S-Nitrosothiols in the Blood
Roles, Amounts, and Methods of Analysis

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In 1991 to 1992, we reported that both endogenous and exogenous nitric oxide (NO) react with thiols in proteins such as albumin to form long-lived S-nitrosothiols (SNOs) with vasodilatory activity. We also described the presence of a circulating pool of S-nitrosoalbumin in plasma whose levels were coupled to NO synthase (NOS) activity. Inhibition of NOS led to a decline in SNO-albumin with concomitant production of low-mass SNOs. We proposed that SNO-albumin provides a reservoir of NO bioactivity that might be utilized in states of NO deficiency, and that vasodilation by SNO-albumin is transduced by the small mass SNOs with which it exists in equilibrium. Shortly thereafter, we determined that a key low-mass SNO in biological systems is S-nitrosoglutathione (GSNO); that GSNO, in contrast to NO, retained smooth muscle relaxant activity in the presence of blood hemoglobin; and that GSNO is a more potent relaxant than SNO-proteins. Subsequently, we demonstrated the existence of intraerythrocytic equilibria between NO bound to the thiol of glutathione and reactive thiols (cysβ93) of hemoglobin on the one hand, and NO bound to thiols of hemoglobin and membrane-associated band 3 protein (AE1), on the other hand. The exchange of NO groups between S-nitrosoglutathione (GSNO) and the red blood cell (RBC) membrane is governed by O2 tension (P02): RBCs dilate blood vessels at low P02, and the production of membrane SNO is required for vasodilation. In peripheral tissues, blood flow is determined by variations in O2 saturation that are coupled to metabolic demand. The mechanism through which the O2 content of blood evokes this response and the basis for its impairment in many diseases (including heart failure, diabetes, and shock) have been major and longstanding questions in vascular physiology. Our studies suggested that the answers reside with hemoglobin’s ability to serve as both an O2 sensor and O2-responsive transducer of vasodilator activity (Figure).

It would later be determined that albumin and hemoglobin are privileged sites of SNO production. In albumin, both a hydrophobic pocket and bound metals (copper and perhaps heme) can facilitate S-nitrosylation by NO, whereas hemoglobin has several channels through which it can react with NO, nitrite, or GSNO to produce SNO-Hb (Figure). Additional studies indicated that S-nitrosylation of blood proteins may be catalyzed by superoxide dismutase (SOD), ceruloplasmin, and nitrite. In particular, ceruloplasmin catalyzes the conversion of NO to GSNO, and NO in solution or derived from GSNO is targeted by SOD to cysβ93 in hemoglobin (rather than heme iron). A similar mechanism (involving SOD and nitrite) may operate in albumin. Numerous laboratories have verified the presence of SNO-albumin, GSNO, and SNO-Hb in blood and tissues of both animals and humans. However, the amounts that form, the suitability of various methods for assaying various SNOs, and the physiological roles of these molecules remain controversial.

Superoxide, Nitric Oxide, and S-Nitrosoalbumin

One physiological benefit of SNOs over NO is their resistance to inactivation by superoxide (O2−). In damaged tissues, increased O2− can react with NO to produce toxic peroxynitrite. But the amounts of peroxynitrite that accrue depend at a minimum on the relative rates of NO/O2− production: NO>O2− in fact favors production of SNO. In this issue of Circulation Research, Ng et al. demonstrate that superoxide, generated by ischemia/reperfusion (I/R) in mesenteric vessels, facilitates the synthesis of SNO-albumin. SNO-albumin is known to protect tissues against I/R-induced damage. Thus, evidently, nature has found a way to exploit superoxide to preserve NO bioactivity. A remaining problem is that the oxidative damage caused by I/R impairs NO production. It is therefore noteworthy that Ng et al. also show that the thiols of albumin can transport inhaled NO to the gut and subserve relaxation of blood vessels. Although relatively high concentrations of SNO-albumin are required to increase blood flow, the amounts that attenuate vasoconstriction are in the physiological range. Furthermore, as evident from the accrual of SNO-albumin in some hypertensive and uremic patients, it is the efficiency of NO group release, not the amount of SNO-protein, that determines bioactivity. In particular, increases in plasma SNOs are associated with high blood pressure and predict adverse cardiovascular outcome. Taken together, these studies suggest that SNO-albumin may dispense NO bioactivity in states characterized by NO deficiency. They also raise the possibility that cysteines in albumin and other key blood proteins may provide a new therapeutic target.

The fact that inhaled NO increases circulating levels of SNO-albumin does not reveal the mechanism by which SNO-albumin is made, where in the circulation it is produced,
or how much NO actually takes this path. Inhaled NO first accumulates in the airways and lung parenchyma in the form of SNOs and other complexes with proteins, and then leaches into the blood. Salient features of this process, including the form in which NO bioactivity enters the blood, are not known. Hemoglobin probably out-competes albumin for NO, but this outcome is not absolute: the relative yield of NO bound to hemoglobin in bioactive form is inversely proportional to the rate and amount of NO administered and exhibits a plateau at low micromolar levels (only nanomolar is required for vasoregulation). With the high amounts of NO administered clinically and by Ng et al, hemoglobin incorporates a brake on the production of SNO by lodging NO on the α-hemes and eliminating it as nitrate.

Controversies

Ng et al measure low nanomolar amounts of SNO-albumin under basal conditions—in agreement with several recent reports—and micromolar concentrations after inhaled NO. The largest single analysis of plasma SNOs, conducted in 250 patients with renal disease, reported mean values of ~2 μmol/L.11,13,23 Five other groups (using four different techniques) have measured 1 to 10 μmol/L levels of SNO-albumin at basal conditions (for examples, see References 2,18,21,24), and other groups have reported concentrations somewhere in-between (~0.5 μmol/L). The largest single analysis of plasma SNOs, conducted in 250 patients with renal disease, reported mean values of ~2 μmol/L.11,13,23,24 Ng et al are to be commended for their thoughtful treatment of these disparities. They point out that they were unable to recover GSNO added to whole blood (no other group has tried). The quantitative aspect of their results, and those of other groups using similar methods, is therefore not very meaningful. Moreover, their study emphasizes that no analysis of plasma SNOs has used appropriate protein standards. The customary method of synthesizing SNO-albumin (acidified nitrite) in fact produces a denatured polypeptide stripped of the cofactors carried by native albumin that may influence SNO reactivity (see below). Similarly, SNO-Hb molecules now routinely used as standards are unlike any SNO-Hb molecule that is found in vivo.

Assays

But, these are the least of the problems. Four assumptions form the basis of currently touted chemical assays for SNOs but arguably, none of them is justified scientifically: (1) that bond dissociation energies (BDEs; a measure of stability) of low-mass SNOs, which are typically used as standards, are representative of SNO-proteins and that protein SNOs have uniform BDEs; (2) that biological fluids and/or cells can be processed in strong acids and other nonphysiological reagents without affecting steady-state levels of SNOs; (3) that alkylating thiols and oxidizing/chelating metals (e.g., N-ethylmaleimide, ferricyanide, and cyanide; often referred to as ‘‘nitrosothiol-stabilizing solutions’’) prevent the decomposition of SNOs; and (4) that nitrite can be removed without disrupting SNO homeostasis.

In fact, homolytic BDEs of SNOs may vary from 20 kcal/mol to 32 kcal/mol—translating into lifetimes of less than seconds to years—and protein nitrosothiols, in particular, may span the range. Indeed, we have clear examples of protein nitrosothiols so unstable that they disappear over the time course of the assay, whereas others last for days. Factors that contribute to the differential reactivity of SNOs include (1) protein conformation, which determines the S-N dihedral angle (cis versus trans isomer versus other), and the accessibility of SNO to solvent; (2) the nature of the SNO moiety, i.e., SNOs may have significant double-bond character (SNO versus S-N=O); may accept an electron (SNO·) or may complex with a nucleophile such as thiol (so-called SNO2, more formally a nitroxyl disulfide/N-hydroxyulfenamide); (3) the proximity of SNOs to transition metals and aromatic residues, with which they can interact or redox-couple in several ways. For example, the BDE of the SNO in hemoglobin, as deduced from the crystal structure, is estimated to be at least 10 kcal lower (ie, less stable) than in GSNO or SNO-cysteine. Thus, the unstable SNO in Hb is evidently stabilized through interactions with an aromatic residue and/or hydrogen bonding. Disruption of hydrogen bonding,
for example with acidification, would lead to immediate loss of the SNO. That some SNO-Hb molecules may survive acidification can be understood by appreciating that SNO-Hb is not one but a family of molecules, whose stability is dependent not only on the conformation of the protein and the nature of the SNO, but also on the ligation and oxidation states of accompanying hemes iron. Overall, SNO-Hb molecules in vivo are far more reactive than those commonly used as standards, as Nagababu et al.29 and Datta et al.24 have recently appreciated, and there is thus a reactive pool of SNO-Hb and iron nitrosyl Hb that is lost in chemical assays. (These problems, it should be noted, do not arise in analysis by photolysis; see below.)

Similarly, the addition of oxidants and thiol alkylators to stabilize SNO and attempts to remove nitrite are not consistent with known SNO chemistry. Thiols can in fact stabilize SNOs,27 and thiol alkylators can, and often do, destabilize them; the effects of oxidants, alkylators and cyanide (which inactivates SOD and may react with oxidized thiol/SNO) alone or in combination cannot be predicted or controlled for. Further, nitrite simply cannot be removed from physiological systems without disrupting SNO homeostasis because it is, in effect, in equilibrium with SNOs. The limitations of such chemical assays are highlighted by the recent report of a novel role for nitrite (as opposed to other species including SNO) in hypoxic vasodilation by RBCs.25 The physiological measurements presented in that study do not support such a role. Specifically, NO inhibitors, which acutely deplete endogenous nitrite in blood, did not block hypoxic vasodilation, and nitrite infusions did not increase blood flow during hypoxia, compared with hypoxia alone. Thus, nitrite (which has been known for over a hundred years to dilate blood vessels) does not mediate the classical hypoxic (and partly RBC-dependent) response that couples blood flow to metabolic demand. In fact, the recently popularized chemical method used by those investigators detects significantly different amounts of nitrite than methods that do not require addition of acids or other reagents (eg, capillary electrophoresis) and has also produced—by comparison with electron paramagnetic resonance spectroscopy gold standard22—errors of 1 to 2 orders of magnitude in the iron nitrosyl content of hemoglobin,26 implicated in nitrite-derived vasodilation.25 Further, in our hands, these methods cannot accurately assay physiologically SNO-Hb molecules nor distinguish them from iron nitrosyl Hb or nitrite.

**Future Prospects**

There is an unfortunate and durable tendency in NO biology to equate amount with importance. If this equation were correct, NO would be unimportant, as it cannot be measured at basal state in blood or tissues, and nitrate would be very important because there is lots of it. In the case of NO/redox systems, it is the molecules that we cannot measure readily (in particular, NO, SNO, O$_2^-$, H$_2$O$_2$, OONO$^-$) and their throughput, not steady state, that matter. The idiosyncrasies of a particular chemical assay under a certain set of conditions may provide a valuable partial view, as achieved by Ng et al.16 But, it is the qualitative aspects of their data, not the quantitative, that should be relied on. New genetic evidence makes it clear that whatever the steady-state amounts of SNO in vivo, they are sufficient to play essential roles in the vasculature.30 In my view, the field will be best served by reliance on assays that preserve the physiological milieu, and by use of standards that best emulate the molecules being measured: photolysis-based methods should use the most stable nitrosothiol standards, thus covering the range of SNO bond quantum yields, whereas chemical assays must utilize the least stable nitrosothiols to verify their recovery. Dose-dependence and reproducibility of assays for SNOs are necessary requirements but not sufficient to ensure against systematic artifacts.

**References**


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