ratio of 1:100. This prediction, however, is drawn from (arbitrarily selected) prior work carried out at Hb concentrations of 100 to 200 µM.\(^3\) (More recent work with similar results at 200 µM was ignored.\(^4\)) In their studies, a substantially higher Hb concentration (> mM) was employed, even though such high concentrations had already been reported to pose technical problems; specifically, such viscous solutions are difficult to oxygenate rapidly and give reduced SNO-Hb yields. The more dilute Hb solutions, similar to Hb in red blood cells (RBCs) traversing the lung, are oxygenated in seconds, as opposed to the approximately 5 minutes needed by the authors to oxygenate their concentrated solutions. This delay allows well-known NO migration and auto-oxidation processes that reduce the efficiency of SNO formation. Thus, the experimental design of Huang et al is flawed. The authors neglect the importance of outpacing competing processes that disarm SNO-Hb production and make unjustified comparisons of results obtained at widely different NO and/or Hb levels. The results of Huang et al are insufficient to contradict findings of others obtained under substantially different conditions, conditions that are arguably more pertinent to the physiologic situation; their general conclusion cannot be validly inferred from the reported work.

Finally, it should be noted that Huang et al\(^7\) actually do form amounts of SNO-Hb that are very similar to those obtained by others at comparable NO levels (~20% of ~10 µM added NO)\(^5-6\) (Figure 1). Interestingly, they report that the SNO-Hb is produced largely prior to oxygenation. This remarkable result raises questions about the composition of their samples. Moreover, given the equilibrium-like regulation of SNO formation, the large SNO-Hb cohort present before oxygenation could also be expected to limit oxygenation-induced SNO-Hb formation.

Andrew J. Gow and David Singel

Correspondence: Andrew Gow, 160 Frelinghuysen Rd, Piscataway, NJ 08854; e-mail: gow@rci.rutgers.edu.

To the editor:

Detecting physiologic fluctuations in the S-nitrosohemoglobin micropopulation: triiodide versus 3C

It is now established that erythrocytes can dilate blood vessels by generating NO bioactivity.\(^1,3\) Singel and Stamler\(^2\) have proposed that this dilator function is conveyed by a micropopulation of hemoglobinins (Hb’s) that have undergone the addition of NO to a critical cysteine (cys\(^\beta\)) via S-nitrosylation, forming S-nitroso Hb (SNO-Hb). In this model, the allosterically governed equilibrium of NO groups between \(\beta\) hemes and cysteine thiols enables erythrocytes to lever oxygen gradients into a graded signal for vasodilation, thereby enhancing perfusion sufficiency. Precise mapping of NO migration via heme-thiol exchanges and attendant oscillations in the SNO-Hb micropopulation, in support of this model, has been generated by 5 distinct assay methods: photolysis chemiluminescence,\(^1,4,7\) reductive chemiluminescence (3C and ascorbate methods),\(^8,9\) modified triiodide,\(^10,11\) and fluorometric (DAF)\(^12\) and colorimetric technique (Greiss-Saville).\(^13,14\) The triiodide assay, however, shows much lower SNO-Hb levels than do the other methods\(^15,18\) and is the only method unable to detect physiologic fluctuations in the SNO-Hb micropopulation\(^19,20\); as such, those who use this assay have challenged the above model.\(^21\) However, there is concern that triiodide is not sufficiently sensitive or precise enough to accurately assay for SNO-Hb within biologic systems.\(^22,23\) In a recent Blood article, Huang et al\(^7\) claimed to validate triiodide by comparison to 3C, then, using triiodide, failed to detect \(O_2\)-responsive fluctuations in SNO-Hb and concluded that the fluctuations do not occur. However, the assays were not shown to be comparable under physiologically relevant conditions, under which they report fundamentally dissimilar results,\(^6,15-19\) so the conclusions are unsupported.

The SNO-Hb micropopulation is composed of a family of molecules with differing reactivities.\(^24,25,27\) Assays for SNO-Hb must detect the entire cohort, including the most evanescent of these adducts. SNO content, heme ligation, and oxidation states are known to alter analyte reactivity. Across all these parameters, the validation standard did not replicate the SNO-Hb that was produced upon oxygenation of FeNO in the experiment. More generally, triiodide’s chemical mechanism remains unknown and there are potential artifacts from the strong acid it uses, including (1) shifting of original NO ligation sites and (2) loss of SNO groups by overt oxidation (or by heme autocapture). The 3C method was developed to overcome these limitations.

References

Published data generated by triiodide and 3C diverge progressively when the physiologic NO/heme ratio is approached (≤1:1000). Triiodide has been shown to suffer from signal attenuation (for SNO) when matrixed with physiologic heme concentrations. In fact, Bryan et al and Rassaf et al claim that triiodide is unable to detect any human HB-bound NO at all. In contrast, the physiologic sample matrix is strictly preserved by 3C, which is selective for SNO-Hb, detects mid-pM SNO-Hb, and has demonstrated signal fidelity across varying heme concentrations and SNO/heme ratios.

The validation did not encompass the range in which the triiodide was employed. The experimental concentration of SNO-Hb was a full log order below validation. Further discrepancies included a validation heme concentration of 125 μM versus an experimental heme concentration of 20 mM (160-fold error) and a validation SNO/heme ratio ranging from 2.5 × 10⁻³ to 3 × 10⁻³ versus an experimental ratio of 2.5 × 10⁻³ (2 log error). Assay sensitivities crossed at approximately 5 μM SNO, with triiodide half as sensitive at 900 nM, the lowest concentration tested. Gross visual correlation between techniques does not constitute assay validation, and, when performed upon an abundant analyte (SNO) matrixed within a scarce sink (heme), cannot be generalized. Such miscalculations are multiplicative, creating a cumulative 1.6 × 10⁻³-fold error in the validation sample range. Thus, critical range-dependent flaws in triiodide would remain masked.

Caution is merited in evaluating Huang et al’s conclusions, which rest substantially on the sensitivity and specificity of the triiodide assay for SNO-Hb.

Allan Doctor and Benjamin Gaston
Correspondence: Allan Doctor, Division of Pediatric Critical Care, Washington University School of Medicine, Campus Box 8116, St. Louis, MO 63110; e-mail: doctor@wustl.edu

References

Response:
SNO-Hb hypothesis
The S-nitrosohemoglobin (SNO-Hb) hypothesis was originally quite simple, and one of its central tenets was that heme-bound NO in T-state HB is transferred to the β-93 cysteine upon oxygenation-induced conversion to R-state HB. Our tests of this allosteric transfer mechanism have failed to confirm its validity. Gow and Singel suggest that our failure to observe allosterically controlled intramolecular transfer is due to the unaccounted-for complex nature of the process. However, their arguments, if true, would leave an SNO-Hb hypothesis that is both unphysical and untestable.

In 2003, we tested the allosteric transfer mechanism in a wide range of conditions including cell-free Hb, red cells, and whole blood. We subsequently argued that the NO/heme ratio used was too high and that there was too much heme-NO present on the alpha chains. Our recent tests of the mechanism were specifically designed to address these concerns, with NO/heme ratios below 0.001 and maximal nitrosylation of the beta chains. Rather than observing a decrease in iron-nitrosyl Hb and an increase in SNO-Hb upon oxygenation, as predicted by the
SNO-Hb hypothesis and misleadingly shown in the letter by Gow and Singel, we observed the opposite trends.3 In all our experiments designed to test the allosteric transfer mechanism, we have employed both a chemiluminescence technique (the triiodide [3I] method) and electron paramagnetic resonance (EPR) spectroscopy.2,3 Doctor and Gaston question our work based on their dissatisfaction with the 3I assay. The SNO-Hb hypothesis predicts that, following oxygenation, we should have detected over 3 μM SNO-Hb, within the range of sensitivities tested in our validation studies. Moreover, previous validation studies of the 3I method showed it could detect 20 nM SNO-Hb in whole blood (0.0002% SNO/heme).5 Our published validation of the 3I assay compared with the 3C assay showed excellent agreement (r = 0.999 258; P < .001). Finally, even without the 3I assay results, our conclusions would still stand firmly on our EPR results.

In carrying out our study, we rigorously addressed factors purported to affect NO intramolecular transfer, namely a low NO/Hb ratio, the time of incubation of the nitrosylated Hb prior to oxygenation, and the absolute amount of NO added. This latter factor, as well as others, does not have a sound physical basis. If intramolecular transfer is allosterically controlled, then whether it occurs or not should depend only on factors affecting an isolated tetramer, specifically its allostatic state. The efficiency of NO transfer within a single Hb tetramer cannot be affected by the absolute number of other tetramers that have NO bound. Likewise, an Hb molecule would not remember that the NO molecule bound to its heme was added as part of a saturated buffer or released from an NO donor. It is reasonable to argue that Hb is complex but not that it is intelligent. Finally, we examined and did not detect any significant heme oxidation or NO migration due to the rate of oxygenation or other factors. We thoroughly characterized the state and amount of heme nitrosylation before and after oxygenation, confirming conditions that should have maximized allosterically controlled transfer.

Strong hypotheses can withstand many different tests under a variety of conditions. The original allosteric transfer mechanism made a simple testable prediction: oxygenation of deoxygenated partially nitrosylated Hb should result in a decrease in heme nitrosylation and an increase in SNO-Hb. In the 10 years since the SNO-Hb hypothesis was first proposed,1 data from simple EPR experiments supporting the hypothesis by showing loss of NO-heme upon oxygenation have never been published, except in cases where exogenous nitrite was added. We have shown that this nitrite is likely to be responsible for perceived intramolecular transfer.2 Our EPR data clearly show that NO is not transferred from the heme to the thiol upon oxygen-induced changes in the allostatic state of normal adult Hb under physiologically relevant conditions. The loosely defined complexities that Gow and Singel discuss only serve to weaken their hypothesis (in a way reminiscent of Ptolemy’s epicycles) and do not apply to our experiments. Gow and Singel admit that “a general model that rationalizes the dependence of SNO formation on these factors has yet to be advanced,” indicating that the once-simple model is now only applicable to poorly defined micropopulations, and a change in allostatic state of the Hb is not sufficient to effect intramolecular transfer. In summary, we find no support for the existence of allosterically controlled NO transfer from heme to thiol in normal physiology or for defects in this transfer serving as a basis of disease.

Daniel B. Kim-Shapiro

Correspondence: Daniel Kim-Shapiro, Olin Physical Lab, Wake Forest University, Winston-Salem, NC 27109; e-mail: shapiro@wfu.edu.

References

Detecting physiologic fluctuations in the S-nitrosohemoglobin micropopulation: triliodide versus 3C

Allan Doctor and Benjamin Gaston