitro studies revealed that, in the presence of oxygen, NO is rapidly oxidized to NO$_2^{-}$, following pseudo-first order kinetics with a strict 1:1 stoichiometry (12). Plasma NO$_2^{-}$ could be taken up by RBCs, where it is oxidized in a Hb-dependent manner to nitrate (NO$_3^{-}$), which may subsequently redistribute into plasma (6). Another potential decomposition pathway for NO is its rapid interaction with superoxide anions to produce the potent oxidant peroxynitrite (ONOO$^{-}$). ONOO$^{-}$ is thought to oxidize thiols or thioethers, nitrating tyrosine residues, nitrating and oxidizing guanosine, degrading carbohydrates, initiating lipid peroxidation, and cleaving DNA. The ONOO$^{-}$ in excess decomposes to yield NO$_3^{-}$.

Alternatively, NO can react with O$_2$ to yield reactive intermediates. It is well appreciated that the autoxidation of NO in an aqueous environment leads to the formation of reactive nitrogen oxide species such as dinitrogen trioxide (N$_2$O$_3$). This
intermediate can nitrosate as well as oxidize different substrates to yield either nitrosamines or S-nitrosothiol adducts (RSNO). Studies in experimental animals revealed that redox-active thiols, which are abundantly present in plasma, can incorporate NO and transport it throughout the mammalian circulation in the form of bioactive RSNOs (10). Plasma RSNOs are subdivided into low-molecular-weight (S-nitroso-glutathione and S-nitrosocysteine) and high-molecular-weight [S-nitrosoalbumin (SNOAlb)] nitrosothiols. In the presence of oxygen, SNOAlb is thought to represent the major reaction product of NO with plasma thiols (10). Although there is no doubt as to its existence in vivo, mechanisms of formation and subsequent release of NO from SNOAlb and other RSNOs are poorly understood.

Metabolism of NO in RBCs

The second major compartment for NO metabolism in blood is represented by the RBCs. NO is metabolized in the RBCs by direct interaction with Hb (see Fig. 2). Depending on the oxygenation state of the heme protein, three routes of NO interactions are envisioned. In aqueous solution, NO reacts rapidly with oxyhemoglobin (oxyHb) to form NO$_3^-$ and methemoglobin with a second-order rate constant of $3.4 \times 10^7$ M$^{-1}$s$^{-1}$ (6). Although this reaction has appreciated widespread recognition as the major inactivation pathway of NO in vivo, recent results obtained in humans suggest that this may not be valid under all conditions (7). Of particular importance may be that the reaction rate of NO with oxyHb within the RBCs is limited by its diffusion into the cell and thus occurs 650 times slower compared with the reaction with free oxyHb. Alternatively, NO may bind to the heme group of deoxyhemoglobin (deoxyHb) to form nitrosylhemoglobin (NOHb) (14). The latter has been detected in the blood of patients receiving nitroglycerin or inhaling NO gas (3, 5) and may interconvert, by reaction with the sulfhydryl group of the Cys93 of the $\beta$-Hb chains, to form $\beta$-nitrosylated Hb (SNOHb) (11) or slowly degrade to NO$_2^-$ (14). The formation of SNOHb may also result from a direct reaction of NO, or of a higher oxidation product such as NO$_2^-$ or N$_2$O$_3^-$, with Cys93 of the $\beta$-Hb chains. The ratio of these three different reactions of NO with Hb is dependent on PO$_2$. Although no systematic investigations, e.g., by stepwise increasing PO$_2$, are available, it has been confirmed that exposure of NO to venous blood results in the formation of more NOHb and less NO$_3^-$ compared with arterIALIZED blood, in which more NO$_3^-$ and less NOHb was measured. Moreover, the oxygenated status of Hb facilitates the formation of SNOHb. However, while the reactions of NO with oxyHb and deoxyHb are well characterized (6, 14), the potential role of $\beta$-Cys93 nitrosation by NO has so far been established in animal models only and challenged in humans (3).

Indexes of NO bioavailability

In general, total NO production is unlikely to be determined at the luminal surface of the endothelium in vivo. However,
stable reaction products that are formed in relation to NO may serve as an index of NO availability. In the following sections, such potential biomarkers in human blood, their respective significance, and their application fields are critically discussed with respect to the present literature (see Table 1).

**Amino acids.** Recently, the problem of assessment of NO synthesis in vivo was approached by measuring the NO-related amino acids L-arginine and L-citrulline and, in particular, the stable intermediate compound NOHA in the plasma of healthy volunteers by using high-performance liquid chromatography analysis (4). Plasma concentrations from different regions were similar and showed no gender- or age-related differences. In contrast, in patients with metabolic syndrome, a disease state known to be associated with endothelial dysfunction and reduced NO availability, plasma concentrations of NOHA were significantly reduced, whereas the plasma concentrations of the NO precursor L-arginine and the end product L-citrulline were unchanged. It was suggested by the authors that this was either caused by a decreased NOS III activity or by an increased breakdown of NOHA by pathways independent of NOS, resulting in a reduced availability of NOHA for NO synthesis. However, to assess the specificity of this approach, additional NOHA measurements under conditions of NOS III stimulation (e.g., with acetylcholine) and inhibition [e.g., N^G^-monomethyl-L-arginine (L-NMMA)] are necessary.

ADMA has been characterized as an endogenous, competitive inhibitor of NOS (13). In young hypercholesterolemic patients, elevated plasma ADMA concentrations were associated with an impaired endothelium-dependent vasodilation and reduced urinary NO_3^- excretion as surrogate parameters of NO bioavailability (1). Others reported a significant correlation between raised ADMA concentrations and intima media thickness in apparently healthy, middle-aged individuals (8).

**TABLE 1. Potential markers of NO bioavailability in human blood**

<table>
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<tr>
<th>Potential Biomarker</th>
<th>Application Field</th>
<th>References</th>
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<tr>
<td>ADMA</td>
<td>Surrogate parameter of endothelial dysfunction and risk indicator for the development of cardiovascular events in patients with renal failure</td>
<td>2, 15</td>
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<tr>
<td>NOHA</td>
<td>Reduced plasma levels in patients with cardiovascular risk factors vs. healthy controls suggest a relation to a decreased endothelial NOS activity; sensitivity and specificity as NO biomarker is still unclear</td>
<td>4</td>
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<tr>
<td>NO_2^-</td>
<td>Sensitive and specific marker of acute changes of NOS III activity in the human forearm vasculature</td>
<td>7</td>
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<td>NO_3^-</td>
<td>Rough estimate of total body nitrogen/NO turnover</td>
<td>6</td>
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<tr>
<td>RSNO, SNOHb, NOHb</td>
<td>Potential role in transport and delivery of NO</td>
<td>5, 10</td>
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ADMA, asymmetric dimethylarginine; NOHA, N^a^-hydroxy-L-arginine; NO_2^-, nitrite; NO_3^-, nitrate; RSNO, S-nitrosothiols; SNOHb, S-nitrosohemoglobin; NOHb, nitrosylhemoglobin.

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**FIGURE 2.** Potential routes of decomposition of NO in human blood. In plasma, NO may react with molecular oxygen to form nitrite (NO_2^-) or with superoxide (O_2^-) to form peroxynitrite (OONO^-), which subsequently decomposes to yield nitrate (NO_3^-). Alternatively, the nitrosonium moiety of NO may react with thiols to form nitrosothiols (RSNO). Furthermore, NO may reach the erythrocytes (RBCs) to react with either oxyhemoglobin to form methemoglobin (metHb) and NO_2^-, with deoxyhemoglobin to form nitrosylhemoglobin (NOHb), or with the Cys93 residue of the b-subunit to form S-nitrosohemoglobin (SNOHb). In addition, plasma NO_2^- could be taken up by RBCs, where it is oxidized in a Hb-dependent manner to NO_3^-; SNOAlb, S-nitrosoalbumin; GSNO, S-nitrosoglutathione; CysNO, S-nitrosocysteine, RSH, sulphydryl group.
Moreover, plasma ADMA concentrations in hemodialysis patients were recently identified as a strong and independent predictor of overall mortality and cardiovascular outcome (15). These studies provide increasing evidence that plasma ADMA levels are related to endothelial dysfunction and represent a risk indicator for the development of cardiovascular diseases, at least in patients with chronic renal failure. However, direct evidence for a link between NO bioavailability and plasma ADMA concentrations in the human circulation is lacking.

**Oxidative metabolites.** Traditionally, NOS II activity was assessed by determining the plasma concentrations of NO\textsubscript{3} or NO\textsubscript{x}, i.e., the sum of NO\textsubscript{2} and NO\textsubscript{3}. The rationale for this approach is based on the findings that NO is converted to NO\textsubscript{3} and NO\textsubscript{2} when inhaled or added to blood and that NO\textsubscript{2} is further oxidized to NO\textsubscript{3} by the Hb contained in red blood cells. Furthermore, circulating NO\textsubscript{3} concentrations are reduced by ~50% in NOS III knockout mice compared with controls. However, plasma NO\textsubscript{3} levels are influenced by a variety of NOS-independent factors, including dietary NO\textsubscript{3} intake, saliva formation, bacterial NO\textsubscript{3} synthesis within the bowels, denitrifying liver enzymes, inhalation of atmospheric gaseous nitrogen oxides, and renal function (6). Moreover, the high background concentration of NO\textsubscript{3} and its relatively long half-life compared with NO\textsubscript{2} raised the question as to the sensitivity of NO\textsubscript{3} and NO\textsubscript{x} for detection of NOS III activity. Therefore, the reliability of this approach was recently reassessed by our group (7). Plasma NO\textsubscript{2} and NO\textsubscript{3} concentrations were measured in blood samples from the antecubital vein and brachial artery of healthy volunteers and compared with forearm blood flow (FBF) during regional acetylcholine-induced NOS III stimulation with and without simultaneous NOS III inhibition with l-NMMA. It was found that NOS III stimulation increased NO\textsubscript{2} levels, which was paralleled by an augmentation of blood flow (Fig. 3), whereas an equieffective dose of papaverine, a NOS III-independent vasodilator, produced no change in NO\textsubscript{2} concentrations. Moreover, NOS inhibition reduced basal NO\textsubscript{2} levels and FBF and blunted acetylcholine-induced vasodilatation and NO release. In contrast, NO\textsubscript{3} and NO\textsubscript{x} levels were unaffected. Thus, whereas plasma NO\textsubscript{3} and/or NO\textsubscript{x} do not generally represent useful markers of endogenous NO production, plasma NO\textsubscript{2} reflects acute changes in regional NOS III activity. In future studies, plasma NO\textsubscript{2} measurements will help to further elucidate the pathophysiological significance of an altered NOS III activity in disease states known to be associated with endothelial dysfunction.

**Nitros(yl)ated metabolites.** It has recently been proposed that NO is stabilized by covalent binding with thiols such as glutathione, cysteine, albumin, and Hb (10, 11). These low- and high-molecular-weight RSNOs are believed to play a role in stabilization and delivery of NO to the vascular bed, where NO may modify vascular tone. Stamler and co-workers (11) have proposed that binding of oxygen to heme irons in Hb promotes the binding of NO to the specific cysteine residue located in the $\beta$-subunits of Hb, forming SNOHb (11). Deoxygenation is accompanied by an allosteric transition in SNOHb that releases the NO group. Therefore, SNOHb has been proposed to participate in the regulation of blood flow (11) and platelet aggregability. However, in volunteers inhaling NO for 1 h, only an increase in NOHb levels was found, whereas the SNOHb levels remained unchanged. Furthermore, an arteriovenous gradient of NOHb occurred, which was associated with a simultaneous increase in FBF (3). This may suggest that NOHb significantly contributed to the observed vasodilatory effects. However, although suggested to have a potential

![FIGURE 3. Time course of acetylcholine-induced effects on forearm blood flow (FBF) and venous NO\textsubscript{2}, NO\textsubscript{3}, and NO\textsubscript{x} levels following infusion of acetylcholine for 40 s into the brachial artery. Increase in NO\textsubscript{2} concentrations closely preceded the increase in FBF, indicating an acute change of regional NOS III activity. In contrast, NO\textsubscript{3} and NO\textsubscript{x} concentrations remained unchanged. *1 indicates the time of maximal NO\textsubscript{2} concentration, and *2 indicates the time of maximum FBF.](https://www.nips.org)
important role in transport and delivery of NO, to date neither qualitative nor quantitative measurements of RSNOs, NOHb, or SNOHb have been demonstrated to represent an index of NO availability in human blood.

In conclusion, although we are still far away from a detailed and complete understanding of the metabolism of NO in human blood, in recent years considerable progress has been made concerning the assessment of NO bioavailability. Depending on the respective aim or purpose, measurement of any of these potential NO biomarkers may help to further elucidate our understanding of the numerous reactions of NO and lead to a change of the current paradigm on NO metabolism and bioavailability. Blood does not constitute only a sink for NO but represents a complex compartment contributing to metabolism, transport, and delivery of NO in human circulation. Future studies should address the relative importance/impact of the respective oxidative and nitros(yl)ated products of NO in human blood. This may lead to new diagnostic tools in the premature assessment of vascular dysfunction and atherosclerosis. Moreover, a better understanding of NO transport and systemic delivery of NO in humans may open an attractive new way to utilize the therapeutic potential of endogenous NO carriers in cardiovascular diseases.

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