Can we just say NO to sickle cell anemia?

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Commentary

In this issue of the JCI, Gladwin et al. (1) describe results contradicting those in another recent JCI article (2) that concerns the effects of nitric oxide (NO) on hemoglobin (Hb). Before we attempt to dissect the controversy, let us step back a bit. NO is generated from L-arginine by NO synthases (NOSs) that are differentially induced by cell-specific (endothelium, neutrophils, adrenal tissue, cerebellum) cofactors such as Ca^{2+}-dependent calmodulin, TNF, and other cytokines (3, 4). NO physiological activity might involve direct cell-to-cell interaction and be only partially mediated by blood NO. The steady-state level of blood NO is the consequence of a balance between the production of NO by NOSs, the binding or scavenging of NO by Hb, and the transformation of NO into NO_2^- and NO_3^-. NO has an affinity for Hb that is 100 times higher than that of CO and 200,000 times higher than that of O_2, which has given NO the reputation of being a very toxic compound lacking physiological importance. Among the functions of NO is the maintenance of vascular tone. Another physiological function might be the formation of S-nitroso Hb, the product of NO– binding to the β93 cysteine. Gow and Stamler (5) propose that S-nitroso Hb generated in the lungs, where Hb is in the oxygenated (R) state, liberates NO in the microcirculation, [...]

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In this issue of the *JCI*, Gladwin et al. (1) describe results contradicting those in another recent *JCI* article (2) that concerns the effects of nitric oxide (NO) on hemoglobin (Hb). Before we attempt to dissect the controversy, let us step back a bit.

NO is generated from L-arginine by NO synthases (NOSs) that are differentially induced by cell-specific (endothelium, neutrophils, adrenal tissue, cerebellum) cofactors such as Ca^{2+}-dependent calmodulin, TNF, and other cytokines (3, 4). NO physiological activity might involve direct cell-to-cell interaction and be only partially mediated by blood NO. The steady-state level of blood NO is the consequence of a balance between the production of NO by NOSs, the binding or scavenging of NO by Hb, and the transformation of NO into NO_2^- and NO_3^-. NO has an affinity for Hb that is 100 times higher than that of CO and 200,000 times higher than that of O_2, which has given NO the reputation of being a very toxic compound lacking physiological importance.

Among the functions of NO is the maintenance of vascular tone. Another physiological function might be the formation of *S*-nitroso Hb, the product of NO^- binding to the β93 cysteine. Gow and Stamler (5) propose that *S*-nitroso Hb generated in the lungs, where Hb is in the oxygenated (R) state, liberates NO in the microcirculation, where the conformational transition (R→T), induced by deoxygenation, liberates NO from the β93Cys site; the NO then acts on subendothelial muscle as a vasodilator. Also, there is crystallographic evidence that NO can bind to β93Cys (6).

The Stamler model, while attractive, leaves several questions unanswered. First, there is no evidence that NO is liberated from the β93Cys site when Hb changes conformation to the T state. Second, as Yakashi Yonetani (personal communication) has pointed out, nitroso compounds do not liberate NO, but rather NO^+, which may then be converted into NO. The mechanisms involved in this conversion in the red cell, and the process by which NO^+ might be exported from the red cell, are not understood. The physiological significance of β93Cys binding of NO requires further research.

Breathing NO raises other issues when considering the clinical applications of NO. Recently, acute-care physicians began using low-concentration gases (generally 5–10 ppm) to manage the acute pulmonary hypertension seen in acute respiratory distress syndrome (ARDS), primary pulmonary hypertension, and bronchospastic disorders. The use of NO is still considered dangerous in sepsis, as a higher production of NO and hypotension is observed in this complication (7). In animal studies, inhalation of 10–40 ppm of NO for 6 months had no ill effect. The common explanation for this is that rapid binding of NO by Hb mops up this dangerous vasodilator. Indeed, when NO is bound to Hb, the affinity for O_2 is significantly reduced. However, NO binds preferentially to the hemes attached to the α-globin chains, inducing a change of conformation of the Hb molecule to a super-T state (8); this is due to the breaking of the bond between the iron and the proximal histidine. While the hemes that have bound NO are excluded from O_2 exchange, the other 2 remaining hemes now have a particularly low O_2 affinity and can deliver O_2 to the tissues more efficiently than in the normal T state. Hence, although the NO tetramer has fewer ligand-carrying hemes (2 out of 4), it exhibits almost the same efficiency of O_2 delivery as does a normal Hb tetramer. The tetramers containing NO will, in a period of about half an hour, be converted to metHb, lose NO, and be reduced by the metHb reductase system of the red cell into normal Hb. For these reasons, NO at limited concentrations is not as toxic as originally feared.

What is the mechanism of metHb formation in Hb's containing heme-bound NO? The rate of NO-dependent oxidation varies linearly with NO concentration, but not with O_2 concentration (9).

Nonreversible NO binding and NO-induced oxidation occur in 2 steps: (a) bimolecular entry of NO into the distal portion of the heme pocket, and (b) rapid reaction of noncovalently bound NO with iron to produce several iron complexes. This unwelcome effect of NO could be particularly problematic in the sickle cell, which already produces more metHb than normal cells, pushing the reductase systems to its limits.

Returning to the *JCI* papers, Head et al.(2) stated that their objective was "to determine if low concentrations of NO gas would augment the O_2 affinity of red cells containing homozygous HbS (SS)." Even if it were possible, many believe that this is not a viable strategy to ameliorate sickle cell anemia, as it will only shift the burden of deoxygenation from the high-affinity tetramers to lower-affinity tetramers (which will still be capable of polymerization). Head et al. found that SS red cells, incubated in vitro with varying concentrations of NO (up to 80 ppm for 5 minutes or longer), decreased their P_{50} by 15% — a change that was correlated with NO concentration. In contrast, the P_{50} of normal red cells was unchanged. Moreover, in SS patients breathing 80 ppm NO for 45 minutes, the P_{50} also decreased significantly. Control subjects breathing NO showed no change in P_{50}. MetHb remained low in all subjects breathing NO. The authors did not provide an explanation for the left-shifted O_2 equilibrium curve found in SS red cells. It initially seemed likely that this left shift arose because of the well-known Darling-Roughton effect arising from metHb. According to this effect, when 1 or 2 hemes are either met or CO-bound, the tetramer tends to adopt the R state (high affinity) in the remaining O_2 binding sites (provided that a sufficient number of chains are modified). This mechanism explains the left shift in chronic methemoglobinemia and CO intoxication. Alternatively, it could have been the melting of the sickle polymers, but

this seems unlikely because of the low concentration of NO. The specificity of this effect for SS cells might be explained by these cells' greater susceptibility to metHb formation.

To be sure, Head et al. measured metHb levels and found them to be low. However, for quantification, the authors used a CO oxymeter whose software does not include NO Hb spectra — hence the possibility of error.

In this issue, Gladwin et al. exposed 3 sickle cell anemia patients in clinical steady state, and 3 controls, to 2 hours of 80 ppm NO (same concentration but longer exposure time than that used by Head et al.) and found no shift of the O_2 equilibrium in sickle cell patients and normal controls. Moreover, they confirmed their metHb determination, obtained with a CO oxymeter with direct spectral analysis: no increase in metHb. Hence, a trivial explanation for the Head et al. findings evaporates. Interestingly, Gladwin et al. found that 0.021% of the Hb was converted to *S*-nitroso Hb, as confirmed by mass spectroscopy.

How do we explain the discrepancy between these 2 papers? Nothing is obvious, except that there could have been problems in the handling of the samples during experimentation. However, another aspect of NO function is raised in the Gladwin et al. paper. The authors propose that the buildup of *S*-nitroso Hb could generate NO, which, by vasodilating the microcirculation, could ameliorate sickle cell obstructive disease. This is not a likely possibility,

for 2 reasons. First, there is no evidence of NO release from *S*-nitroso Hb inside of red cells reaching the basal side of the endothelium. Second, and more importantly, there is evidence that the microcirculation is already vasodilated, a finding based on the low peripheral resistance in sickle cell patients (9). There is data on increased expression of eNOS in S + S-Antilles transgenic mice (11), as well as increased eNOS and in vivo vasodilation in the Paszty-Rubin knockout/transgenic mice (12). This phenomenon is compatible with the relative hypotension in sickle cell anemia.

However, we should not lose sight of other potential benefits that breathing NO at low concentrations may hold for sickle cell anemia patients. The bronchodilation effect and the potential vasodilation effect of the lung microcirculation may be useful tools to combat acute chest syndrome, the most frequent fatal complication in young adults with this disease. The lung microcirculation vasoconstricts with hypoxia, in contrast with the rest of the circulation. Aldrich et al. (13) have demonstrated, in an animal model, that this event traps dense sickle red cells and could be a part of the pathophysiological cascade resulting in the potentially fatal acute chest syndrome. One potential problem of this approach could be the copresence of sepsis. Gladwin et al. establish an important new fact: up to 0.021% *S*-nitroso Hb is not accompanied by changes on the O_2 equilibrium curve. This level of Hb *S*-nitrosylation (compatible with safety) does not inhib-

it the sickle polymer. Thus, there seems to be no great prospect for NO as an anti-sickling agent per se.

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