Red Blood Cell Nitric Oxide as an Endocrine Vasoregulator
A Potential Role in Congestive Heart Failure

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Background—A respiratory cycle for nitric oxide (NO) would involve the formation of vasoactive metabolites between NO and hemoglobin during pulmonary oxygenation. We investigated the role of these metabolites in hypoxic tissue in vitro and in vivo in healthy subjects and patients with congestive heart failure (CHF).

Methods and Results—We investigated the capacity for red blood cells (RBCs) to dilate preconstricted aortic rings under various O₂ tensions. RBCs induced cyclic guanylyl monophosphate–dependent vasorelaxation during hypoxia (35±4% at 1% O₂, 4.7±1.6% at 95% O₂; P<0.05). RBC-induced relaxations during hypoxia correlated with S-nitrosohemoglobin (SNO-Hb) (R²=0.88) but not iron nitrosylhemoglobin (HbFeNO) content. Relaxation responses for RBCs were compared with S-nitrosoglutathione across a range of O₂ tensions. The fold increases in relaxation evoked by RBCs were significantly greater at 1% and 2% O₂ compared with relaxations induced at 95% (P<0.05), consistent with an allosteric mechanism of hypoxic vasodilation. We also measured transpulmonary gradients of NO metabolites in healthy control subjects and in patients with CHF. In CHF patients but not control subjects, levels of SNO-Hb increase from 0.0029±0.00089 to 0.00585±0.00137 mol NO/mol hemoglobin tetramer (P=0.005), whereas HbFeNO decreases from 0.00361±0.00109 to 0.00081±0.00040 mol NO/mol hemoglobin tetramer (P=0.03) as hemoglobin is oxygenated in the pulmonary circulation. These metabolite gradients correlated with the hemoglobin O₂ saturation gradient (P<0.05) and inversely with cardiac index (P<0.05) for both CHF patients and control subjects.

Conclusions—We confirm that RBC-bound NO mediates hypoxic vasodilation in vitro. Transpulmonary gradients of hemoglobin-bound NO are evident in CHF patients and are inversely dependent on cardiac index. Hemoglobin may transport and release NO bioactivity to areas of tissue hypoxia or during increased peripheral oxygen extraction via an allosteric mechanism. (Circulation. 2004;109:1339-1342.)

Key Words: heart failure ■ hypoxia ■ nitric oxide ■ vasodilation

Nitric oxide (NO) generated by the vascular endothelium has traditionally been attributed a purely paracrine role, the reaction of NO with heme groups forming methemoglobin and nitrate considered its key metabolic fate.1 Recent evidence shows that NO also reacts with hemoglobin (Hb) to form stable metabolites, which may transport and subsequently release NO distant to its site of production.2-5 In this model, NO binds to the heme group of deoxygenated Hb to produce iron nitrosylhemoglobin (HbFeNO), and during pulmonary oxygenation, some NO transfers to the highly conserved β chain cysteine 93 residue of Hb to produce S-nitrosylhemoglobin (SNO-Hb). During deoxygenation in the peripheral circulation, SNO-Hb is able to transfer and subsequently release NO bioactivity. In vitro, it has been shown that red blood cells (RBCs) dilate blood vessels in proportion to the degree of hypoxia.3 However, the sensitivity and specificity of the techniques used to make these measurements vary.6-8 and it is unclear whether the amounts of NO released are sufficient to provide a physiological reserve in health or disease. We hypothesized that if hypoxia triggers release of NO from SNO-Hb to cause vasorelaxation, then AV gradients of NO metabolites may exist in patients with congestive heart failure (CHF) because of enhanced peripheral O₂ extraction. This is a potential autoregulatory mechanism mediating peripheral vasodilation during low cardiac output states. We performed an in vitro study as previously described9 to demonstrate hypoxia-induced release of vasoactive NO from RBCs and a clinical study involving measurement of NO metabolites in healthy control subjects and patients with CHF.

Methods

In Vitro Study

Aortic Ring Preparation

Thoracic aortas were removed from euthanized male New Zealand White rabbits (Charles River, UK). Endothelium-intact rings were...
mounted in tissue baths filled with Krebs’ buffer (KB) (pH 7.4 at 37°C) and a resting tension set to 2g. This was recorded continuously using PowerLab software. After an equilibration period, tissues were repeatedly constricted with phenylephrine (PE) 10⁻⁶ mol/L and relaxed with acetylcholine 10⁻⁶ mol/L until reproducible responses were achieved. Under O₂ tensions of 1%, 2%, 5%, 21%, or 95%, the tissues were then preconstricted with PE, and when a plateau in tension was reached, RBCs were added. Studies were carried out at low O₂ tensions to investigate the potential of RBCs dilating blood vessels through an allosteric mechanism. At the end of each experiment, the exogenous NO donor S-nitrosothiolamine (GSNO) 10⁻⁷ mol/L was added as a positive standard. After correction for preconstriction, relaxations and subsequent constrictions were calculated as percent tension induced by PE. Relaxations were retrospectively corrected for Hb concentration.

The concentration of O₂ in the KB was controlled by adjusting the gas inflow into the bottom of the tissue bath and was monitored online with an O₂ electrode (World Precision Instruments). In some experiments, to inhibit soluble guanylate cyclase and consequent smooth muscle relaxation, 1H-[1,2,4]oxadiazole[4,3-a]quinoxalin-1-one (ODQ) 10⁻⁵ mol/L (Alexis) was added to the chamber for 1 hour before PE. In a separate group of experiments, GSNO 10⁻⁹ to 10⁻⁵ mol/L was added to study the effect of O₂ tension on vessel responsiveness to a standard amount of NO, with release of NO bioactivity from GSNO being independent of O₂ concentration.¹⁰

Preparation of RBCs
RBCs from the rabbit aorta were separated, washed, and made up to the original hematocrit with KB. Then, RBCs (80 μL) were added to a final volume of 8 mL KB surrounding the aortic ring. Experiments were carried out with and without exogenous addition of NO (NOC-9; Alexis) to give a final NO concentration of 0.24 to 24×10⁻⁶ mol/L. Hb concentration was measured with the hemoglobin-cyanide method. Hb-bound NO was measured in RBCs as described below. Four aortic ring preparations were subjected to 95% O₂ and 4 to 1%, 2%, 5%, or 21% O₂, with the results from each group averaged to give a single data point. This was repeated 8 times for each study at different O₂ concentrations.

Clinical Studies
Studies were performed in 10 CHF patients and 8 healthy control subjects without left ventricular systolic dysfunction or conventional cardiac risk factors. All subjects gave fully informed written consent for the studies, which were approved by the relevant local research ethics committees. CHF patients were undergoing clinically indicated cardiac catheterization, and healthy subjects were undergoing cardiac electrophysiological assessment. All CHF patients and control subjects were in sinus rhythm. Heart failure therapies were omitted the morning of the study in CHF patients, and antiarrhythmic medication was omitted 48 hours before studies in healthy subjects. Catheters were inserted into the left ventricle (LV) via the right femoral artery and into the pulmonary artery (PA) via the right femoral vein. After the catheters had both remained in situ for 5 minutes, 2 mL blood from each site was analyzed for O₂ saturation (OSM3 Hemoximeter, Radiometer), and 5 mL was injected into a 4-mL EDTA collection tube. This was centrifuged at 3500 rpm for 5 minutes. Both the red cell fraction and plasma were snap-frozen in liquid nitrogen and then stored at −80°C for subsequent analysis.

Cardiac index was calculated by the Fick method.¹¹ Measurement of Hb-bound NO was achieved by modifying the method described by Gladwin et al.¹² Briefly, lyed blood samples were incubated with or without cyanide for 30 minutes before purification of the Hb fraction on a G25 column. Bound NO was liberated with triiodide in acid and detected in the carrying gas with an AMI 700 NO electrode.
LV NOx, PA NOx, with the relaxation observed at 95% O2. An unpaired striction, the enhancement in relaxation was calculated by comparing changes in vessel responsiveness to NO, after correction for precon-

LV SNO-Hb, mol NO/mol Hb tetramer
PA SNO-Hb, mol NO/mol Hb tetramer
LV HbFeNO, mol NO/mol Hb tetramer
PA HbFeNO, mol NO/mol Hb tetramer
LV NOx, μmol/L
PA NOx, μmol/L
LV NO2−, μmol/L
PA NO2−, μmol/L
LV RSN0, μmol/L
PA RSN0, μmol/L

Cardiac index

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Statistical Analysis
One-way ANOVA using a Bonferroni post hoc test was used to compare changes in mean values between RBC- and GSNO-induced relaxations at each O2 concentration. To account for O2-induced changes in vessel responsiveness to NO2, after correction for preconstriction, the enhancement in relaxation was calculated by comparing with the relaxation observed at 95% O2. An unpaired t test was used to compare metabolite values between patient groups, and a paired t test was used to compare values within individuals. A bivariate correlation (Pearson’s correlation coefficient) assessed the relationships between total metabolite flux (defined as the sum of HbFeNO and SNO-Hb loss or gain) and cardiac index ALTERATION IN Hb O2 saturation gradient in vivo and vasorelaxation and Hb-bound NO in vitro. The SPSS statistics package was used.

Results

In Vitro Study
RBCs containing SNO-Hb and HbFeNO (0.0059±0.0006 and 0.0098±0.001 mol NO/mol Hb tetramer, respectively) added to PE constricted aortic rings incubated in 95% O2 resulted in a further 35±6% constriction. RBCs added to rings incubated in 1% O2 resulted in an initial 35±4% relaxation, followed by a 22±8% constriction (Figure 1A). Preincubation at 1% O2 of rings with ODQ abolished relaxation with no impact on constriction (34±7%; P=NS).

Preincubation at 1% O2 of rings with ODQ abolished relaxation with no impact on constriction (34±7%; P=NS). RBC-induced vessel relaxations were greater at 1% and 2% O2 (P<0.05 versus 95%). GSNO-induced vessel relaxations were greater under hypoxic conditions, depending on GSNO concentrations (Figure 1B). The enhancement of relaxation as a result of hypoxia was assessed for GSNO and RBCs by calculating the fold difference at 1% compared with 95% O2. This mean enhancement was 2.6±0.8-fold for GSNO and was independent of GSNO concentration, whereas the enhancement in RBC-induced relaxations was found to be 7.6±1.7-fold (P<0.05 compared with GSNO enhancement), suggesting that hypoxic hyperresponsiveness of vessels alone did not account for the increased RBC-
induced relaxations observed at lower $O_2$. Relaxations at 1% for RBCs (with and without exogenous NO added) correlated with RBC SNO-Hb concentration ($R^2=0.88$) but not with HbFeNO.

**Clinical Studies**

Subject characteristics are described in the Table. Ejection fraction by echocardiogram and/or LV angiogram was $\geq40\%$ for CHF patients and $\geq45\%$ for control subjects. Higher PA levels of HbFeNO were found in CHF patients ($P<0.05$) (Figure 2A). In CHF patients, SNO-Hb levels increased with oxygenation across the pulmonary circulation ($P=0.005$). The converse was true for HbFeNO ($P=0.03$). In control subjects, there was no significant difference in concentration of either metabolite across the circulation, although levels of HbFeNO were nonsignificantly greater in the LV. The total metabolite flux correlated inversely with cardiac index ($P<0.05$) and correlated positively with the AV $O_2$ saturation change across the pulmonary circulation ($P<0.05$) for the total study population (Figure 2B and 2C, respectively).

**Discussion**

Our findings confirm that Hb-bound NO metabolites are vasoactive under hypoxic conditions and are supportive of an allosteric mechanism effecting NO release. Although in agreement with others, we find that vessels exhibit increased responsiveness to nitrovasodilators during hypoxia, and this alone does not account for RBC-mediated hypoxic vasodilatation. We also found that RBC-induced vasodilation correlated strongly with SNO-Hb content (but not with HbFeNO). One explanation for this phenomenon is that SNO-Hb passes on its vasodilatory potential to smaller-molecular-weight proteins in the RBC membrane that in turn release NO bioactivity in the microcirculation.

Our clinical data show levels of Hb-bound NO to be in the 0.008- to 0.005-mol NO/mol Hb range, in close agreement with other studies using different methodologies. We establish that transpulmonary gradients of Hb-bound NO exist in patients with CHF and that the magnitudes of these gradients correlate with the Hb $O_2$ saturation gradient and correlate inversely with cardiac index. An NO gradient does not represent the amount of NO delivered to tissue, most likely to be nanomolar or less under physiological conditions, but may reflect the transfer of NO from HbFeNO to SNO-Hb. In control subjects, no measurable metabolite flux across the circulation was detected, although baseline gradients have previously been described under different experimental conditions. The tendency for higher levels of total HbFeNO in the LV relative to PA in control subjects may indicate loss of Hb(FeIII)/NO, which is reactive and unstable, although we are unable to substantiate this hypothesis because of limitations of current methodologies. Speculatively, SNO-Hb may be used peripherally in CHF, a condition characterized by increased $O_2$ extraction and altered microvascular $O_2$ kinetics. Taken together, our results suggest a role for NO as an endocrine vasoregulator in CHF. We acknowledge that we do not demonstrate transfer of NO between metabolites or define the mechanism of delivery of NO from its metabolites; the precise mechanism is still under debate. However, our in vitro and in vivo data are consistent with an allosteric mechanism of NO delivery.

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**References**

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