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UNIVERSITY OF CALIFORNIA, SAN DIEGO

# The Interplay of Nitric Oxide and Hemoglobin in Hemolytic Anemia: Consequences for Parasite and Host During Malaria.

A Dissertation submitted in partial satisfaction of the requirements for the degree
Doctor of Philosophy

in

Bioengineering

by

Peter Sobolewski

Committee in Charge:

Dr. Marcos Intaglietta, Chair
Dr. Roland Blantz
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Dr. Wayne Giles
Dr. Paul Johnson
Dr. Robert Winslow

2006

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The Dissertation of Peter Sobolewski is approved and it is of acceptable quality and form for publication:

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Chair

University of California, San Diego

2006

# DEDICATION

I dedicate this dissertation to my parents, Roman and Bożena. From an early age they stressed the importance of education and pushed me to achieve. Even though at the time it often felt like punishment or tedium, I greatly appreciate their efforts; they never let me settle for less then I was capable of. As such, while this is a milestone achievement for me, I see it as new beginning rather than an end.

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LIST OF ABBREVIATIONS

**CO** carbon monoxide

**deoxyHb** deoxyhemoglobin

**ECM** experimental cerebral malaria

**ESM** experimental severe malaria

**Hb** hemoglobin

**metHb** methemoglobin

**NO** nitric oxide

**NOS** nitric oxide synthase

**oxyHb** oxyhemoglobin

**RBC** red blood cell

**ROS** reactive oxygen species

LIST OF FIGURES

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I would like to acknowledge Dr. Frangos for giving me a lab to aim for in selecting graduate programs and accepting me into his lab upon my arrival in San Diego. He continually challenged me with new ideas and responsibilities, while leaving me with a great deal of independence.

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Finally, I would like to acknowledge my parents, my fiancée Karen Landon, and all my friends for supporting me along the way, as well as my doctor, Dr. Bernstein, for getting me through a difficult last year.

Chapter 3 is, in full, a reprint of the material as it was published in the Journal of Investigative Medicine, 2005 (Sobolewski P, Gramaglia I, Frangos JA, Intaglietta M, van der Heyde HC. "Hemoglobin serves to protect Plasmodium parasites from nitric oxide and reactive oxygen species." *J Investig Med*. 2005 Jul ; 53(5): 246-52). I would like to acknowledge the BC Decker for permitting me to include this work in my dissertation and all of the co-authors for their intellectual and written contributions.

Chapter 4 is, in full, a reprint of the material as it was published in Infection & Immunity, 2005 (Sobolewski P, Gramaglia I, Frangos JA, Intaglietta M, van der Heyde

HC. “Plasmodium berghei resists killing by reactive oxygen species.” *Infect Immun.* 2005 Oct ; 73(10): 6704-10). I would like to acknowledge the American Society for Microbiology for permitting me to include this work in my dissertation. I, the dissertation author, performed the in vitro NO and ROS treatments and Hb measurements. Dr. Gramaglia was responsible for all animal aspects of the work, tracked parasitemia and survival, and performed the flow cytometric analysis. Drs. Frangos, Intaglietta, and van der Heyde directed and supervised the research.

Chapter 5 is, in full, a reprint of the material as it was published in Trends in Parasitology, 2005 (Sobolewski P, Gramaglia I, Frangos J, Intaglietta M, van der Heyde HC . "Nitric oxide bioavailability in malaria." *Trends Parasitol*. 2005 Sep ; 21(9): 415-22). I would like to acknowledge Elsevier for permitting me to include this work in my dissertation and all of the co-authors for their intellectual and written contributions.

Chapter 6 is, in full, a reprint of the material as it was submitted to Nature Medicine, 2006 (Irene Gramaglia*, Peter Sobolewski*, Diana Meays, Ramiro Contreras, John P. Nolan, John A. Frangos, Marcos Intaglietta, and Henri C. van der Heyde (*contributed equally). "Low nitric oxide bioavailability contributes to the genesis of experimental cerebral malaria." *Nature Medicine*, 2006.). I would like to acknowledge the Nature Publishing Group for permitting me to include this work in my dissertation. I, the dissertation author, was responsible for the analytic aspects of the cGMP, vascular leak, plasma Hb, NO quenching, and nitrite measurements. Dr. Gramaglia, in addition to handling all animal aspects of the work, tracked parasitemia, survival and MABP, and assessed thrombocytopenia and plasma cytokine levels. Diana Meays implanted blood pressure telemetry devices. Ramiro Contreras directed the layout of the NO/air gas mixing system and built the NO gas treatment chamber. Guang Sun assessed parasitemia and survival in NOS knockout mice. Figure 4 was prepared by Dr. van der Heyde. Drs. Nolan, Frangos, Intaglietta, and van der Heyde directed and supervised the research.

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"Plasmodium berghei resists killing by reactive oxygen species."

Sobolewski P, Gramaglia I, Frangos JA, Intaglietta M, van der Heyde HC

*Infect Immun*. 2005 Oct ; 73(10): 6704-10

"Nitric oxide bioavailability in malaria." (Review)

Sobolewski P, Gramaglia I, Frangos J, Intaglietta M, van der Heyde HC

*Trends Parasitol*. 2005 Sep ; 21(9): 415-22

"Hemoglobin serves to protect Plasmodium parasites from nitric oxide and reactive oxy-

gen species.**”** (Review)

Sobolewski P, Gramaglia I, Frangos JA, Intaglietta M, van der Heyde HC

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**Manuscripts in Preparation**

“Internal hemoglobin concentration modulates nitric oxide scavenging by red blood cells.”

Sobolewski P, Intaglietta M, Frangos JA.

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“Low levels of bioavailable nitric oxide lead to experimental severe malaria.”

Sobolewski P, Gramaglia I, Frangos JA, Intaglietta M, van der Heyde HC

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“Superoxide functions in malarial pathogenesis, but not parasite killing”

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“Nitric oxide has no detectable effect on malarial parasite viability**.**”

Sobolewski P, Gramaglia I, Frangos JA, Intaglietta M, van der Heyde HC

FASEB EB2005 (Poster)

“Reactive oxygen species have no detectable effect on malarial parasite viability. ”

Sobolewski P, Gramaglia I, Frangos JA, van der Heyde HC

BMES 2004 (Poster)

“Internal red blood cell microviscosity impairs nitric oxide scavenging by hemoglobin.”

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ABSTRACT OF THE DISSERTATION

# The Interplay of Nitric Oxide and Hemoglobin in Hemolytic Anemia: Consequences for Parasite and Host During Malaria.

by

Peter Sobolewski

Doctor of Philosophy in Bioengineering

University of California, San Diego, 2006

Professor Marcos Intaglietta, Chair

Hemoglobin (Hb) is the protein responsible for the oxygen carrying capacity of red blood cells (RBCs). However, its iron-containing, heme prosthetic group is also capable of generating radical species and reacting with nitric oxide (NO). If oxygen is present, the latter reaction results in the oxidation of the heme and the conversion of NO to nitrate. Within the RBC, Hb redox reactivity is minimized by the presence of protective enzyme systems and NO consumption by RBCs is >600 lower than that of free Hb. However, in hemolytic anemia, cell-free Hb released into the blood, which results in vasoconstriction and vascular dysfunction. The case of malaria is particularly interesting, because the *Plasmodium* parasite resides within the RBC and is surrounded by Hb, but also ruptures RBCs as part of its lifecycle, releasing cell-free Hb into the blood.

In this dissertation the dual role of Hb during malaria is examined: intracellular Hb is evaluated in the context of immune-system-generated, anti-parasitic oxidative spe-

cies (NO, superoxide, peroxynitrite), while the Hb released into the blood is considered in the context of pathogenesis. It is established that NO and reactive oxygen species (ROS) are unlikely to play an anti-parasitic role, due to the presence of large amounts of Hb within RBCs, as well as the possible existance of intrinsic protective mechanism. The cell-free Hb, on the other hand, participates in pathogenesis by scavenging NO in experimental severe malaria (ESM). It is determined that low NO bioavailability contributes to malaria pathogenesis and that NO restoration can protect animals from development of disease.

In conclusion, the study of malaria in the context of hemolytic anemia, taking into account the toxic nature of cell-free Hb and its potency as an NO scavenger, may to lead to the development of new adjunct therapies.

# Chapter 1: Introduction

## Blood

Blood is a unique, circulating tissue, accounting for ~8% of the body weight of a human. As the primary fluid of the circulatory system, it plays an integral role in the maintenance of homeostasis; it is responsible for the delivery of oxygen, metabolites, and signaling molecules and the clearance of carbon dioxide and metabolic waste. By its nature, blood is ubiquitous, permeating the other tissues and blood disorders frequently result in the dysfunction of other tissues. [1]

Blood is composed plasma, the fluid component (~55% of volume), and cells: platelets, white blood cells (WBCs), and red blood cells (RBCs). Plasma is an aqueous solution of proteins (predominantly albumin and immunoglobulin), small molecules, and electrolytes. Platelets and WBCs play key roles in thrombosis and the immune system, respectively, however the focus of this work will be on RBCs, which, by volume, are the dominant cellular component of blood and fulfill its oxygen carrying role. [1]

## Red Blood Cells

Red blood cells are biconcave discs, with human RBCs being approximately 8μm in diameter and 90fl in volume, with a 135$\mu m^2$ surface area [2]. Red blood cells are unique cells, because they lack the organelles typically associated with mammalian cells; RBCs do not possess a nucleus, mitochondria, nor ribosomes [3]. RBCs are composed of a cell membrane, cytoskeleton, and cytoplasmic compartment. The cell membrane is predominantly composed of palmitic (41%), oleic (19%), linoleic (15%), stearic (8%), and arachidonic (8%) fatty acids, as well as stroma proteins (<2% of total protein content) [4]. The cytoskeleton consists of a spectrin network, which is attached to band 3, a transmembrane anion exchange protein, by anakyrin [3]. The cytoplasmic compartment of an RBC is composed of 72% (m/v) water and 37% (m/v) protein, of which 98% is hemoglo-

bin [4]. Hemoglobin (Hb) is the iron-containing protein that binds oxygen, allowing the RBC to fulfill its oxygen carrying role. Given that a human RBC contains ~30pg of Hb, an adult human will have about 900g of Hb in the blood [1].

The metabolic pathways of a mature RBC revolve around the maintenance of its hemoglobin. In addition to ATP, the RBC glycolysis pathway generates NADH and contains a pentose shunt to generate NADPH, as well as the Rapoport-Luebering shunt to generate 2,3-bis(phosphoglyceric acid) (2,3 BPG). The NADH is utilized to maintain Hb in the ferrous (oxygen binding) state by reducing any oxidized ferric Hb (methemoglobin) via cytochrome $b_5$ (methemoglobin reductase). The NADPH is utilized in glutathione reduction, protecting against oxidation, while the 2,3 BPG is an allosteric factor modulating oxygen Hb affinity. [5]

**Hemoglobin**

Hemoglobin is a tetrameric molecule with a molecular mass of approximately 64kDa, consisting of two dimers, each typically composed of one alpha and one beta subunit. Each globular subunit possesses one heme prosthetic group, consisting of a porphyrin ring and ferrous iron atom ($Fe^{2+}$), where oxygen binding occurs, however the tertiary and quaternary structure, as well as a conformational shift influence the oxygen binding properties. The oxygen binding curve of hemoglobin is sigmoidal, with normal hemoglobin being half-saturated with oxygen at 27mm Hg (this value is referred to as $P_{50}$). When it is deoxygenated, Hb is in the tense (T) state, with salt-bridges stabilizing the contact area between opposite alpha and beta subunits. Upon the binding of oxygen, the dimers rotate, shifting the molecule into the relaxed (R) state. This structural change is allosterically regulated by 2,3 BPG, which preferentially binds to and stabilizes the T state. [3]

While the primary role of Hb is to carry oxygen, it can also react with nitric oxide (NO) and carbon monoxide (CO), due to the similarity of these molecules to oxygen.

All three of these gasses are composed of small molecules that are neutrally charged and hydrophobic. All three of these molecules bind reversibly with the ferrous iron of the Hb heme group and have similar on rates (on the order of $10^7$ $M^{-1}$ $s^{-1}$), however NO has a much slower off rate (on the order of $10^{-5}$ $s^{-1}$, as compared to 15 $s^{-1}$ and .01 $s^{-1}$ for oxygen and CO, respectively). [6]

Further, because iron is a transition metal, it can also be involved in oxidation and/or reduction (redox) reactions. Oxygenated Hb (oxyHb) can auto-oxidize to produce superoxide and methemoglobin (metHb), which has its heme iron in the ferric state ($Fe^{3+}$). The reaction of oxyHb with NO also oxidizes Hb, resulting in metHb and nitrate. Superoxide can play a dual role, because it can oxidize oxyHb and reduce metHb at a similar rate, resulting in ~40% metHb at steady state. Similarly, both metHb and oxyHb can react with hydrogen peroxide, resulting in ferrylhemoglobin (ferrylHb), which has its iron in the $Fe^{4+}$ state. [7] These redox reactions result in a dramatic reduction in oxygen binding (metHb has a $P_{50}$ of approx. 10mm Hg) and are the reason for the presence of catalase, superoxide dismutase, and glutathione in the RBC, which serve to protect the Hb, and cytochrome $b_5$, which reduces metHb back to ferrous Hb [5].

**Cell-free Hb**

While the redox activity of Hb is well regulated within the RBC, cell-free Hb present in the plasma is not afforded the same level of protection. A great deal of recent research has been spent on studying the effects of cell-free Hb in the context of blood substitutes [8] and hemolytic disorders [9]. Briefly, cell-free Hb is more than 600 times as efficient at reacting with and consuming NO [10], making it a potent vasoconstrictor [11]. Further, the uncontrolled redox reactivity of cell-free Hb results in the generation of radicals and increased oxidative stress, leading to cellular damage [8]. Clearly, the compartmentalization of Hb within the RBC is an important mechanism in maintaining

vascular homeostasis.

**Hemolytic anemia**

While new strategies are being developed by the blood substitute field to address the problems associated with cell-free Hb [12, 13], options such as chemical modification or liposome encapsulation are not available in the context of hemolytic disorders. As a result, there is a great need to understand the mechanisms of pathogenesis in hemolytic disorders and the role cell-free Hb, in order to develop appropriate therapy.

Intravascular hemolysis can occur as a result of physical trauma to the RBC (e.g. cardiac heart valve hemolysis), immune-system mediated RBC destruction (e.g. paroxysmal nocturnal hemoglobinuria), hemoglobin disorders (e.g. sickle cell anemia), the effect of chemicals, or the action of infectious agents. While these disorders have diverse symptoms and causes, the common etiology of their vascular complications (e.g. pain, vasoconstriction, endothelial dysfunction) is likely to be oxidative damage and consumption of NO caused by cell-free Hb. [9] In this context, malaria is one of the biggest causes of intravascular hemolysis in the world [14], however up until now the role of Hb during malaria has not been investigated.

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# Chapter 2: Internal hemoglobin concentration modulates nitric oxide scavenging by red blood cells.

**Abstract**

Hemoglobin (Hb) is a potent nitric oxide (NO) scavenger, however the scavenging of NO by erythrocytes is reduced compared to a solution containing equivalent amount of free Hb. It is proposed that higher internal Hb concentration within the RBC is one factor that accounts for this reduced NO scavenging, due to high microviscosity and decreased NO diffusivity. This study utilized changes in solution osmolarity to affect internal Hb concentration and the effects of solution osmolarity on the rate of NO consumption were examined via NO electrode. It was observed that the rate of NO decay in the RBC solution depends inversely on the osmolarity. These findings support the hypothesis that an intrinsic factor contributes to reduced NO consumption by by Hb when encapsulated in RBCs.

**Introduction**

A great deal of research has been focused on determining why hemoglobin (Hb) encapsulated in red blood cells (RBCs) is two orders of magnitude less effective at scavenging nitric oxide (NO), as compared to cell-free Hb [1]. Among the proposed mechanisms are diffusional limitations due to unstirred external boundary layers around the RBCs [1], a cell-free region due to intravascular flow [2], membrane barriers to NO diffusion [3], and the possibility of NO transport via SNOHb [4, 5] or nitrite [6]. However, recent simulations demonstrate that no one set of parameters can account for all of the published data, with the greatest uncertainty being in the degree of NO membrane permeability [7]. We propose that NO consumption by RBCs is limited not by cytoskeletal/membrane resistance to diffusion, but is due to the compartmentalization of Hb in RBCs and resultant higher internal concentration of Hb causing reduction in diffusivity of NO within the RBC.

## Methods

### Nitric oxide solution

Saturated NO stock solution (~1.8mM) was prepared by bubbling NO gas (2.0 CP grade, Airgas West, San Diego, CA) through degassed PBS for 10 min [8].

### Buffers

HEPES buffers (pH=7.4, 40mM HEPES, 5mM glucose, Sigma, St. Louis, MO) were prepared with a range of osmolarities (200-400 mOsmol) and were verified via osmometer (3MOPlus, Advanced Instruments, Inc., Norwood, MA).

### Blood cell preparation

Blood was harvested from female C57BL/129 mice in accordance with IACUC protocol. RBCs were pelleted at 800g for 30min and washed with isotonic HEPES buffer. RBC suspensions were prepared at concentration of $2x10^6$ cells/mL (.93μM Hb).

### Experimental setup

At room temperature, 3mL samples of RBC suspensions of different osmolarities were stirred using a magnetic stir bar and injected with a bolus of NO. The decay of the NO was monitored via amiNO-700 probe and inNO-T sensor (Innovative Instruments, Inc., Tampa, FL).

### Data Analysis and Statistics

Data was fitted (first-order decay) using Synergy KaleidaGraph 3.6 and correlation was determined by Spearman Rank Correlation Test, using StatView 5.

## Results and Discussion

The principal finding of this study is that the rate of NO quenching by a solution of RBCs is modulated by changes in solution osmolarity (Fig. 1A). Over a range of os-

molarities (200-400 mOsmol), we find that the rate of NO quenching is inversely proportional to and correlates well (Spearman Correlation Rank Test Rho = .60, $p<.0001$) with solution osmolarity (Fig. 1B).

It is well established that changing solution osmolarity results in an inversely proportional RBC volume change [9]. Because the amount of Hb in RBCs remains fixed, change in osmolarity results in a proportional change in internal Hb concentration and a proportional change in internal microviscosity [10, 11]. Additionally, Kreuzer demonstrated an approximately linear, inverse dependence of oxygen diffusivity on Hb concentration [12]. Increasing internal microviscosity by shrinking RBCs may serve to limit the availability of the heme pocket to NO by reducing rotational diffusion, as hypothesized by Perutz to occur if Hb concentration is increased beyond that occurring naturally inside RBCs [13]. Modeling of bimolecular reactions incorporating the importance of rotational diffusion by Solc and Stockmayer and others [14], predicts a reduction in reaction rate between NO and Hb upon shrinkage of the RBC. Conversely, swelling RBCs, should facilitate NO reaction with Hb by increasing the diffusivity of NO, because of the reduced Hb concentration. A similar effect of internal Hb concentration on oxygen uptake has been noted by Vandegriff [15] and Carlsen and Comroe observed an inverse relationship between NO uptake and solution osmolarity in deoxygenated RBCs [16].

Our data suggests the existence of an intrinsic effect, although the osmotic changes likely also affect external diffusion, because the cell size and surface-to-volume ratio change. The boundary layer surrounding an RBC should depend on the cube root of the cell radius [17] and modeling suggests that the external diffusional resistance should increase with RBC size, decreasing NO consumption [7]. However, our observations show the opposite effect, because NO consumption increased with swelling, suggesting that

an internal diffusional change compensates for the change in boundary layer thickness. Similarly, one would predict that the reduction in surface-to-volume ratio caused by the swelling of a discoid to a sphere would result in reduced NO consumption. However, Carlsen and Comroe did not observe NO uptake changes by deoxygenated RBCs converted to spherocytes with no change in cell volume [16]. We conclude that osmolarity changes modulate an intrinsic limitation to NO consumption by erythrocytes.

It is important to consider that the osmolarity treatments may be affecting the cytoskeleton or membrane of the RBCs and that the changes in NO consumption may be caused by changes in the cytoskeletal diffusion barriers [3]. Huang et al observed that 4,4'-diisothiocyanatosilbene-2,2'-disulfonic acid (DIDS) treatment of RBCs increased NO consumption and bis(sulfosuccinimidul)-suberate ($BS^3$) treatment decreased NO consumption. These results were explained by the existence of a cytoskeletal diffusion barrier, because DIDS promotes band 3 dimerization and decreases ankyrin binding (reducing the diffusional barrier) and $BS^3$ promotes tetramerization and ankyrin binding (increasing the diffusional barrier). However, DIDS treatments are known to change RBC morphology, increasing cell volume [18], which could account for the ~15% increase in NO consumption observed by Huang following DIDS treatment [3].

Our findings support the hypothesis that reduced RBC intracellular diffusion is one of several factors that accounts for the discrepancy in NO consumption between RBC-encapsulated Hb and free Hb. We would predict, based on the diffusion coefficient data for oxygen in hemoglobin solutions [12], that the ~50% difference in concentration between the same amount of hemoglobin encapsulated in RBCs or free in solution accounts for ~40% difference in the diffusion coefficient and, correspondingly, reaction rate. Although this difference does not account for the full difference in NO quenching

between the two solutions, a 40% reaction rate difference is similar in magnitude to the cytoskeletal barrier effects (~15-50%) observed by Huang [3].

The effect of solution osmolarity on NO quenching by RBCs may also be physiologically significant in the kidney, where NO plays a key role in sodium and water homeostasis [19]. Recent modeling by Zhang suggests that NO bioavailability in the renal medulla is determined primarily by the NO consumption rate of RBCs [20]. However, current NO consumption parameters result in 2 to 5-fold underestimate of medullary NO concentration [20]. We propose that the reduction in NO consumption by RBCs exposed to high osmolarity (>700 mOsmol [21]) can contribute to this difference between calculated and measured NO bioavailability in the medulla.

In conclusion, our finding that solution osmolarity modulates NO consumption by RBCs suggests that high internal Hb concentration may be a contributing factor in reducing NO consumption by RBC-encapsulated Hb. This effect may be of particular relevance to the blood substitute field, because encapsulating Hb in liposomes [22] provides a simple mechanism to reduce NO consumption. Additionally, it may contribute to the preservation of NO bioavailability in the kidney, where RBCs are exposed to high osmolarities.

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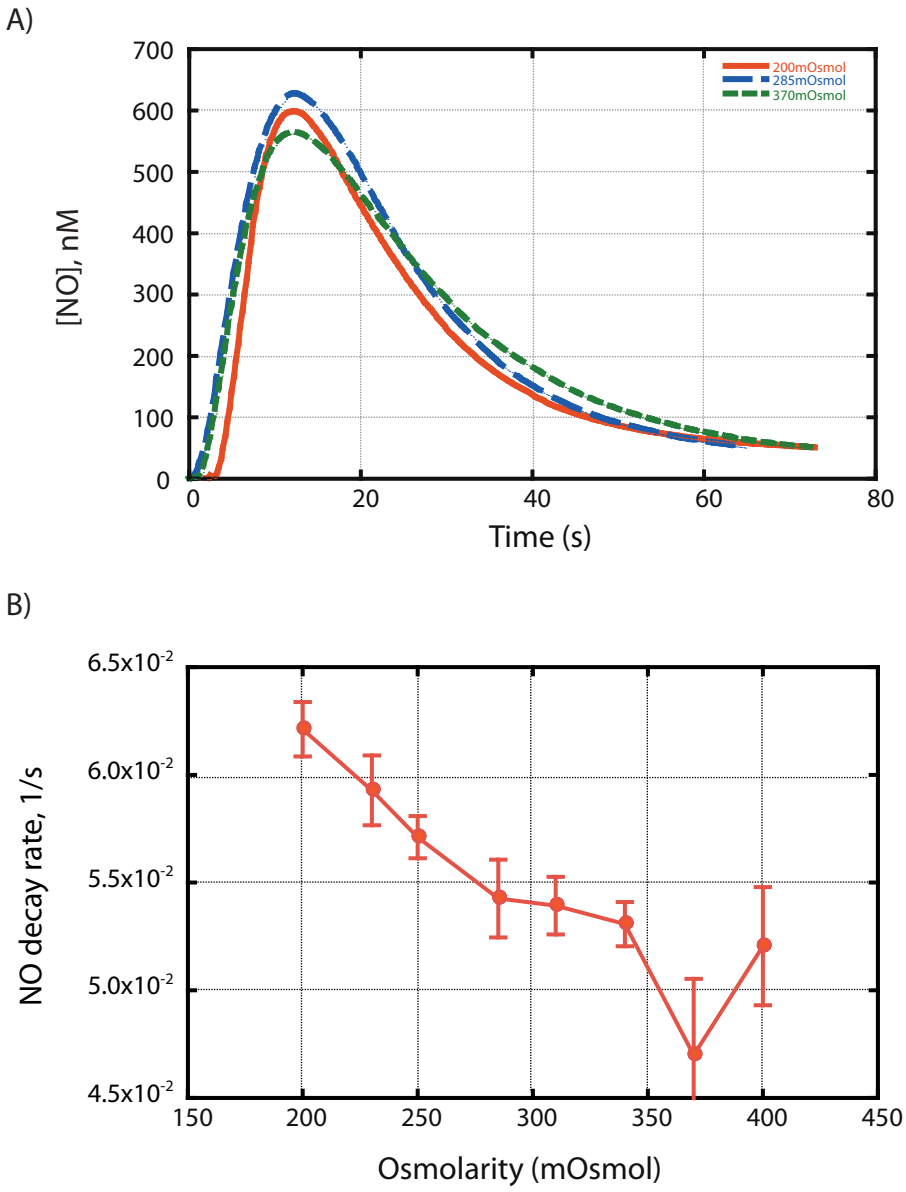


**Figure 2.1: NO quenching by a solution of RBCs is modulated by solution osmolarity.** A) Representative tracings of NO concentration vs. time in three solutions of different osmolarities containing $2x10^6$ RBC/mL (.93μM Hb). B) Mean reaction rate and standard error vs. osmolarity (minimum n=6). The reaction rate vs. osmolarity data shows statistically significant correlation (Spearman Rank Correlation Test Rho=.60, $p<.0001$).

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# Chapter 3: Hemoglobin serves to protect *Plasmodium* parasites from nitric oxide and reactive oxygen species.

REVIEW ARTICLE

# Hemoglobin Serves to Protect *Plasmodium* Parasites from Nitric Oxide and Reactive Oxygen Species

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**ABSTRACT**

Our understanding of how the host immune response kills *Plasmodium*, the causative agent of malaria, is limited and controversial. One widely held belief is that reactive oxygen species are crucial for controlling parasite replication. One of the hallmarks of blood-stage malaria is the cyclic rupture of erythrocytes by the parasite, which releases free hemoglobin into the circulation. We propose that this free hemoglobin, as well as the hemoglobin within the erythrocyte and surrounding the parasite, effectively shields *Plasmodium* from reactive oxygen species well in excess of those achievable in vivo.

**Key Words:** nitric oxide, reactive oxygen species, malaria, *Plasmodium*, hemoglobin

Malaria, a devastating disease that kills over 2 million people each year, is caused by infection with parasites of the genus *Plasmodium*.[1,2] *Plasmodium* is transmitted by the bite of an infected mosquito vector, and this initiates first the liver and then the blood stage of the infection. The liver stage is asymptomatic and is beyond the scope of this article. The blood stage comprises parasites (merozoites) invading erythrocytes, developing within the erythrocyte, and producing new progeny every 48 or 72 hours, depending on the species. The blood stage of the infection causes clinical complications, such as fever and chills, as well as life-threatening multiorgan failure (brain, lungs, and kidney). *Plasmodium falciparum* is the most virulent of the species of *Plasmodium* that infect humans, and this species adheres to activated venular endothelium to sequester itself from the lymphoid and filtration organs.[3] Despite considerable research effort, an effective vaccine against *Plasmodium* infection remains elusive perhaps because our understanding of the mechanism(s) underlying parasite killing by the immune system is poorly defined and controversial. The observation by Jensen and colleagues of crisis forms of the parasite (parasites exhibiting deteriorating morphology) immediately prior to declines in parasitemia[4] suggests that a soluble factor is responsible for parasite killing, rather than erythrophagocytosis. One potential effector mechanism that is widely believed to be critical for this parasite killing is the immune production of reactive oxygen species (ROS), including nitric oxide ($NO^{\bullet}$), superoxide, and peroxynitrite. These ROS are produced mainly by activated phagocytic cells, including macrophages and neutrophils, although T helper 1 cells are also reported to produce $NO^{\bullet}$ during experimental malaria.[5]

$NO^{\bullet}$, a highly toxic free radical, is produced during the enzymatic conversion of L-arginine to L-citrulline by members of the nitric oxide synthase (NOS) family.[6] To date, three members of the NOS family have been identified, specifically endothelial (eNOS or NOS3), neuronal (nNOS or NOS1), and inducible (iNOS or NOS2).[7–9] All three isoforms have similar $NO^{\bullet}$ production rates of about 1 µM/min/mg protein.[10] The iNOS isoform is responsible for the high-level production of $NO^{\bullet}$ because it is highly expressed after activation of phagocytes, comprising up to 1% of total protein.[11]

Activated phagocytes also produce superoxide by the enzymatic reduction of molecular oxygen by reduced nicotinamide adenine dinucleotide phosphate (NADPH) oxidase (pHox family). On activation, the NADPH oxidase is assembled from its membrane and cytosolic pHox subunits (p91, p22, p67, p47, p40) and superoxide is formed inside a phagosome or outside the cell.[12] When both $NO^{\bullet}$ and superoxide are produced in close proximity, for example, by activated phagocytes, this results in the formation of peroxynitrite by the rapid reaction between $NO^{\bullet}$ and superoxide.[10]

$NO^{\bullet}$ is well established as a major cytotoxic molecule in infectious diseases.[13] Because $NO^{\bullet}$ and ROS are important in the control of other parasitic diseases, it is generally accepted that they play a critical role in killing the malarial parasite. In fact, this hypothesis is supported in all 15 recent reviews addressing the role of $NO^{\bullet}$ or ROS in malaria. However, evidence from animal models challenges this notion: mice deficient in the iNOS gene or p91phox gene exhibit similar *Plasmodium berghei* para-

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sitemia as controls, indicating that iNOS-derived NO•, pHox-derived superoxide, and peroxynitrite do not serve to control parasitemia.[14–18] Further, the role of hemoglobin (Hb) as a ROS quencher has not been taken into account, leading us to propose that NO• and ROS do not play a significant role in malarial parasite killing.

## ROLE OF Hb

Hb is released when *Plasmodium* ruptures the erythrocyte, and our preliminary experimental data suggest that the plasma concentration of free Hb on day 6 of *P. berghei* infection (when the animal becomes moribund) can reach 100 μM. The in vivo effects owing to free natural molecular Hb in blood are well defined in the research community interested in the development of molecular Hb–based oxygen carrying blood substitutes.[19,20] Free Hb is a powerful in vivo scavenger of NO•, leading to vasoconstriction and impaired microvascular blood perfusion, which, in turn, are major determinants of tissue and organism survival.[21] Free Hb is almost 1,000-fold more efficient at scavenging NO• than Hb packaged in red blood cells (RBCs),[22] so this free Hb may be protective by significantly quenching ROS before they can diffuse into the erythrocyte. Besides free Hb, *Plasmodium* resides in a parasitophorus vacuole within the erythrocyte and is surrounded by about 0.5 fmol Hb. The combination of free molecular Hb in blood plasma and Hb in the erythrocyte likely protects the parasite because of complex ROS scavenging reactions.

The chemical reactions of NO• with Hb depend on the form and state of the Hb molecule. When Hb passes through the lung, it changes from the taut or T state without oxygen (deoxyhemoblogin) to the relaxed or R state with oxygen bound to heme moiety (oxyhemoglobin).[23,24] In the venous microcirculation, in which sequestered parasites such as *P. falciparum* and murine-infecting *P. berghei* and *Plasmodium yoelii* reside, about 60% of Hb within erythrocytes is oxyhemoglobin and 40% is deoxyhemoglobin. In the arterial microcirculation, most of the Hb is oxyHb (> 99%). NO• reacts with the oxygen in oxyHb, generating methemoglobin [Hb($Fe^{3+}$)] and nitrate (Figure 1, reaction 1). NO• also reacts with deoxyhemoglobin, forming Hb($Fe^{2+}$)NO (see Figure 1, reaction 2), which, in turn, reacts with oxygen to form methemoglobin and nitrate (see Figure 1, reaction 3). Further, oxyhemoglobin is capable of quenching superoxide to form molecular oxygen, hydrogen peroxide, and methemoglobin (see Figure 1, reaction 4).[25] Oxyhemoglobin can also rapidly react with peroxynitrite to ultimately form nitrate and methemoglobin (see Figure 1, reaction 5).[26] Thus, Hb effectively renders exogenous free radicals incapable of directly damaging the parasite.

Hb not only acts as a scavenger of radical species, it can also undergo redox transitions to higher oxidation states, making it a potent oxidant.[27] First, oxyhemoglobin can slowly auto-oxidize, forming methemoglobin and superoxide, and the latter can form hydrogen peroxide via dismutation. However, this process within the erythrocyte is balanced within the erythrocyte by the presence of superoxide dismutase, catalase, and methemoglobin reductase.[28,29] Second, both oxyhemoglobin and methemoglobin can react with peroxides to form the highly oxidant ferryl species, which damages many biomolecules.[30] Again, the presence of catalase within the erythrocyte is likely to mitigate this. Third, Hb may be able to act as a Fenton reagent, catalyzing the formation of the hydroxyl radical from hydrogen peroxide,[31,32] but it is likely that the heme must be free (ie, released from the Hb molecule) for this to occur.[33,34] Finally, recently Cosby and colleagues and Nagababu and colleagues proposed that deoxyhemoglobin within the erythrocyte can reduce nitrite, resulting in NO• production.[35,36] However, this reaction is limited by the influx of nitrite into the erythrocyte and has a low yield of NO•, which may bind to the excess deoxyhemoglobin, according to Figure 1, reaction 2.

Although the intraerythrocytic Hb may generate oxidants and radicals, the extent of exposure of the *Plasmodium* parasite to oxidant stress is mitigated by the presence of superoxide dismutase, catalase, and methemoglobin reductase within the erythrocyte. However, Clark and colleagues argue that the intraerythrocytic environment is not favorable to the parasite and that the requirement of 3% oxygen tension for parasite culture in vitro is also indicative of the parasite being susceptible to oxidant stress.[37] Indeed, in vivo the *Plasmodium* parasite sequesters itself on the venous endothelium, where oxygen tension is low. However, hypoxic conditions actually result in greater radical production by Hb auto-oxidation.[38] This observation, combined with the nitrite reductase role of deoxyhemoglobin proposed by Gladwin and Rifkind, suggests that oxidative stress may be increased when the parasite sequesters. A more likely explanation for parasite sequestration in venules is that cell adhesion molecules (CAMs) are increased under proinflammatory conditions in venules but not arterioles. To combat ROS exposure, *Plasmodium* has adapted by acquiring host superoxide dismutase[39] and possibly synthesizing its own.[40] We conclude that within the erythrocyte, Hb is more likely to act as a ROS scavenger than a source of oxidant stress, thus serving to protect the parasite.

$$Hb(Fe^{2+})O_2 + NO^{\bullet} \rightarrow Hb(Fe^{3+}) + NO_3^- \quad (1)$$

$$Hb(Fe^{2+}) + NO^{\bullet} \rightarrow Hb(Fe^{2+})NO \quad (2)$$

$$Hb(Fe^{2+})NO + O_2 \rightarrow Hb(Fe^{3+}) + NO_3^- \quad (3)$$

$$Hb(Fe^{2+})O_2 + 2H^+ + O_2^{\bullet -} \rightarrow Hb(Fe^{3+}) + O_2 + H_2O_2 \quad (4)$$

$$Hb(Fe^{2+})O_2 + OONO^- \rightarrow \text{intermediates} \rightarrow Hb(Fe^{3+}) + O_2 + NO_3^- \quad (5)$$

FIGURE 1 Chemical reactions of hemoglobin and reactive oxygen species.

## MAXIMAL IN VIVO LEVELS OF NO• AND ROS DURING MALARIA

We envision two maximal scenarios for exposure of *P. falciparum* parasitized erythrocytes to NO•: (1) a parasitized erythrocyte passing through the splenic shunt between arterioles and venules in the red pulp and between activated red pulp macrophages[41] and (2) a sequestered parasite adherent in the venous microcirculation to an activated endothelial cell with a macrophage adherent close by. For the case of the splenic red pulp, the erythrocyte passes within 20 to 30 µm of two layers of macrophages.[41] Assuming a monolayer of cells surrounding the shunt, the NO• production of the macrophages should be about 2 µM.[42] However, the splenic red pulp is filled with erythrocytes in close proximity to the macrophages, resulting in tremendous NO• scavenging capacity, which likely would prevent the majority of the NO• from reaching the parasitized RBC. Thus, the effective dose of NO• experienced by the parasite is likely lower than the 1 µM.

The likely worst-case scenario for the parasite occurs when the erythrocyte is sequestered and there is an activated macrophage in very close proximity (Figure 2). The first source of NO• is the activated venous endothelial cell, which can produce approximately 0.17 fmol/h of NO• in venules exposed to shear stress of 1.8 dyne/cm$^2$.[43] In arterioles, the blood flow results in a 1 to 10% cell-free region near the luminal surface of the endothelium, where NO• is scavenged only by the reaction with oxygen, resulting in a NO• half-life in the range of several minutes to hours, depending on the NO• and oxygen concentrations.[44] In venules, on the other hand, no axial migration of erythrocytes is seen,[45] so NO• is quenched by Hb throughout the vessel. Although models estimate that the arterial endothelium can maintain approximately 100 nM NO• at its luminal surface primarily owing to the cell-free region,[46] a fraction of this concentration is likely at the luminal surface of venules because of the presence of erythrocytes and free Hb. In C57BL/6 mice, we have detected a marked increase in plasma free Hb on day 6 of *P. berghei* infection compared with healthy animals. Therefore, the bulk of the NO• will come from the area of direct contact of the erythrocyte with the single endothelial cell. Based on the endothelial cell to erythrocyte surface area ratio of ~50, as well as the fact that half of the 0.17 fmol/h NO• produced will diffuse away from the lumen, we estimate that 1.7 amol/h NO• will be bioavailable within the RBC (0.17/50/2 fmol/h). If the *P. falciparum*–infected erythrocyte is sequestered for 24 hours, this yields 40.8 amol of NO•, which is well below the 2 fmol of heme within an RBC. Thus, it appears that the endothelium alone cannot produce sufficient levels of NO• to overcome the quenching by the Hb inside the erythrocyte.

Any scenario involving a macrophage is highly dependent on the relative positioning of the macrophage and erythrocyte with respect to the flow (see Figure 2). The macrophage must be extremely close and upstream to have an effect, but if the macrophage touches the parasitized erythrocyte, it will likely phagocytize the erythrocyte, and the parasite will not be able to replicate because its progeny merozoites cannot reach new erythrocytes. An activated macrophage sustains up to 10 fmol/h of NO• production and 1 fmol/h of superoxide production for several hours.[42] This yields an NO• concentration of about 1 µM of NO• near the surface of the macrophage,[42] but the resultant flow-driven NO• "plume" is rapidly scavenged by the surrounding erythrocytes and free Hb. Thus, it is unlikely that the sequestered erythrocyte will experience any marked elevation of NO• levels, and it will remain sheltered from NO• by erythrocytic Hb.

Activated macrophages also produce superoxide, but at a 10-fold lower level than NO•.[47] Owing to the much higher NO• levels and the fact that both radicals come from the same source, all of the superoxide rapidly reacts with the NO•, yielding ~1.5 nM peroxynitrite at the macrophage surface. In addition, Hb in other erythrocytes and plasma

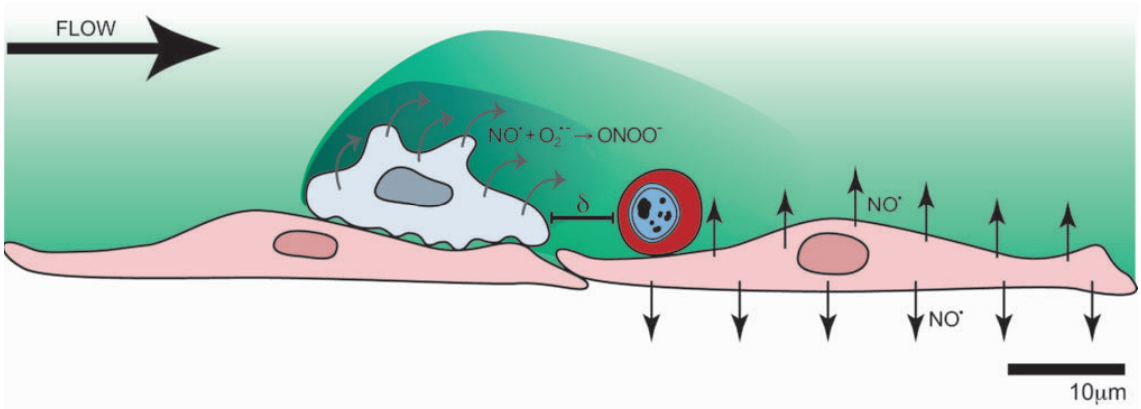


FIGURE 2 Exposure of parasitized erythrocytes to reactive oxygen species derived from endothelial cells or from an activated adherent monocyte/macrophage. NO• = nitric oxide; $O_2^{\bullet-}$ =: superoxide; $ONOO^-$ = peroxynitrite. δ = distance between the upstream monocyte/macrophage and the parasitized erythrocyte. Flow = blood flow in venules. Green shading represents nitric oxide; blue represents peroxynitrite.

Hb quench superoxide, making the likelihood of superoxide reaching the parasite almost nil.

The peroxynitrite resultant from the reaction of superoxide and NO• produced by the macrophage is a highly reactive radical and at physiologic pH has an extremely short half-life.[47] This results in a very short diffusion distance and near-zero concentration (< 0.2 nM) at 10 µm from the macrophage. Thus, a sequestered erythrocyte is unlikely to be exposed to significant concentrations of peroxynitrite (< 1 nM), and peroxynitrite is quenched by oxyhemoglobin, to form methemoglobin, nitrite, and oxygen. Based on the low production and its scavenging by Hb, we propose that peroxynitrite does not play a significant role in parasite killing in vivo.

## CURRENT EVIDENCE IN SUPPORT OF NO•- AND ROS-MEDIATED *PLASMODIUM* KILLING

NO• is reportedly produced at high levels during the course of *P. falciparum* malaria in humans.[48] Further, the blood from patients with uncomplicated malaria contains high levels of iNOS messenger ribonucleic acid (mRNA), with the increased expression primarily in monocytes, compared with those with severe malaria.[49] One concern with this study is that the two patient groups also received different drug treatments, and antimalarial agents have been shown to affect monocyte function.[50] Several studies report that polymorphisms in the promotor region of the *NOS2* gene (CCTTT repeat, G-945C or Lambarene mutation, and C-1173T) result in higher baseline NOS activity in the peripheral blood mononuclear cells and are associated with protection from malaria.[51–54] On the one hand, these investigators proposed that NO• killing of the parasite explains this genetic association with protection from malaria. On the other hand, the results have been inconsistent,[55–58] and we speculate that these findings may be explained by the possible anti-inflammatory, homeostatic, and vasodilatory roles of NO• rather than direct parasite killing. NO• is a potent anti-inflammatory molecule that plays a role in *P. falciparum* sequestration, so increased NO• may down-regulate endothelial activation and CAM expression.[59] Further, NO• quenching by Hb during sickle cell crisis results in vasoconstriction, which can be attenuated by NO• gas or sodium nitroprusside treatment.[60] The reduced NO• bioavailability during sickle cell anemia may also be responsible for the observed increase in soluble vascular cell adhesion molecule 1 expression.[60] Similarly, NO• quenching by Hb is likely to occur during malaria, and increased NO• production may ameliorate microcirculatory complications. Finally, it is possible that the chronic exposure to increased levels of free Hb and NO• quenching during sickle cell anemia serves as an adaptive mechanism, contributing to the resistance to malaria conferred by sickle cell anemia.

Several in vitro coculture studies are also cited as evidence for NO• and ROS killing the malarial parasite. One in vitro study reported that interferon-γ–treated macrophages kill *P. falciparum* during coculture, and this parasite killing is markedly reduced by NG-monomethyl-L-arginine, a NOS inhibitor.[61] However, another in vitro study with cultured *P. falciparum* reported that a saturated solution of NO• does not kill the parasite.[62]

Similarly, superoxide reportedly kills *P. falciparum* when produced in vitro by monocyte-derived macrophages.[63,64] Peroxynitrite also reportedly kills *P. falciparum* in vitro.[65]

However, these studies do not take into account the role of free Hb in the in vivo situation. Ockenhouse and colleagues used indirect methods ($^3$H-hypoxanthine incorporation when the parasite replicates its deoxyribonucleic acid [DNA] at the trophozoite stage) to assess parasitemia.[64] Because coculture of *P. falciparum* with activated monocyte-derived macrophages shortens the replication time from 48 hours to 24 hours, it is likely that lower $^3$H-hypoxanthine incorporation reflects poor labeling, despite the presence of viable parasites, because the parasites are past the stage of DNA incorporation when the radiolabel is added.[66] Fritsche and colleagues detected parasite killing only when they added to the parasite-macrophage cocultures, which are already producing ROS, additional supraphysiologic concentrations (1 mM) of SIN-1 (3-morpholinosydnonimine, a NO• and superoxide donor that reacts to produce peroxynitrite) for 24 hours, resulting in > 1 mM cumulative peroxynitrite production.[65]

## EVIDENCE AGAINST THE ROLE OF NO• AND ROS KILLING OF THE MALARIAL PARASITE

Direct evidence using animal models of malaria argues against the role of NO• in parasite killing. Although NO• is produced at high levels during experimental malaria, mice deficient in the iNOS gene exhibit similar *P. berghei* and *Plasmodium chabaudi* parasitemia as controls, indicating that iNOS-derived NO• and peroxynitrite are not required in parasite killing.[14–16,67] Treatment with the iNOS inhibitor aminoguanidine does not alter the time course of *P. chabaudi* or *P. berghei* parasitemia.[14,16,67,68] However, a compensatory mechanism by one or both of the remaining NOS genes in these mice may account for the lack of an effect. Injection of killed *Propionibacterium acnes* prior to *P. chabaudi* infection results in a marked (> 100-fold) increase in NO• production that is sustained for more than a week, but this high level of NO• has no detectable effect on the time course of parasitemia in mice when compared with similarly treated iNOS knockout mice lacking the elevated NO• production.[16]

Similar to the lack of detectable in vivo killing of malarial parasites by NO•, the results from animal models also question the in vivo role of superoxide and peroxynitrite in parasite killing. P-mice (a strain of mice that have a spontaneous mutation in the pHox gene, resulting in reduced

superoxide production) and p91pHox knockout mice have similar *P. chabaudi* and *P. berghei* parasitemias as controls,[17,18,67] indicating that superoxide and peroxynitrite are not required for parasite killing.

In fact, the results obtained from both the NOS- and pHox-deficient animals can be explained by the ROS quenching capability of Hb. Because Hb is present at such a high concentration within the blood, it can easily quench even the elevated ROS production that occurs during malaria,[69–73] resulting in the effective amount of ROS reaching the parasite being near zero. Thus, modulating ROS production via gene knockout or inhibitor treatment cannot further lower the already low effective dose of ROS to which the parasite is exposed.

Finally, we assessed the ability of ROS to kill the *P. berghei* parasites in a defined ex vivo situation with careful analysis of the Hb oxidation state.[74] Parasitized RBCs were treated ex vivo with 1.5 µM $NO^{\bullet}$, 15 µM $NO^{\bullet}$, 150 µM $NO^{\bullet}$, 1.2 mM $NO^{\bullet}$, 1.5 mM $NO^{\bullet}$, or 1 mM SIN-1 for 10 minutes, and the ROS-treated inoculum (1 million pRBC) was injected into groups of mice. Supraphysiologic $NO^{\bullet}$ treatments up to and including 150 µM do not reduce parasite viability in the inocula. The 150 µM $NO^{\bullet}$ treatment was bioactive inside the erythrocyte because it converts all of the oxyhemoglobin to methemoglobin. The fact that the parasite remains viable, even if treated with $NO^{\bullet}$ (up to 150 µM) or peroxynitrite (up to 220 µM cumulative production), far in excess of the Hb concentration indicates that (1) the theoretical in vivo levels of ROS are insufficient to kill and (2) *Plasmodium* likely has an intrinsic protective mechanism to deal with exogenous (immune) ROS.

## SUMMARY

Despite its complex redox chemistry, Hb, which is a potent $NO^{\bullet}$ and ROS scavenger and is present in abundance around the parasite, is likely to protect the malarial parasite. The high levels of free Hb in the plasma during the course of disease are likely to minimize the ability of $NO^{\bullet}$ and ROS to enter the erythrocyte. Intraerythrocytic Hb is also likely to be protective because oxidant stress relating to Hb redox chemistry is enzymatically controlled within the red cell. Finally, the malarial parasite has developed mechanisms to cope with the oxidant nature of the free heme it produces, such as packaging it in the hemozoin granule and confiscating erythrocyte superoxide dismutase.

Although $NO^{\bullet}$ does not kill the parasite, it may protect against the development of disease. $NO^{\bullet}$ plays important roles in maintaining the homeostasis of (1) the immune system, (2) the endothelium, and (3) the coagulation system, whereas pathologic activation of these systems is required for malarial pathogenesis.[3,75,76] Thus, $NO^{\bullet}$'s function may be as an antioxidant and protective molecule,[77] as well as a potent anti-inflammatory molecule that minimizes *P. falciparum* sequestration by down-regulating endothelial activation and CAM expression.[59]

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Figure 2 was generated by Karen Landon.

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# Chapter 4: *P. berghei* resists killing by reactive oxygen species.

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# *Plasmodium berghei* Resists Killing by Reactive Oxygen Species

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**Reactive oxygen species (ROS) are widely believed to kill malarial parasites. C57BL/6 mice injected with *P. berghei* inocula incubated with supraphysiological doses of NO (≤150 μM) or with peroxynitrite (220 μM), however, exhibited parasitemia similar to that seen with those given control inocula, and there was no difference in disease development. Only treatment of inocula with NO doses nearing saturation (≥1.2 mM) resulted in no detectable parasitemia in the recipients; flow cytometric analysis with a vital dye (hydroethidine) indicated that 1.5 mM NO lysed the erythrocytes rather than killing the parasites. The hemoglobin level in the inocula was about 8 μM; the hemoglobin was mainly oxyhemoglobin (oxyHb) (96%), which was converted to methemoglobin (>95%) after treatment with 150 μM NO. The concentrations of 150 μM of NO and 220 μM of peroxynitrite were far in excess of the hemoglobin concentration (~8 μM), and yet no parasite killing was detected. We therefore conclude that hemoglobin protects *Plasmodium* parasites from ROS, but the parasite likely possesses intrinsic defense mechanisms against ROS.**

Malaria, a reemerging disease (20) caused by the parasite of the genus *Plasmodium*, remains refractory to the development of a vaccine in part due to incomplete understanding of the mechanism(s) underlying parasite killing by the immune system. It is generally accepted that reactive oxygen species (ROS), including nitric oxide (NO), superoxide, and peroxynitrite, kill intraerythrocytic malarial parasites (5, 8, 29). The most cited mechanism for parasite killing is that acute *Plasmodium* infection induces gamma interferon-producing Th1 cells, which in turn activate macrophages to secrete parasiticidal NO and ROS (29, 30).

We propose, however, that the blood stage *Plasmodium* parasite is virtually immune to the cytotoxic effects of NO and ROS as a consequence of hemoglobin (Hb) NO scavenging and ROS suppression within red blood cells (RBCs). The *Plasmodium* parasite is surrounded by hemoglobin through most of its asexual blood cycle, because it resides within a parasitophorous vacuole inside erythrocytes. *Plasmodium falciparum* parasites rupture erythrocytes, releasing progeny merozoites, which invade new RBCs after completing their 48-hour blood stage cycle. This extracellular excursion constitutes a brief period in which the parasite is in principle vulnerable to higher ROS concentrations induced by the infection. However, the disruption of the RBC membrane inevitably releases molecular hemoglobin into the circulation, enhancing ROS scavenging; thus, both inside and outside the red cells the parasite is protected from ROS because ROS are scavenged by hemoglobin.

Malaria, therefore, is fundamentally different from most infections, because the parasite is surrounded by hemoglobin and can evade the ROS-based protective mechanism as a consequence of ROS quenching by Hb (2), an antioxidant mechanism that has been overlooked. Although Hb's heme group can undergo redox transitions to higher oxidation states and it can auto-oxidize naturally to form methemoglobin (metHb) and superoxide ions, this oxidation process is controlled within RBCs by the metHb reductase system (1). The biochemical basis for the in vivo hemoglobin redox reactions has been extensively reviewed by Alayash (2).

To test the hypothesis that the *Plasmodium* parasite evades the ROS-based protective mechanisms because the parasite is surrounded by hemoglobin, we analyzed NO and ROS effects on *Plasmodium berghei* ex vivo, with particular attention to the molecular state of hemoglobin. Although there are differences between human and experimental malaria which limit extrapolation to the human condition, ex vivo treatments of *P. berghei* can assess the effect of NO and ROS on common features of parasite viability and replication as well as its ability to elicit disease.

## MATERIALS AND METHODS

**Evaluation of NO solution bioactivity. (i) Nitric oxide stock preparation.** Phosphate-buffered saline (PBS) solution (Invitrogen, Carlsbad, CA) (pH 7.4) was degassed under vacuum overnight and placed in line between a 2 M NaOH solution and a 1 M $KMnO_4$ solution. Prepurified grade 4.8 (99.998% pure) nitrogen was bubbled through the system for 30 min followed by UHP nitric oxide (Airgas, San Diego, CA) (99.5% pure) for 5 min (13). A saturated solution of NO has a concentration of 1.8 mM at room temperature and 1 atm (13), which we verified using the amperometric inno-T nitric oxide system (Innovative Instruments, Inc.). NO stock solutions were prepared in an anaerobic chamber filled with grade 4.8 nitrogen gas, 2 μl or 5 μl (1:10,000 and 1:4,000 dilution, respectively) was injected into a stirred 20 ml sample of PBS, and the resultant NO concentrations were monitored as a function of time and were 176.9 ± 6.6 nM for the 10,000 dilution and 435.6 ± 2.3 nM for the 4,000 dilution.

**(ii) Hemoglobin solution preparation and NO concentration analysis.** Hemoglobin solutions were prepared by lysing freshly isolated murine RBCs. Blood was obtained from anesthetized C57BL/6 mice (Jackson Laboratories) and centrifuged at 500 × *g* for 10 min to pellet the erythrocytes. The supernatant was aspirated, and then the cells were lysed with 0.9 ml distilled water. The solution was later osmotically balanced with the addition of 0.1 ml 10× PBS. The oxyhemoglobin (oxyHb) and methemoglobin concentration of this stock was determined using the Winterbourn spectrophotometric method (35). The oxyhemoglobin assay for NO (13, 22) was used to verify the effective dose of NO treatments. Hemoglobin solutions of 1.2 mM, 600 μM, 60 μM, and 20 μM were prepared and were treated three times using Hamilton syringes with 0.7 ml, 125 μl, 12.5 μl, and 1.25 μl of saturated NO solutions, respectively. Following each

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injection, the change in oxyHb level was determined by monitoring the change in absorption at 577 nm with a μQuant spectrophotometer (BioTek, Winooski, VT).

**Infection of mice with ROS-treated *P. berghei*.** *Plasmodium berghei* ANKA was stored as a frozen stabilate. The stabilate was thawed and then injected into a source mouse to generate an inoculum for the experiments. Blood from the source mouse was diluted with PBS to prepare aliquots of the inoculum. In the first series of experiments, the blood was diluted 1:6 in PBS upon collection from the source mouse. The number of RBC per milliliter was determined by using a hemocytometer. This value and the percent parasitemia were used to calculate the final dilution necessary to obtain $7.5 \times 10^6$ parasitized erythrocytes (pRBCs) in 1.5 ml PBS ($5 \times 10^6$ pRBC/ml), and this dilution (~100-fold) was then achieved. In subsequent experiments, the blood cells were kept in a 1:6 dilution of PBS and then diluted to the final concentration just before they were exposed to either NO or SIN-1 (3-morpholino-sydnonimine) as described below. A 200-μl volume of the NO- or SIN-1-exposed inoculum ($1 \times 10^6$ pRBC) was injected intraperitoneally (i.p.) into groups of three to five C57BL/6J mice for each treatment group. The mice were obtained from Jackson Laboratories (Bar Harbor, ME) at 6 to 8 weeks of age and were injected with parasitized RBCs (pRBCs) when they were 8 to 10 weeks of age. The animals were housed in microisolator cages and provided food ad libitum. Parasitemia was assessed in each group of mice by counting the number of infected erythrocytes in Giemsa-stained thin blood films. The effects of NO treatments on the survival of severe malaria were also assessed. The *P. berghei* model of malaria is a very reproducible model of cerebral malaria in which virtually all C57BL/6J mice develop neurologic impairment and obtundation and then succumb to infection between days 6 and 8 of infection (14, 15). The Institutional Animal Care and Use Committee committee at the La Jolla Bioengineering Institute approved all procedures.

**(i) Nitric oxide treatment.** Experiments were carried out in a water-jacketed reaction chamber (WPI), preheated to 37°C, which was placed inside a custom anaerobic chamber that was degassed with grade 4.8 (99.998% pure) nitrogen for 1 h. Inoculum samples containing 7.5 million pRBCs were pipetted into the reaction chamber and treated with a bolus of nitric oxide stock solution (1,250 μl, 1,000 μl, 125 μl, 12.5 μl, or 1.25 μl) delivered via a gas-tight Hamilton syringe. The final sample volume was 1.5 ml, contained 7.5 million pRBC, and had an NO concentration of 1.5 mM, 1.2 mM, 150 μM, 15 μM, or 1.5 μM. The chamber was capped to minimize NO escape from the solution into the gas headspace, and the solution was maintained at 37°C for 10 min. The control treatment consisted of pipetting 1,250 μl of degassed PBS into the 250 μl inoculum sample containing 7.5 million pRBC. At the end of the treatment period, the treated inoculum was aspirated from the sample chamber via syringe, and 200 μl of the sample (1 million pRBC per sample) was injected i.p. into mice. The oxyhemoglobin content of inoculum was determined by lysing the erythrocytes and analyzing the lysate by use of the Cripps method (9).

**(ii) NO bioactivity assessment.** The inoculum was treated with 1.5 mM NO, 150 μM NO, or PBS, as described above. Instead of splitting the inoculum into samples and injecting the samples into mice, it was then centrifuged and lysed, as described above, and the Winterbourn spectrophotometric assay was used to determine oxyHb, metHb, and choleglobin levels.

**(iii) SIN-1 treatment.** A SIN-1 stock solution (250 mM) was prepared by dissolving 50 mg SIN-1 chloride (Alexis, San Diego, CA) in PBS and immediately freezing the solution. SIN-1 treatments consisted of adding 8 μl of SIN-1 stock to 1 ml PBS and mixing this resultant 2 mM SIN-1 solution with 1 ml of inoculum containing 10 million pRBC in PBS, yielding 2 ml of 1 mM SIN-1 solution with 5 million pRBC/ml and production rates of superoxide and nitric oxide of 7.0 μM/min and 3.7 μM/min, respectively (17). The treated inoculum was maintained in room air for either 1 h at room temperature or 10 min at 37°C. Following treatment, the inoculum was centrifuged at $500 \times g$ for 10 min and resuspended in 2 ml of PBS, and 200 μl samples (1 million pRBC) were injected i.p. into mice as described above.

**Flow cytometric assessment of parasite viability and permeability of RBCs.** Inoculum samples were labeled with the vital dye hydroethidine immediately after NO treatment or following a 4-h incubation in fetal bovine serum at 37°C by the use of a previously described technique (10). Hydroethidine (Molecular Probes) at 10 mg/ml in dimethyl sulfoxide (Invitrogen Corporation, Carlsbad, CA) was diluted 1:600 in PBS. Parasite inocula (10 million) treated with 1.5 mM NO or PBS were washed once in PBS, resuspended in 0.5 ml of the hydroethidine solution, and incubated for 20 min at 37°C in the dark. Following the incubation, all hydroethidine-stained samples were washed twice in PBS and resuspended in 1 ml of PBS. Propidium iodide was added to replicates of the treated inocula to assess the number of erythrocytes that were permeable. Spherotech beads were added to all samples prior to being processed using a FACSCalibur system (Beckton Dickinson, San Diego, CA) to assess cell numbers. These beads are distinguished from cells on the basis of their unique forward and side scatter and fluorescence on all three channels. The acquisition of data was performed using the CellQuest (Becton-Dickinson) program on 10,000 cells identified by characteristic forward and side scatter. The Attractors (Becton-Dickinson) program was used to analyze the data. Cell numbers were determined using the following formula:

$$\text{number of cells per microliter} = \frac{\text{number of positive events}}{\text{number of beads collected}} \times \frac{\text{bead count per test}}{\text{sample volume (in microliters)}}$$

**Statistical analysis.** Analysis of variance with the Statview program (SAS Institute, Cary, NC) and Fisher's post hoc test was performed to statistically compare all parasitemia with a $P$ value cutoff of 0.05. The means and standard deviations of the results are reported in the text and figures.

## RESULTS

**Verification of nitric oxide stock concentration.** The decay of NO concentration with time was monitored with a NO amperometric method, showing that our stock was saturated with NO and that dilutions of the stock provided reproducible treatments of NO. The recording of NO levels in the solution diluted 1:4,000 showed that the exponential decay constant for NO in the anaerobic chamber was 20.9 ± 0.6 min. It was not possible to eliminate the headspace above the solution, so the most-rapid loss of NO was due to NO diffusion from the solution into the atmosphere. This NO diffusion was minimized in the capped reaction chamber used in the treatment of parasite inocula, so we estimate that the NO decay time constant for parasite treatments is at least twice that measured (41.8 ± 1.2 min). Either time constant indicates that most of the NO was present during the 10-min treatment period in the anaerobic chamber.

The amount of NO delivered by our treatments and by the treatment variations was determined using the oxyhemoglobin NO assay because this is the NO experienced by the RBC-based parasite, and concentrations were beyond the operating range of our NO electrode. The Hb solutions were treated with the selected NO doses, and then the amount of bioactive NO actually delivered was assessed by spectroscopically monitoring the reduction in oxyHb (13, 22). Before treatment, most (90.7 ± 2.5%) of the Hb in the test solution was oxyHb. Measurement of oxyHb concentration following treatment showed that the selected doses of NO were similar to the actual concentrations for bioactive NO (Fig. 1B).

**Almost-saturated solutions of nitric oxide are needed to impair the viability of *P. berghei* parasites.** There was no detectable effect of NO on parasitemia or development of disease in groups of mice injected i.p. with $1 \times 10^6$ *P. berghei* pRBCs treated with either 15 μM or 1.5 μM NO compared to the control group results (Fig. 2A). In this experiment, blood was immediately diluted (~1:600) to obtain the parasite inoculum ($7.5 \times 10^6$ pRBC/1.5 ml) and was kept diluted in PBS for about 2 h before the injection of $1 \times 10^6$ pRBCs into the mice. Mice injected with parasitized RBCs treated with 150 μM NO showed a modest but not significant decrease in parasitemia at days 4 and 6 ($P = 0.17$ and $P = 0.63$, respectively) and slightly delayed development of experimental cerebral malaria (Fig. 2B). Thus, there appeared to be an increase in survival in groups of mice injected with parasitized RBCs treated with 150 μM NO (Fig. 2B). Only mice infected with parasitized RBCs

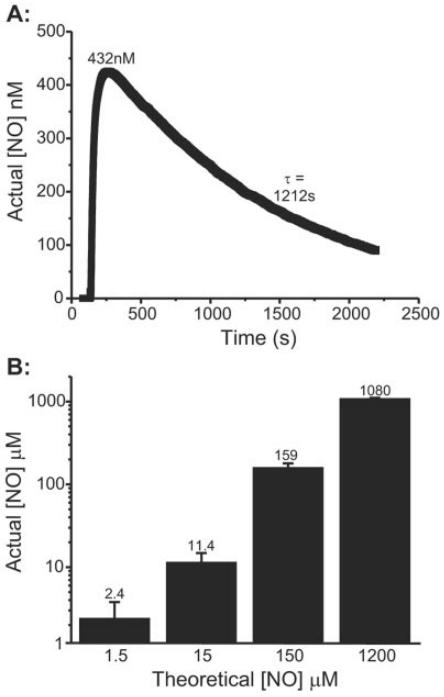


FIG. 1. Verification of NO stock solution, treatment concentrations, and NO decay in the anaerobic chamber. (A) Representative ($n$ = 8) trace of NO concentration versus time resulting from a 1:4,000 dilution of NO stock. The peak NO concentration was 432 nM compared with a theoretical concentration of 450 nM, and the decay constant $\tau$ after exponential curve fitting was 1,212 s. Similar results were obtained with a 1:10,000 dilution ($n$ = 3). (B) Hemoglobin-assayed concentrations of NO treatments compared to calculated values.

treated with 1.5 mM NO exhibited no viable parasites and consequently no development of disease. This experiment was repeated twice with similar results.

We assessed the amount of Hb in the parasitized erythrocyte sample and assessed the state of the Hb within the erythrocytes prior to our NO treatment. The oxyheme concentration of the samples assessed by the Cripps spectrophotometric method was 29.3 ± 1.4 μM. Thus, the samples treated with 150 μM and 1.5 mM NO had sufficient NO in principle to overcome the Hb quenching of NO.

We repeated the above-described experiment except that instead of diluting the blood immediately to its final concentration (7.5 $\times 10^6$ pRBC/1.5 ml) for NO treatment, the blood was kept at a 1:6 dilution and then diluted further (~1:100) to the final inoculum concentration just before exposure to NO. Mice were injected i.p. with $1 \times 10^6$ pRBCs from the inoculum immediately after it was exposed to NO. We performed this assay because hydroethidine results suggested that prolonged incubation in PBS alone lowered parasite viability compared with the results seen with parasite samples kept in a 1:6 dilution and then diluted immediately before use; this suggests that the lack of nutrients may have affected parasite viability. Under the new condition of parasite storage prior to NO treatment, no change in parasite viability or disease-eliciting capability was now detected at the 150 μM NO concentration (Fig. 2C). This observation indicated that a weakening of the parasite by prolonged (1 to 2 h) incubation in the absence of serum had occurred, rendering it artificially more susceptible to NO killing. The 1.2 mM NO treatment resulted in no viable parasites, and no infected mice developed disease (Fig. 2D). The oxyheme concentration in the inoculum samples was 45.6 ± 1.3 μM, indicating that the 150 μM and 1.2 mM NO treatments should have overcome the NO-quenching capacity of the oxyHb. We repeated this experiment twice with similar results. In all cases, the concentrations used for the NO treatments (1.2 mM and 150 μM) were far in excess of the oxyhemoglobin concentration of the samples.

**Nitric oxide is bioactive following diffusion into erythrocytes.** Because no parasite killing was detected even when the NO treatment concentration (150 μM) far exceeded the sample hemoglobin concentration (30 to 45 μM), it is possible that sufficient NO did not diffuse into the RBC during the course of the 10-min treatments. In fact, Liao and coworkers (33) have proposed that diffusional barriers slow the kinetics of NO consumption by RBCs, thereby explaining why NO consumption by free Hb is about 1,000-fold greater than that seen with the same amount of Hb packaged in RBCs. To verify that on the 10-min time scale of our treatments the diffusional barriers did not markedly inhibit NO bioavailability within the erythrocyte and that the parasite was exposed to NO, we determined the Hb oxidation state before and immediately after NO treatment of the experimental samples. Before NO treatment and placement in the anaerobic chamber, the samples were mainly oxyHb (96.1 ± 1.1%), with little metHb (3.9 ± 1.1%) or choleglobin (0.0 ± 0.0%), a denatured form of Hb. Following NO treatment of the parasitized erythrocytes, most of the oxyHb (~95%) was converted into either metHb or choleglobin (Fig. 3). Maintaining the erythrocyte sample in the anaerobic chamber during the NO experiment had no effect on the oxyHb levels (95.6 ± 1.0%), indicating that the conversion of oxyHb was related to the NO treatment rather than to the effects of the anaerobic chamber. This experiment was repeated twice with similar results.

**Supraphysiological NO treatment results in erythrocyte lysis that diminishes *P. berghei* viability.** To assess whether a high dose of NO rapidly kills malarial parasites ex vivo, parasite viability and red cell permeability were assessed after 10 min of incubation with 1.5 mM NO in the anaerobic chamber. Hydroethidine is a vital dye that becomes fluorescent when cleaved to ethidium by the intracellular esterases of a live cell; this conversion within cells was detected by flow cytometry. Propidium iodide is excluded when the cell membrane is intact and consequently was used to assess cell permeability. In the untreated samples ($n$ = 3), the number of erythrocytes was 10.9 ± 0.8 $\times 10^6$; the parasitemia was 10.4 ± 0.1%; and the percentage of permeable erythrocytes was 0.7 ± 0.1%. The parasitemia assessed in Giemsa-stained thin blood films was 10.5%, and the theoretical number of erythrocytes in the inoculum was $10 \times 10^6$. Immediately following incubation in PBS at 37°C for 10 min, there was no detectable change ($P > 0.9$) in any of the above-named three parameters (Fig. 4). Incubation of the parasite inoculum with 1.5 mM NO ($n$ = 3) resulted in a significant ($P < 0.0001$) decline in the number of eryth-

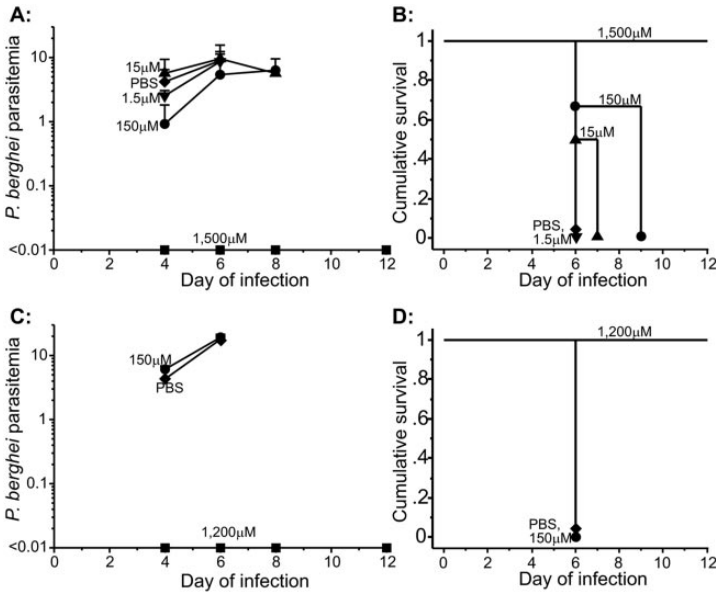


FIG. 2. Nitric oxide treatments below 150 μM have no detectable effect on parasite viability and its ability to elicit disease. (A and C) The average parasitemia and standard deviation of the mice (three per group) injected i.p. with parasites incubated with selected doses of NO. (B and D) The cumulative survival of mice injected with NO-treated parasites. In panels A and B parasitized erythrocytes were stored in PBS prior to the experiment, whereas in panels C and D the parasites were stored in a minimally diluted blood stock and dilutions were prepared immediately prior to NO treatment. All NO treatments were performed following dilution in PBS to form the working solutions.

rocytes (NO, $0.93\times 10^6 \pm 0.08 \times 10^6$), but the parasitized erythrocytes were preferentially spared from NO-induced erythrocyte lysis because the parasitemia had increased significantly ($P < 0.0001$) to 93.9 ± 0.8%. Analysis of the forward and side scatter of the samples showed a marked increase in cell debris after NO treatment of the inocula that was not observed in the PBS controls (Fig. 4) and a significant ($P < 0.0001$) increase in the percentage of permeable erythrocytes (PBS, 0.7% ± 0.1%; NO, 91.0% ± 3.7%). In a replicate experiment, two out of three samples treated with 1.5 mM NO exhibited severe RBC lysis compared with PBS-treated controls at 10 min.

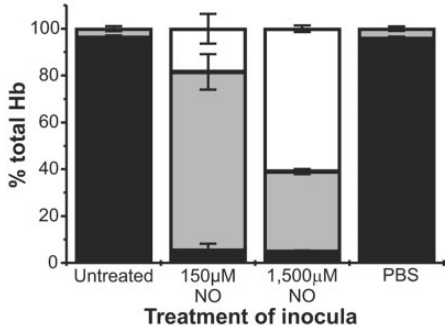


FIG. 3. NO treatment of inocula containing parasitized erythrocytes results in the conversion of oxyHb to metHb and consequently is bioavailable within the erythrocyte. Percentages and standard errors ($n = 3$) for the different forms of hemoglobin present in inocula are shown. Solid bars, oxyhemoglobin; shaded bars, methemoglobin; open bars, choleglobin.

**SIN-1 treatment has no effect on parasite viability and host survival.** Because (i) macrophages during malaria produce high levels of NO and superoxide (11, 18, 19, 26, 28), which react to form peroxynitrite (4), and (ii) superoxide and peroxynitrite reportedly kill the *P. falciparum* parasite in vitro (16), SIN-1 treatment was used to determine whether superoxide, nitric oxide, and peroxynitrite collectively have an effect on parasite viability. Parasitized RBCs were treated with 1 mM SIN-1 for 1 h in room air and at room temperature and then injected into mice. The oxyheme concentration of the inoculum was 13.3 ± 1.5 μM. Despite supraphysiological levels of ROS, the levels of parasitemia in the mice injected with the SIN-1-treated inoculum on day 4 or day 6 were similar to those seen with the PBS control group (day 4, $P = 1.0$; day 6, $P = 0.6$) (Fig. 5A). Further, the ROS treatment had no detectable effect on the parasite's ability to elicit disease because the host survival rates for mice injected with SIN-1-treated parasites were similar to those of mice injected with control parasites (Fig. 5B). This experiment was repeated with similar results, and the SIN-1 treatment for 10 min at 37°C also did not reduce parasitemia or increase survival.

## DISCUSSION

There is generally equivocal evidence that reactive oxygen species produced by phagocytes play a crucial role in controlling *Plasmodium* replication in vivo. However, few malarial studies take into account the role of hemoglobin as a potent

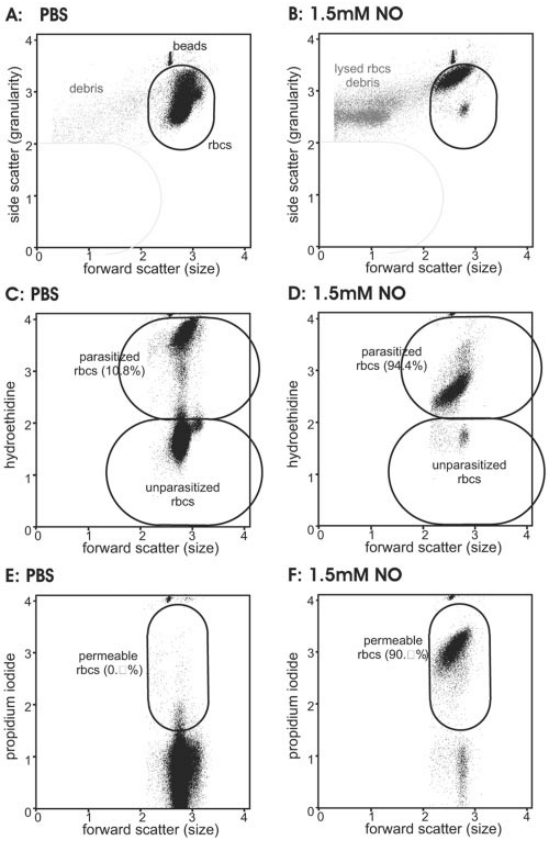


FIG. 4. *P. berghei* killing by 1.5 mM NO treatment is likely due to RBC lysis. Representative ($n = 3$) flow-cytometric analysis plots of parasite inoculum incubated either with PBS for 10 min at 37°C (A, C, and E) or with PBS and 1.5 mM NO (B, D, and F) are shown. Each dot represents a single cell. The various cell populations and counting beads are indicated on the dot plots. Forward scatter and side scatter (A and B) are approximate measures of size and granularity, respectively. (C and D) Hydroethidine labeling of parasitized erythrocytes. (E and F) Propidium iodide labeling of the inoculum. The parasite inoculum in PBS was indistinguishable from untreated inoculum ($P > 0.9$).

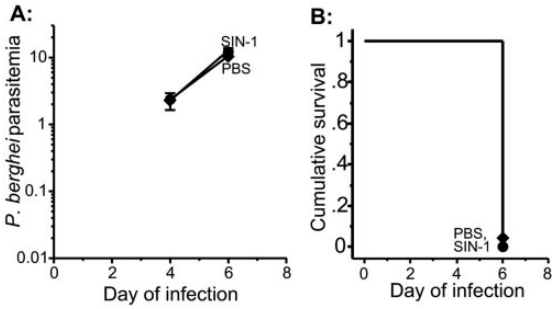


FIG. 5. Supraphysiological peroxynitrite production by SIN-1 does not affect viability of *P. berghei* or its ability to elicit disease. (A) The average *P. berghei* parasitemia and standard deviations of mice (five mice per group) injected with inocula that were treated with 1 mM SIN-1 or PBS. (B) Cumulative survival.

scavenger of reactive oxygen species and the physiological levels of ROS that a parasitized erythrocyte might be exposed to in vivo. The malarial parasite resides in a parasitophorous vacuole within the erythrocyte and is surrounded by about 0.25 fmol of Hb within the murine erythrocyte and an additional 2.5 mM in other erythrocytes. Thus, to reach and kill an intraerythrocytic malarial parasite the level of ROS must exceed this Hb concentration. Additionally, Hb is released from the erythrocyte into blood during schizogony (erythrocyte rupture and parasite progeny release). Our preliminary data suggest that the plasma concentration of free Hb on day 6 of *P. berghei* infection is markedly elevated and can reach up to 100 μM just prior to death. Free hemoglobin is about 1,000-fold more efficient at scavenging NO than Hb packaged in RBCs (21), so this free Hb may also be protective by significantly quenching ROS before they can diffuse to the erythrocyte.

We observed that the Hb state in inoculum samples from day 6 animals is usually 96% oxyHb and <4% of the other forms combined. There is about 15 pg of hemoglobin or about 1 fmol of heme in a single healthy murine erythrocyte (7). The inoculum containing 7.5 million parasitized erythrocytes at about 10% parasitemia had an assayed hemoglobin concentration of 7.5 μM (30 μM total heme), translating into about 10 pg of Hb/RBC or 0.6 fmol of heme/RBC. Despite treatment with high doses of NO, no significant effect of 1.5 μM or 15 μM NO treatment upon parasitemia is detected after injection of the inoculum into mice, indicating that intraerythrocytic *Plasmodium* parasites are protected against ROS at up to the concentration of Hb within the erythrocytes.

Treating the parasite with 150 μM nitric oxide also has no significant effect on the parasitemia, despite the NO concentration being far in excess of the Hb concentration in the inoculum. Because these nitric oxide treatments do not kill the parasite, it is possible that some factor(s), such as the cytoskeletal diffusional barrier proposed by Liao, limits the bioavailability of the nitric oxide inside the erythrocytes by increasing the time it takes to achieve equilibrium. If the nitric oxide was able to diffuse into the erythrocyte and the Hb within the erythrocyte was protecting the parasite from NO exposure, then the NO should have converted the erythrocytic oxyHb to metHb. After 150 μM NO treatment of a sample containing parasitized erythrocytes and with about 8 μM hemoglobin, little oxyHb (5.2%) was now detectable, with the Hb being mainly in the metHb state, with some choleglobin. Thus, the ineffectiveness of the NO treatments in reducing parasite viability is not due to a failure of delivery—the 10-min treatments were sufficient to allow NO to diffuse into the RBC—but to the presence of protective mechanism(s), including hemoglobin scavenging of NO. Because there is no detectable impact on *P. berghei* viability after the conversion of almost all of the Hb within the erythrocyte to metHb, the parasite either uses alternate food sources or is capable of processing and storing metHb in a manner analogous to that seen with oxyHb.

Based on current research, a single parasitized erythrocyte is likely exposed to much less than 1 μM of NO in vivo (23, 34).

Our data indicate that exposure to up to 150 μM NO has little or no effect on parasite viability, indicating that NO is unlikely to inhibit *P. berghei* replication. This conclusion is supported by the observation that the characteristics of *P. berghei* parasitemia and *P. chabaudi* parasitemia in iNOS$^{-/-}$ mice are similar to those seen with NOS-intact controls (12, 32, 36). One possibility is that NO toxicity is a component of a multihit sequence and that other immune mechanisms exhibit greater effects against parasites weakened by NO. This issue is difficult to address in the *P. berghei* model because the parasite replicates similarly in strains of mice that are resistant or susceptible to experimental severe malaria and all mice succumb to anemia secondary to hyperparasitemia, indicating the absence of a protective immune response in *P. berghei*-infected mice. Nevertheless, *P. chabaudi*-infected mice injected with heat-killed *Propionibacterium acnes* parasites that elicit high levels of NO production for more than a week exhibit parasitemia similar to that seen with *P. acnes*-injected iNOS$^{-/-}$ mice that lack elevated NO production, indicating that there is no detectable effect of NO toxicity with respect to rendering the parasite more susceptible to other immune mechanisms.

Besides nitric oxide, activated macrophages also produce superoxide, but at a much (~10 times) lower level. Due to the much higher NO levels and the fact that both radicals come from the same source, all of the superoxide rapidly reacts with the nitric oxide, yielding ~1.5 nM peroxynitrite at the macrophage surface (24). Thus, in vivo, there is little likelihood of superoxide reaching the parasite, particularly when one considers the presence of other erythrocytes and free Hb in the blood. Indeed, the observation that P mice (mice of a strain that has defective macrophage function) exhibit *P. chabaudi* parasitemia similar to that seen with superoxide-producing controls supports this conclusion (6) but contrasts with findings of increased *P. berghei* parasitemia in p91pHox$^{-/-}$ mice (27).

The peroxynitrite resulting from the reaction of superoxide and nitric oxide and produced by the macrophage is a highly reactive radical and at physiological pH has an extremely short half-life (24). This results in a very short diffusion distance and a near-zero concentration (<0.2 nM) at 10 μm from the macrophage (24). Thus, a sequestered erythrocyte is unlikely to be exposed to significant concentrations of peroxynitrite (<1 nM) and peroxynitrite is quenched by oxyhemoglobin to form metHb, nitrite, and oxygen. Based on the low production and its scavenging by hemoglobin, we propose that peroxynitrite does not play a significant role in parasite killing in vivo.

This conclusion is supported by our observation that 1 mM SIN-1 producing 220 μM (3.7 μM/min × 60 min) of peroxynitrite over the course of an hour had no detectable effect on *P. berghei* viability. This conclusion is not supported by the results of studies coculturing *P. falciparum*-infected erythrocytes with gamma interferon-activated macrophages; these studies have shown that nitric oxide, superoxide, and peroxynitrite reduce parasitemia and do play a role in parasite killing. However, there are concerns regarding the interpretation of these studies. First, the investigators added supraphysiological concentrations (1 mM) of SIN-1 for over 24 h to the cocultures already producing additional ROS (16), resulting in >1 mM cumulative peroxynitrite production. Second, these studies used approximate methods to assess parasitemia. Ockenhouse et al. used [$^3$H]hypoxanthine incorporation (hypoxanthine is incorporated when the parasite replicates its DNA at trophozoite stage) to assess parasitemia (25). We subsequently reported that coculture of *P. falciparum* with activated monocyte-derived macrophages shortens the replication time from 48 h to 24 h; this decreased replication time results in minimal [$^3$H]hypoxanthine incorporation despite the presence of viable parasites, because the parasites are past the stage of DNA incorporation when the radiolabel is added (31). Indeed, Fritsche et al. did not observe significant parasite killing until they added the supraphysiological levels of SIN-1 (16).

Collectively, our results indicate that *P. berghei* parasites remain viable even after being treated with NO (150 μM) or peroxynitrite (220 μM) at concentrations far in excess of the hemoglobin concentration (~8 μM) in the inoculum. Indeed, even after we treat the parasite inoculum with a nearly saturated NO solution (1.5 mM), some parasites remain intact and are labeled with the vital dye hydroethidine. Because NO and peroxynitrite concentrations exceed the scavenging capacity of Hb in our defined ex vivo experiment by more than 1 order of magnitude, these observations indicate that *P. berghei* is exposed to significant concentrations of NO without exhibiting any detectable toxicity. That *Plasmodium* parasites likely have intrinsic protective mechanisms against NO toxicity is not surprising in light of the fact that *Plasmodium* parasites possess a host of antioxidant and antiradical mechanisms inherent in the pro-oxidant nature of their redox metabolism, their hemozoin granules, and their intraerythrocytic environment (3). Because all *Plasmodium* parasites reside within erythrocytes, the basis for our proposed mechanism of protection of *P. berghei* parasites from ROS, the ROS-quenching potential of hemoglobin, as well as any intrinsic protection should be conserved across all species of *Plasmodium*. Further, the hemoglobin content of the blood is so high compared to the NO and ROS production capabilities of the immune system that we speculate that our results can be applied to other species of parasite.

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# Chapter 5: Nitric oxide bioavailability in malaria.

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# Nitric oxide bioavailability in malaria

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**Rational development of adjunct or anti-disease therapy for severe *Plasmodium falciparum* malaria requires cellular and molecular definition of malarial pathogenesis. Nitric oxide (NO) is a potential target for such therapy but its role during malaria is controversial. It has been proposed that NO is produced at high levels to kill *Plasmodium* parasites, although the unfortunate consequence of elevated NO levels might be impaired neuronal signaling, oxidant damage and red blood cell damage that leads to anemia. In this case, inhibitors of NO production or NO scavengers might be an effective adjunct therapy. However, increasing amounts of evidence support the alternate hypothesis that NO production is limited during malaria. Furthermore, the well-documented NO scavenging by cell-free plasma hemoglobin and superoxide, the levels of which are elevated during malaria, has not been considered. Low NO bioavailability in the vasculature during malaria might contribute to pathologic activation of the immune system, the endothelium and the coagulation system: factors required for malarial pathogenesis. Therefore, restoring NO bioavailability might represent an effective anti-disease therapy.**

## Pathogenesis of malaria

Malaria is one of the leading causes of morbidity and mortality, annually killing more than two million people worldwide [1]. Furthermore, patients with severe *Plasmodium falciparum* malaria still exhibit a 20–30% risk of mortality when given effective antiparasite therapy, providing the rationale for developing adjunct or anti-disease therapy that rescues ill patients from pathogenesis [2]. To develop adjunct therapy rationally, cellular and molecular definitions of the pathogenesis of *P. falciparum* malaria are needed. Nitric oxide (NO) is a molecule that has been proposed to have a crucial role in malaria pathogenesis [3] but its mechanism of action during the disease is controversial. On the one hand, it has been proposed that NO is produced in excess and kills the *Plasmodium* parasite [4], with the unfortunate side effects of mediating pathogenesis through oxidative damage or aberrant signaling in the brain [3], and contributing to anemia [5]. In this case, nitric oxide synthase (NOS) inhibitors or extracellular scavengers of NO might function as an adjunct therapy for severe *P. falciparum* malaria.

On the other hand, the findings that hypoargininemia [6] and low levels of plasma nitrates and nitrites (NOx) [7] – the stable degradation products of NO – correlate with the development of severe *P. falciparum* malaria pathogenesis suggest that levels of NO production might be low during malaria. In addition, the increased levels of free hemoglobin (Hb) in the blood of patients with severe *P. falciparum* malaria [8,9], an unavoidable consequence of parasite replication in red blood cells (RBCs), and the elevated levels of superoxide elicited by the infection, together with the well-documented scavenging of NO by Hb and superoxide, indicate that NO produced during malaria in the vasculature is probably removed rapidly. Studies of other diseases [10] report that low NO bioavailability contributes to pathologic activation of the immune system, the endothelium and the coagulation system; indeed, these factors are observed during severe *P. falciparum* malaria [11]. Therefore, restoring NO bioavailability or reversing the consequences of low NO bioavailability might represent an effective adjunct therapy for severe malaria.

## Physiological role of NO

NO is an uncharged free radical that is produced during the enzymatic conversion of L-arginine to L-citrulline by members of the NOS family of proteins [12]. Three members of the family have been identified: endothelial NOS (eNOS or NOS3), neuronal NOS (nNOS or NOS1) and inducible NOS (iNOS or NOS2), with eNOS and nNOS both functioning in a calcium-dependent and -independent fashion, and iNOS functioning in a calcium-independent fashion [12].

NO was identified originally as the endothelium-derived relaxation factor that mediates vasodilation in a soluble guanylate cyclase (sGC)-dependent manner [13]. Because NO readily diffuses through cell membranes and can bind with high affinity to heme-containing proteins (e.g. sGC) or nitrosylate proteins, it has been reported to exhibit complex pleiotropic effects and is central to many aspects of physiology and pathology [14]. Adding to the complexity, proteins nitrosylated by NO can release NO, thus serving as a NO-transport mechanism [15], and Gladwin has proposed that the nitrite – the stable end-product of NO degradation – can be converted by deoxyhemoglobin (deoxyHb) to NO and that this NO has an important role in ischemia–reperfusion injury in the heart and liver [10]. Besides NO concentration, the state of the NO species, its cellular location and the presence of superoxide, which reacts with NO to produce peroxynitrite, all determine which of the many pleiotropic roles NO has.

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Many of the physiological effects of NO (Figure 1), such as vasodilation, inhibition of platelet aggregation and endothelial-cell activation, are mediated through the activation of sGC by NO, leading to increased production of guanosine 3′,5′-phosphate (cGMP) [16]. In the vasculature, NO is produced by endothelial cell eNOS and targets smooth-muscle cells, causing vasodilation through the sGC–cGMP pathway. In addition, eNOS-derived NO has an important role in maintaining homeostasis by inhibiting the activation of endothelial cells and by modulating their expression of cell-adhesion molecules (CAMs) [17]. NO in the vasculature also modulates platelet function; platelets express eNOS, and NO inhibits platelet adhesion, activation and aggregation by both cGMP-dependent and cGMP-independent pathways [18].

In the immune system, NO is a component of the innate immune response, and iNOS-derived NO is required for the control of several viral, bacterial and parasitic infections [19]. Despite all three NOS isoforms having similar NO-production rates of $\sim 1\ \mu M\ min^{-1}\ mg^{-1}$ protein [20], iNOS is responsible for the high-level production of NO by phagocytes because it is highly expressed after activation, comprising up to 1% of total protein [21]. Although the initial controversy regarding differing iNOS functions in human versus murine phagocytes has been settled [22] and the initial comparison of human and murine iNOS genes and promoter regions has shown them to be extremely similar [23], further detailed analysis of the iNOS genes of both species [24] is necessary to determine differences in their transcriptional regulation.

In addition to its antimicrobial role, NO can regulate immune-system function through cGMP-dependent and -independent mechanisms. In particular, several anti-inflammatory effects of NO have been established, including inhibition of T-cell and B-cell proliferation, and leukocyte rolling and adhesion on microvascular endothelial cells [19,25]. Furthermore, NO also modulates the production of cytokines, generally decreasing the amounts of pro-inflammatory cytokines [interleukin (IL)-1, IL-2, tumor necrosis factor (TNF) and interferon (IFN)-γ] and increasing the amounts of anti-inflammatory cytokines (IL-4, IL-13 and transforming growth factor-β) [19].

Finally, NO functions in both the peripheral nervous system (PNS) and the central nervous system (CNS). In the PNS, neurons use nNOS to produce NO that functions as a neurotransmitter, targeting smooth muscle and causing relaxation through the sGC–cGMP pathway. In the CNS, however, the role of NO is not fully understood, although it has been implicated in neuronal excitability (through both sGC–cGMP-dependent and -independent pathways), synaptic plasticity (through the sGC–cGMP pathway) and neurotransmitter release (through the sGC–cGMP pathway) [26]. Although nNOS is the primary isoform responsible for CNS NO production, eNOS and iNOS might also be involved, particularly in glial cells. Considering the involvement of NO in processes that are profoundly affected by malaria pathogenesis (e.g. endothelial activation, expression of CAMs, vascular leak, severe inflammatory response and coagulopathy) (Figure 1), it is easy to see why elucidating the mechanisms with which NO functions in malaria pathogenesis is an important line of research.

## The role of NO during malaria

Several hypotheses have been proposed regarding the role of NO during malaria pathogenesis. First, virtually all review articles about the killing of *Plasmodium* by the immune system, including a recent review by Riley and Stevenson [4], state that NO derived from iNOS-expressing monocytes or macrophages kills *Plasmodium* as part

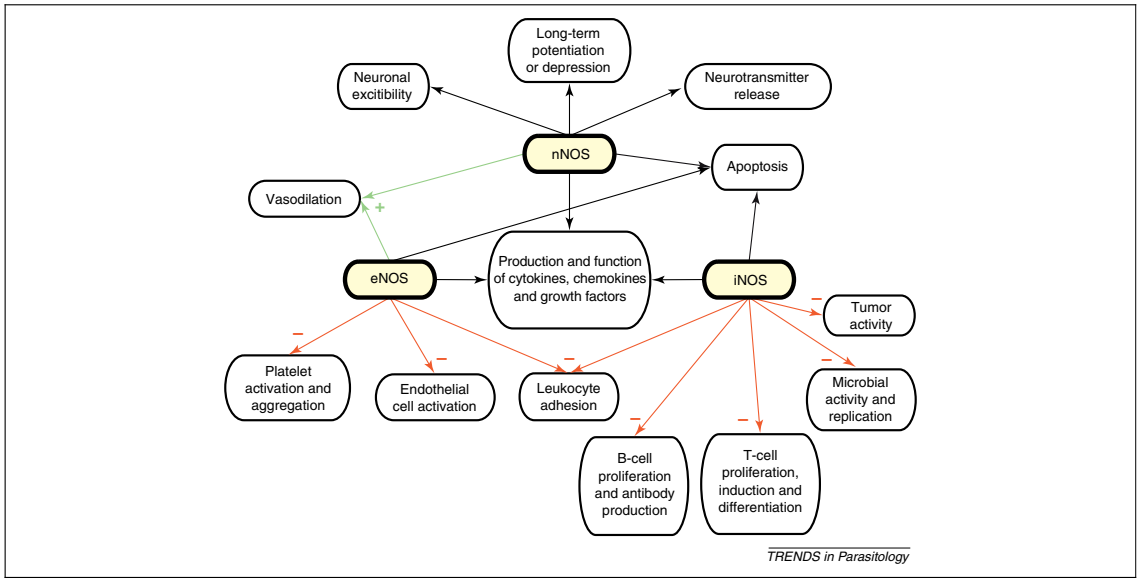


**Figure 1.** Physiological roles of NO that are relevant to malaria pathogenesis. Of particular importance are the homeostatic and anti-inflammatory pathways (red arrows) that can be disrupted by NO quenching during malaria. Green lines indicate enhancement, red lines indicate depression and black lines indicate modulation.

**Table 1. Effect of NO on *Plasmodium* parasites and its involvement in malaria pathogenesis**

| *Plasmodium* species | Effect of NO on parasite | Effect of NO on pathogenesis |
|---|---|---|
| *Plasmodium falciparum* | No killing *in vitro* [29] or *in vivo* [69,70] | **NO is beneficial**<br>iNOS protein levels and mRNA correlate inversely with disease [7,36,37]; plasma and urine NOx levels correlate inversely with disease [7,34]; hypoargininemia correlates with *P. falciparum* disease severity [6]<br>**NO is harmful**<br>CSF NOx levels correlate with death [31]; elevated iNOS levels in CM brains [3]; NOx levels and iNOS activity correlate inversely with total Hb levels [5,32,33]<br>**Controversial**<br>Role of NOS promoter mutations [38] |
| *Plasmodium berghei* | No killing [39,71][a] | iNOS or eNOS deficiency or inhibition has no effect [39,71][a] |
| *Plasmodium chabaudi* | Killing [72] | iNOS deficiency or inhibition increases mortality rate [72,73] |
| | No killing [73–76] | iNOS deficiency or inhibition has no effect [74–76] |
| *Plasmodium yoelii* | No killing [77] | NOS inhibition increases mortality rate [77] |

[a]P. Sobolewski *et al.*, unpublished.

of innate immunity. Whereas the case for NO killing in leishmaniasis is well documented [27], there is little evidence to support its role in the killing of *Plasmodium* [28]. By contrast, most evidence (Table 1), including the lack of killing after incubation of *P. falciparum* with a saturated NO solution [29], suggests that the parasite possesses innate resistance to killing by NO and other reactive oxygen species (ROS). Rockett *et al.* observed that nitrosylated cysteine or glutathione kills *P. falciparum* – with 50% killing occurring at 40 μM and killing with NOx occurring in the range 10–30 mM – suggesting that NO-derived products rather than NO itself might be toxic to the parasite [29]. However, the amount of nitrosylated cysteine or glutathione required is two orders of magnitude greater than the total free-nitrosothiol levels observed in human plasma [30], and the NOx levels required are three orders of magnitude greater than the levels observed in *P. falciparum* malaria patients [7].

The second hypothesis proposes that NO is overproduced during *Plasmodium* infection and that NO has a role in pathogenesis. For example, Clark *et al.* proposed that iNOS-derived overproduction of NO in the brain might disrupt the regulatory role of NO in the CNS, leading to the impaired consciousness of cerebral malaria (CM) [3]. Indeed, elevated levels of iNOS protein are detected in the cerebral-blood-vessel walls of patients who have succumbed to CM [3]. In addition, markedly increased levels of NOx have been observed in the cerebrospinal fluid (CSF) of children who died from malaria [31]. NO has also been implicated in the pathology of malaria anemia; plasma [32] and urine [5] NOx levels and *ex vivo* peripheral blood mononuclear cell (PBMC) iNOS activity [33] have been reported to correlate inversely with Hb levels.

Several lines of evidence argue against the hypothesis that NO is overproduced during malaria and that the excess NO functions systemically to mediate severe malaria pathogenesis. First, the presence of hypoargininemia in malaria patients suggests that NO production might be limited in malaria [6]. Human erythrocytes contain arginase and, consequently, the rupture of RBCs by the parasite will increase plasma arginase levels; this is a mechanism of hypoargininemia in sickle-cell patients [10]. Second, plasma and urine NOx levels correlate inversely with disease severity [7,34]. The results of some earlier studies seem to contradict this finding but these studies did not properly account for the effects of diet, dehydration and renal impairment [35]. It is important to note that the endpoint of all NO reactions is NOx, so it cannot be discerned what impact, if any, the NO had on physiological processes (e.g. binding to sGC or nitrosylation of a protein). Finally, PBMC iNOS levels [7,36] and *iNOS* mRNA levels [37] correlate inversely with disease severity. Collectively, these observations suggest that NO is not overproduced and that iNOS-derived NO might be beneficial rather than harmful.

Although controversial because studies in different regions report opposing results, several investigations report that polymorphisms in the promoter region of the *iNOS* gene (G-945C and/or C-1173T) of human malaria patients, which result in higher baseline NOS activity in PBMCs, are associated with protection from severe *P. falciparum* malaria [38]. If correct, these findings indicate that increased production of NO might be beneficial, either by killing the parasite or by preventing the development of disease. We propose that prevention of disease development is more likely because of the minimal evidence supporting parasite killing by NO (Table 1).

Furthermore, studies in animal models do not support the hypothesis that NO overproduction mediates malaria pathogenesis or is antiparasitic (Table 1). Neither *Plasmodium berghei* ANKA (PbA)-infected iNOS-deficient mice nor mice treated with aminoguanidine (AG), a NOS inhibitor, are protected from severe malaria, both exhibiting similar parasitemia, pathogenesis and mortality to controls [39]. These observations indicate that overproduction of NO does not have a pathogenic role during malaria pathogenesis.

One interpretation of the data from animal models is that NO overproduction has a minimal role, if any, in the development of severe experimental malaria because neither inhibition of NOS nor use of NOS-deficient mice had a measurable effect on pathogenesis. However, it is rare for the rate of malaria mortality to be increased; to our knowledge, there is only one publication reporting more-rapid mortality in experimental (treated or knockout) mice compared with controls [40], and that article challenges a report of opposing results using the same

polyclonal antiplatelet serum [41]. In addition, mice lacking other molecules with demonstrated efficacy at ameliorating experimental severe malaria (ESM) do not exhibit increased mortality rates. For example, we observed that IL-10 is present in the plasma of PbA-infected mice and that IL-$10^{-/-}$-knockout mice become moribund at the same time as control mice [42,43]. However, treatment with this anti-inflammatory cytokine ameliorates the development of ESM [44]. Finally, an increased mortality rate is not expected in NOS-deficient mice if NO bioavailability during malaria is low owing to NO scavenging or low production; this is because decreasing NO production through knockout of NOS would not further diminish already minimal NO levels. To date, the preponderance of evidence indicates that NO production is diminished in subjects with severe *P. falciparum* malaria compared with in uncomplicated controls. It remains to be determined whether low NO bioavailability – caused by NO scavenging – during malaria contributes to malaria pathogenesis. There are two main candidates for NO scavenging: Hb and superoxide.

### NO bioavailability is limited by free Hb

Based on the extensive literature about the role of free Hb both as a blood substitute [45] and during sickle-cell anemia [46], free Hb released during the asexual cycle of blood-stage *Plasmodium* might quench NO, thereby having an important role in limiting NO bioavailability during malaria. Low NO production as a consequence of hypoargininemia represents another mechanism [6]. During the course of malaria pathogenesis, there are two possible mechanisms of hemolysis. First, *Plasmodium* parasites rupture erythrocytes after completing their development within them, thus releasing progeny merozoites. The rupture will also release the remaining contents of the erythrocyte, which includes Hb. Second, there are more-complex, immune-system-mediated mechanisms of erythrocyte lysis leading to malaria anemia [47]. Complement-mediated erythrophagocytosis has long been postulated as a major mechanism of malaria anemia [48] but we speculate that other, non-phagocytic mechanisms such as T-cell-mediated and TNF-mediated cytotoxicity [49] might also be involved.

Free-Hb levels are markedly increased in patients with *P. falciparum* malaria [8], by up to an order of magnitude (15-μM heme) in acute cases [9], and we have observed elevated levels of cell-free Hb in the plasma in experimental *P. berghei* malaria. The *in vivo* effects due to free, natural molecular Hb in blood are well defined within the research community interested in the development of molecular-Hb-based oxygen-carrying blood substitutes [45]. Free Hb is a powerful *in vivo* scavenger of NO, leading to vasoconstriction and impaired microvascular blood perfusion, which are major determinants of tissue and organism survival [50]. The efficiency of free Hb at scavenging NO is almost 1000-fold that of Hb packaged in RBCs [51]. NO reacts with the oxygen in oxyhemoglobin (oxyHb), generating methemoglobin [metHb or Hb($Fe^{3+}$)] and nitrate (Equation 1). NO also reacts with deoxyHb to form Hb($Fe^{2+}$)NO, which then reacts with oxygen to form metHb and nitrate (Equation 2).

$$Hb(Fe^{2+})O_2 + NO^{\bullet} \rightarrow Hb(Fe^{3+}) + NO_{3^-} \quad (1)$$

$$Hb(Fe^{2+}) + NO^{\bullet} \rightarrow Hb(Fe^{2+})NO \xrightarrow{+O_2} Hb(Fe^{3+}) + NO_{3^-} \quad (2)$$

Parasite-derived hemozoin (Hz) granules, which are vacuoles within the parasite where it stores the heme moiety of consumed Hb, are released with Hb during rupture of parasitized RBCs. Hz granules are unlikely to scavenge NO because the iron moiety of Hz is in the ferric $Fe^{3+}$ state – as with metHb – which has a lower affinity (10 000 times) than the ferrous $Fe^{2+}$ state for NO [20]. Recent evidence suggests that Hz increases the levels of lipopolysaccharide-induced and IFN-γ-induced iNOS transcription and NO production in PBMCs *in vitro* [33]; it remains to be determined whether this mechanism leads to increased NO production *in vivo* in *P. falciparum* patients.

Confirming the role of free Hb in scavenging NO during ESM, we have observed marked *in vitro* quenching of NO by the plasma of mice six days after infection with *P. berghei*, and this quenching correlated well with the increase in plasma Hb levels. Moreover, the quenching was abrogated by cyanide–ferricyanide inactivation of the Hb, indicating that free Hb in plasma during malaria quenches NO. These results clarify previous observations that NOS inhibition and NOS deficiency did not affect ESM pathogenesis; NO bioavailability is already limited by NO quenching in the plasma and, thus, treatment with NOS inhibitors or the use of NOS-deficient mice does not decrease NO bioavailability further.

An argument against the role of free Hb in malaria pathogenesis is that free Hb is released during other diseases such as congenital hemolytic anemias without eliciting the impaired mental status and respiratory distress observed during severe *P. falciparum* malaria. However, less inflammation is elicited during these congenital anemia syndromes than during malaria, and there is no sequestration of parasites to cerebral and lung endothelium, as observed during *P. falciparum* infection. Gladwin's group proposes that many of the vascular complications common in hemolytic disorders are attributable to NO quenching by free Hb [10]. Indeed, the amelioration of sickle-cell anemia by administration of the NO donor sodium nitroprusside or by inhalation of NO gas indicates that NO quenching by Hb has a crucial role in the pathogenesis of sickle-cell anemia, which is another inflammatory disease [46].

If the low-NO-bioavailability hypothesis is correct, the malaria inflammatory response, in addition to increased endothelial activation (Figure 2), lacks feedback regulation by NO. This inflammatory response also results in increased production of superoxide, which rapidly reacts with NO to form the labile radical peroxynitrite, thus decreasing NO bioavailability [52]. Furthermore, peroxynitrite is an extremely damaging oxidant [53], particularly in the brain [54] where its formation might reconcile Clark's hypothesis that NO is involved in pathogenesis, with low NO bioavailability functioning in the vasculature. Another potential toxic consequence of free Hb in plasma is the potent ability of Hb to generate radicals,

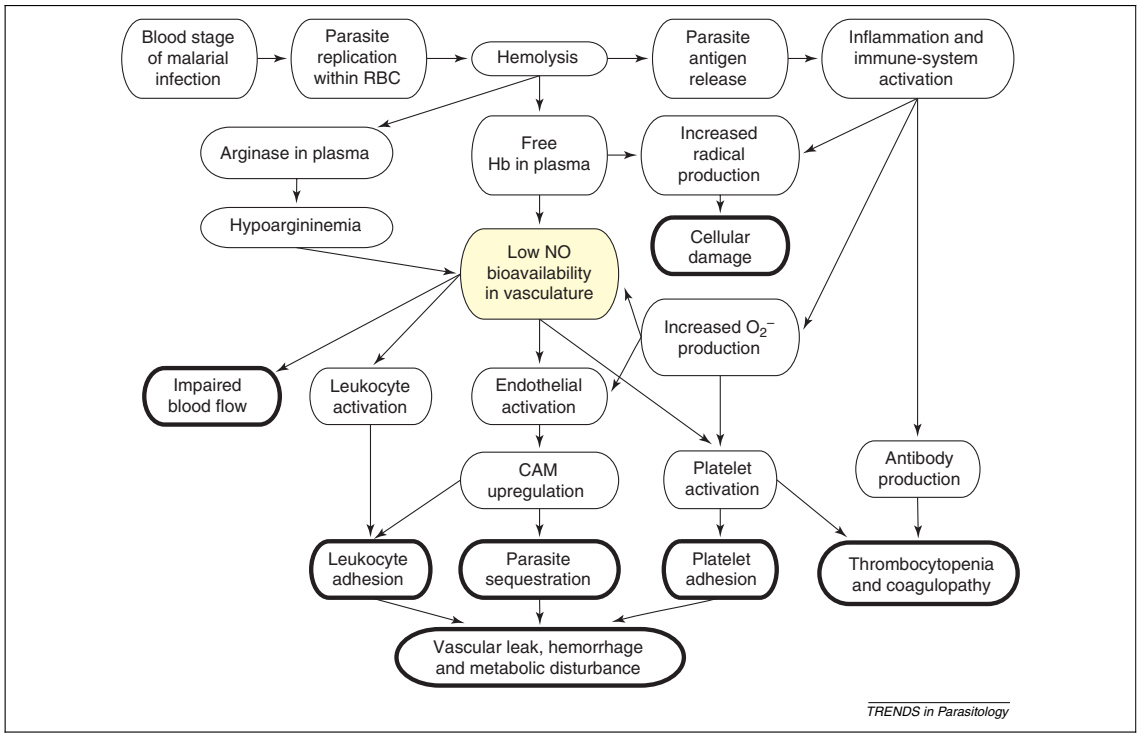


**Figure 2.** Hypothesis of low NO bioavailability. Image illustrates the key role that NO bioavailability might have in malaria. Restoring NO bioavailability by reducing quenching or providing exogenous NO might ameliorate the downstream consequences (e.g. vascular leak and thrombocytopenia) during malaria of impaired NO bioavailability due to Hb and superoxide scavenging. Thick ovals represent end-stage hallmark effects of malaria pathogenesis.

which have been shown to cause vascular leak [45]. Similarly, metHb, produced as a result of NO scavenging by Hb, is labile and readily dissociates into free heme, which is a highly pro-inflammatory molecule [55]. A buildup of heme at sites of hemorrhage is a potent recruitment signal for inflammation at that site [55]. Thus, the low NO bioavailability, in concert with the malaria-specific inflammatory response, probably mediates the impaired consciousness that is observed in malaria but not in the other hemolytic disorders.

### NO bioavailability is limited by superoxide

The rupture of infected erythrocytes caused by the release of parasite progeny also results in the release of malaria glycosylphosphatidylinositol (GPI)-anchored proteins and Hz. Both GPI and Hz elicit a strong pro-inflammatory response in monocytes and neutrophils [56,57] that leads to increased production of ROS, including superoxide, causing oxidative stress [58]. Studies of several fields, including ischemia–reperfusion injury, cardiovascular disease [59], diabetes [60] and sickle-cell anemia [61], have implicated oxidative stress in reducing the bioavailability of NO [62] and in pathogenesis. Of particular relevance to malaria is the fact that NO scavenging by superoxide has been implicated in endothelial dysfunction [59], platelet activation [63] and regulation of immune response [25].

Possible sources of vascular superoxide include phagocyte NAD(P)H oxidase (NOX2), endothelium NAD(P)H oxidase (NOX4), xanthine oxidase and uncoupled members of the NOS family. Increased phagocytic NAD(P)H oxidase activity is expected to occur as a result of the host inflammatory response. Increased endothelial production of superoxide has been observed in response to pro-inflammatory cytokines such as TNF [64] and IL-1 [65] that are upregulated during malaria pathogenesis [43]. Xanthine oxidase functions in the pathology of sickle-cell anemia [61] and might be an important component of innate immunity [66]. Finally, members of the NOS family can produce superoxide following depletion of arginine or tetrahydrobiopterin (a necessary cofactor) [12], which might exacerbate the effects of the hypoargininemia observed during *P. falciparum* malaria [6]. Superoxide might also mediate Hb scavenging of NO because Hb is not only a potent NO scavenger by itself but also a generator of superoxide by autooxidation of oxyHb. Within the erythrocyte, autooxidation of oxyHb is balanced by the presence of superoxide dismutase (SOD), catalase and metHb reductase [67] but, in plasma, this process is uncontrolled. Thus, it is likely that malaria inflammation results in increased NO quenching by superoxide.

In confirmation of the role of superoxide in limiting NO bioavailability during malaria pathogenesis, our group and others have observed marked protection of mice infected

with *P. berghei* by treatment with pegylated SOD (PEG–SOD) and pegylated catalase (PEG–CAT), compared with PEG–CAT treatment alone or vehicle controls [68]. All three groups had similar parasitemia and, thus, the results cannot be attributed to differences in infection or parasite replication. Furthermore, we observed a marked reduction in the profound thrombocytopenia that is a hallmark of malaria, suggesting either that NO bioavailability is increased by the PEG–CAT + PEG–SOD treatment (because NO is a potent inhibitor of platelet activation) or that superoxide contributes to malaria thrombocytopenia.

### NO bioavailability and resistance to severe malaria

Any hypothesis regarding malaria pathogenesis must account for the fact that only 1–2% of the individuals infected with *P. falciparum* develop severe malaria and succumb to the disease. The aforementioned clinical studies correlating iNOS promoter polymorphisms with NO production and survival from severe malaria suggest that elevating NO production might be a mechanism to overcome NO scavenging and prevent the development of disease. However, the presence of hypoargininemia and low levels of plasma NOx in patients with severe *P. falciparum* malaria indicates that these patients might have insufficient amounts of NOS substrate to elevate NO production. A variety of other factors such as malarial inflammation probably functions in concert with free-Hb toxicity to mediate malaria pathogenesis. Differences in the inflammatory response (e.g. decreased production of pro-inflammatory cytokines) might inhibit the pathogenic process (Figure 2) independently of free-Hb levels. Furthermore, decreasing the immune response might reduce hemolysis, thus decreasing free-Hb levels and ameliorating free-Hb exacerbation of the inflammatory response. Differences in activation of the coagulation cascade might inhibit the pathogenic process independently of free-Hb levels but might also decrease vascular leak and petechial hemorrhage, thus decreasing both inflammation at the hemorrhage site and release of free Hb. Finally, more-efficient means of clearing plasma Hb or increased expression of antioxidants such as SOD and catalase might be beneficial by reducing NO scavenging. Thus, increasing the levels of bioavailable NO, either by increasing the release of NO in the vasculature or by mitigating NO scavenging, might serve as an adjunct or anti-disease treatment for malaria.

Restoration of NO bioavailability is being tested therapeutically for diseases such as acute respiratory distress, cardiovascular disease, diabetes and sickle-cell anemia. We have observed that treatment with a NO donor results in marked protection of mice infected with *P. berghei* compared with vehicle controls. Both groups had similar parasitemia; thus, the results cannot be attributed to differences in infection, parasite replication or parasite killing by NO. We conclude that treatment with the NO donor restored NO bioavailability by overcoming the quenching that occurs during the course of malaria. The NO can reach one of its targets (sGC) and carry out its homeostatic role. Overall, our interpretation of data from malaria and other diseases is that NO bioavailability is limited during malaria and exacerbates malaria inflammation, endothelial activation and coagulopathy (Figure 2).

### Concluding remarks

To develop effective adjunct or anti-disease treatment for malaria, the mechanisms of malaria pathogenesis must be understood. We believe that the data indicate that NO bioavailability is low within the vasculature during malaria because levels of NO production are low owing to hypoargininemia and increased quenching of NO by free Hb and superoxide. NO has a potent role in vascular homeostasis that is disrupted during malaria pathogenesis. Restoring NO bioavailability by diminishing NO quenching, providing exogenous NO or restoring arginine levels might, therefore, be an effective anti-disease therapy. It remains to be determined whether restoring NO bioavailability within the vasculature late in the disease is beneficial. Finally, it is still unclear whether, as proposed by Clark *et al.* [3], NO produced within tissue such as the brain might function in pathogenesis.

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# Chapter 6: Low nitric oxide bioavailability contributes to the genesis of experimental cerebral malaria.

**Abstract**

The role of nitric oxide (NO) in the genesis of cerebral malaria is controversial with most investigators proposing that the unfortunate consequence of elevated NO levels produced to kill the parasite is the development of cerebral malaria. However, vascular NO synthase-deficient mice were not protected from experimental cerebral malaria (ECM) and exogenous NO provided marked protection by restoring NO-mediated signaling in the brain, decreasing proinflammatory biomarkers, and markedly reducing vascular leak and petechial hemorrhage into the brain. Low NO bioavailability in the vasculature during ECM was caused in part by (1) elevated levels of NO scavenging free hemoglobin in the blood, which was inactivated by NO donor treatment, and (2) hypoargininemia. Low levels of nitrite in erythrocytes during ECM suggest that the conversion of nitrite to NO by deoxyhemoglobin has a low potential to increase NO bioavailability. Thus, low rather than high NO bioavailability contributes to ECM pathogenesis.

**Introduction**

The contribution of NO bioavailability to cerebral malaria (CM) pathogenesis is controversial. On the one hand, nitric oxide (NO) is proposed to be produced by the protective immune response and control parasite replication[1]; the unfortunate side effect of this is the development of CM[2]. On the other hand, NO in vitro and in vivo does not inhibit bloodstage *Plasmodium* replication[3-7]. Hypoargininemia, decreased plasma nitrite levels in patients with *P. falciparum* malaria[8], and the increased levels of NO scavenging cell-free hemoglobin (Hb) in the blood as a consequence of parasite replication[9-12] suggest that NO bioavailability is low[13].

This controversy about the role of NO bioavailability in CM pathogenesis provides the impetus to determine whether (i) lack of vascular NOS or exogenous NO ad-

ministration affects development of ECM, and (ii) low NO bioavailability occurs during CM. To test these questions in a pre clinical animal model, we selected the *P. berghei* ANKA (PbA) infection of mice, a well-recognized model of severe human CM[14] with animals (like humans) exhibiting neurological dysfunction, acute respiratory distress with lactic acidosis, and nephritis[15-18]. The results from animal models should be extrapolated cautiously to the human condition because *P. berghei* is distinct from *P. falciparum* and mice differ from humans. Nevertheless, PbA-infected erythrocytes adhere to tissue endothelium (also termed sequestration) via CD36 like *P. falciparum*[19,20] and sequestration in the brain has been documented in some but not all studies[19,21]. The role of parasitized RBC sequestration and leukocyte adhesion in human CM pathogenesis is also controversial with the relative roles of leukocyte and parasitized RBC adhesion debated[2,22,23]. CM is a syndrome with the largest group of patients exhibiting parasitized RBC sequestration plus inflammation in the brain leading to extravascular pathology[24-26]. Platelets may contribute to this pathology because platelet accumulation in the brains of African children correlate with the development of disease[26]. Platelet adhesion occurs in experimental cerebral malaria (ECM)[21] and platelets are required for the development of ECM[27,28]. Thus, key cell adhesion events of human CM, namely leukocyte, platelet, and possibly parasitized RBC adhesion, are recapitulated in ECM. In addition, ECM mice exhibit a marked proinflammatory response, profound thrombocytopenia, hypotension, vascular leak and petechial hemorrhaging in the brain[16]; which are the key impairments in humans; the effects of NO on these impairments is the focus of this study.

## Results

### Lack of vascular NOS isoforms does not protect against the development of ECM

The elevated levels of NO functioning in ECM hypothesis predicts that lack of

either iNOS or eNOS should protect against the genesis of ECM. However, there was no significant difference in the survival or parasitemia between groups (n=10) of iNOS- and eNOS-deficient mice and C57BL/6 controls (Figure 1A, B).

**NO donor protects C57BL/6 mice from the development of ECM and restores cGMP levels in brain**

To determine the converse, we injected PbA-infected mice twice daily with an NO donor (1mg Dipropylenetriamine NONOate [DPTA/NO]) or saline vehicle. All 5/5 saline-injected mice were moribund on day 6 post infection (PI) whereas only 1/5 NO donor treated mice became moribund on day 9 PI (Figure 1C). Because the parasite completes its life-cycle every 24 hours, it is continually replicating during the experiment. However, the parasitemia in NO donor injected mice was similar to saline controls (Figure 1D), indicating that NO had no effect on parasite replication or viability[29]. The NO donor treated group on day 6 of infection were clinically indistinguishable from uninfected control mice (score: 0±0) whereas the saline treated group were moribund with clinical scores of 8.8±1.6 (Figure 1E). Mice with a clinical score of >7 are considered moribund and are euthanized. Similar results were obtained in a replicate experiment and when mice were treated with NO gas (40ppm±5), confirming that exogenous NO protects against ECM.

To estimate the amount and the duration that DPTA/NO was releasing NO in the circulation, we assessed the pharmacokinetics of DPTA/NO by monitoring its plasma concentration over time (Figure 1F). Maximum concentration in plasma was achieved after 1 hour and by 4 hours post-injection the compound was cleared. The decay of the compound was fitted with an exponential function ($R^2$=0.999), and the time constant was determined to be 1.6±0.3 hours. From the first order kinetics of decomposition and clearance, we estimate for a 1 mg injection of DPTA/NO that only about one third of the

DPTA/NO molecules decompose within the plasma, resulting in the cumulative production of 100μM NO. The pharmacokinetics of DPTA/NO described above correlated well with the kinetics of the 61% decline in mean arterial blood pressure (MAP) elicited by DPTA/NO injection (Figure 1F). Although this dose caused a marked decline in MAP, none of treated animals exhibited any clinical signs, did not appear in cardiac distress, and no animals died.

To determine whether the low NO bioavailability during ECM decreased NO-dependent signaling through soluble guanylate cyclase (sGC) and whether NO donor administration restored these levels, we assessed cGMP levels by enzyme-linked immunoassay in brains and plasma on day 6 of ECM in animals (n=5) 4 hours after injection with NO donor or saline, the timepoint when the pharmacokinetics indicated that DPTA/NO had been cleared from the circulation and the MAP had returned to baseline. The saline-injected mice with clinical signs of ECM on day 6 exhibited significantly ($P<0.05$) lower levels of cGMP in the brain compared with uninfected controls or NO donor treated mice with no clinical signs of ECM (Figure 1G). The levels of plasma cGMP were significantly ($P<0.005$) lower both in ECM-susceptible saline-injected mice and in ECM protected, NO donor treated mice compared with uninfected controls (Figure 1H).

**NO donor administration inhibits the development of vascular leak and hypotension during ECM, and decreases the production of MMP-9 and proinflammatory biomarkers**

Vascular leak in the brain may be an important component of ECM pathogenesis[30]. We observed significantly ($P=0.0008$) lower extravasation of Evans blue/albumin complex into the brains in groups (n=5) of DPTA/NO treated animals as compared to infected saline injected controls on day 6 of ECM (Figure 2A). In fact, the levels of Evans blue in brain were similar in NO donor treated animals on day 6 of PbA and uninfected

controls ($P$=0.62). In addition, marked petechial hemorrhaging was observed visually on brain surfaces and in brain sections on day 6 of ECM in saline injected mice, whereas none were detected in the NO donor treated group or uninfected controls.

Thrombocytopenia occurs during ECM[31,32] and exogenous NO inhibits the activation of platelets through sGC-dependent mechanisms[33], suggesting that NO may inhibit ECM hemorrhage by ameliorating thrombocytopenia or coagulopathy during ECM. However, the NO donor injections did not ameliorate thrombocytopenia assessed by flow cytometry on day 4 or day 6 of infection when compared with saline treated control mice (NO donor day 4: 530,403±126,055 vs saline day 4: 474,626±173,564; NO donor day 6: 29,637±8,133 vs saline: 10,488±6,977). Although NO donor treatment significantly increased levels of circulating fibrinogen (NO donor day 6: 6,255±116 ng/ml vs saline: 5,122±369), it did not markedly affect the levels of other coagulation factors (fVII, tissue factor, and thrombopoeitin, von Willebrand factor) assessed by microsphere fluorescence assay in blood on day-6 compared with saline-treated controls (supplemental data).

Inflammation elicits MMP-9, a protease that contributes to the disruption of endothelial barrier integrity and MMP-9 is inhibited by tissue inhibitor metalloproteinase-1 (TIMP-1); the ratio of these 2 molecules determines whether MMP-9 can affect endothelial barrier integrity. The levels of IL-18 (Figure 2B), a proinflammatory cytokine, and sCD40 (Figure 2C), an inflammatory marker, were significantly ($P$<0.005) decreased in plasma of NO donor treated mice on day 6 of ECM compared with saline-treated controls, suggesting some amelioration of inflammation in these mice by the NO donor. The levels of other cytokines were at or below the limit of detection (IL-2,-4,-5, -12p70, and TNF), or similar (IL-1β,-6,-10, and IFN-γ) in both groups of mice on day 6 of ECM (supplemental data), indicating not all pathways were equally affected by NO donor treatment. The levels of MMP-9 were significantly ($P$=0.0001) decreased in NO-donor treated mice on day 6 of ECM compared with saline injected controls (Figure 2D), while

TIMP-1 levels were similar (supplemental data).

Because loss of fluids via vascular leak into tissue can contribute to hypotension, which occurs in ECM[15], we determined whether the administration of NO donor to C57BL/6 mice (n=4) during ECM maintains the baseline MAP (before NO donor administration) assessed by telemetric blood pressure transducers implanted in the carotid artery. The vehicle control animals developed hypotension starting on day 4 which progressively worsened on day 6 (Figure 2E). In contrast, the NO donor treated animals maintained their baseline MAP and on day 6 of PbA were significantly ($P$=0.03) protected from the development of hypotension.

**Free Hb in plasma and hypoargininemia contribute to low NO bioavailability during ECM**

The protection against ECM by DPTA/NO suggests that NO bioavailability is low during ECM; this low NO bioavailability may be caused by (i) free Hb released during parasite replication and non-specific RBC lysis, (ii) hypoargininemia, or (iii) low nitrite levels.

*Free Hb:* No significant increase in plasma Hb concentration was observed on day 4 of infection as compared to day 0. However, plasma from C57BL/6 mice on day 6 of the infection contained significantly ($P$=0.0001) higher levels of oxyHb: 7.4±0.7μM as compared to 2.8±0.2μM on day 0 (Figure 3A). The increase in free Hb correlated with disease severity but parasitemia correlated poorly when NO treated mice were included because they exhibit similar parasitemia as controls but no disease (supplemental data).

The hypothesis of high NO bioavailability predicts that free Hb is in the NO inert metHb state. Consequently, we related the free Hb levels in plasma to its ability to quench NO and to oxyHb standards (supplemental data). A marked increase in NO quenching was detected when plasma from an infected mouse on day 6 of infection was

injected into the NO sample container compared with an uninfected control (Figure 3B). Degassed PBS exhibited no detectable NO quenching, whereas the 3.75μM oxyHb standard rapidly decreased the NO level. The free Hb levels in the plasma of infected mice on day 6 or 7 of infection and uninfected controls correlated (Spearman rank correlation R=0.85, *P*=0.000003) with NO quenching by plasma from these mice; the NO quenching in the infected animals was significantly (*P*=0.005) increased in infected mice versus controls (13.7±2.0μM in controls and 23.0±2.0μM in infected; Figure 3C).

To verify that Hb in the plasma from infected mice was responsible for the NO quenching, we treated the plasma samples with cyanide/ferricyanide, which inactivates the NO scavenging of Hb, or with KCl as a control. Inactivation of free Hb of infected plasma with cyanide/ferricyanide (n=3) significantly (*P*=0.0005) reduced NO quenching of plasma, virtually eliminating it (Figure 3D). In contrast, there was no significant change in NO quenching caused by the control KCl treatment of plasma (n=3) and untreated plasma.

In order to restore NO bioavailability and exhibit efficacy beyond the short release of NO, the donor must inactivate free Hb. To determine whether NO from the DPTA/NO donor (1) converts free NO-scavenging oxyHb present in the plasma of sick animals to NO unreactive metHb and (2) does not induce methemoglobinemia in RBC Hb, we collected blood from PbA-infected mice and uninfected controls (n=3) before and 1.5 hours after injection with 1mg DPTA/NO on day 6 of infection, isolated plasma and lysed RBCs and then assessed the levels of oxyHb and metHb spectrophotometrically. Following NO donor treatment, the plasma exhibited a significant (*P*<0.05) conversion of free oxyheme to metheme in the PbA-infected and uninfected groups of mice (Figure 3E). The baseline level of free metHb before NO administration was higher than in the uninfected group, indicating increased conversion of oxyHb to metHb during infection. The NO donor caused a further significant (*P*<0.05) increase in RBC metHb and NO gas

caused a significant ($P$=0.04) decrease in free Hb levels (Figure 3F).

*Hypoargininemia:* To determine whether hypoargininemia occurs during ECM, we assessed the levels of arginine and its NOS-inhibiting analogs (homoarginine, asymmetric and symmetric dimethylarginine) in the plasma of groups (n=5) of ECM mice and uninfected controls. The levels of arginine and its analogs declined markedly and significantly ($P$<0.0001) by day 4 and even further by day 6 in animals with ECM (Figure 3G).

*Nitrite*: One of the in vivo footprints of NO is nitrite, which during hypoxia can be reduced back to NO by deoxyHb[34]; this mechanism of NO generation protects against ischemia/reperfusion injury[35]. However, the levels of nitrite were significantly ($P$<0.05) lower in erythrocytes, and in plasma on day 6 of ECM compared with uninfected controls (Figure 3H). NO gas treatment during infection restored plasma but not erythrocyte nitrite levels (Figure 3H).

**Discussion**

Others' and our data do not support the hypothesis that elevated NO bioavailability in the vasculature contributes to the genesis of ECM. In fact, they support the converse that NO bioavailability is low during ECM. First, mice lacking iNOS or eNOS exhibit similar parasitemia as controls and are not protected from ECM[5]. A more rapid time course of mortality in $iNOS^{-/-}$ or $eNOS^{-/-}$ mice is not expected if NO bioavailability is minimal because decreasing NO production through gene targeted knockout should not further lower NO bioavailability. Second, exogenous NO administration protects against the development of ECM, which precludes NO being pathogenic. Third, animals with ECM exhibit marked hypoargininemia, indicating that NO production is likely compromised. Fourth, the in vivo footprints of labile NO (brain cGMP and nitrite) are markedly decreased rather than increased as predicted by the elevated NO bioavailability hypothesis. Our findings that exogenous NO administration significantly protects against the

development of ECM, decreases cerebral vascular leak and hemorrhage, converts free oxyHb to metHb, decreases circulating free Hb levels, and restores the levels of cGMP in brain and nitrite in plasma collectively indicate that restoring NO bioavailability is protective.

This low NO bioavailability in ECM-susceptible strains caused in part by elevated free Hb and arginase I due to parasite replication needs to be reconciled with the existence of ECM-resistant strains with similar parasitemia and hence elevated free Hb and arginase I levels. Hypoargininemia is significantly associated with the development of CM in Tanzanian children[8]. Although the clinical study of free Hb levels in humans correlating free Hb levels with CM remains to be performed, the levels reported to date are higher in *P. falciparum* infections compared with less virulent *P. vivax* and elevated free Hb in *P. falciparum* patients correlated with increased levels of myoglobin and creatine kinase, biomarkers for disease[12]. Thus, clinical studies in humans support the role of low NO bioavailability in pathogenesis.

On the other hand, free Hb in the blood by itself and hence low NO bioavailability is not necessarily pathogenic. Indeed, elevated levels of free Hb are observed not only in patients with virulent *P. falciparum* but also in *P vivax* malaria and in hemolytic uremic syndrome where CM does not develop. Hemoglobin based oxygen carriers do not exhibit toxicity in control subjects but contribute to mortality in patients with hemorrhagic shock[36]. Thus, we posit that the free Hb and hypoargininemia limit NO bioavailability during ECM; the low NO bioavailability likely exacerbates the underlying pathogenic processes as observed in sickle cell disease[37]. Indeed, Serirom et al. report that exogenous NO downregulates endothelial cell adhesion molecules, decreasing parasite adhesion in vitro[38]. Low NO bioavailability is likely an essential cofactor that is necessary but not sufficient for the development of CM. Thus, an altered immune response in ECM-resistant mice[39] may explain why low NO bioavailability does not cause disease in these

mice. Given that CM is a syndrome, it is unlikely that one single factor mediates disease.

There are three primary mechanisms that may account for the administration of the NO donor ameliorating ECM pathogenesis. First, the NO donor reduced the level of circulating free Hb and inactivated the free Hb by converting it to NO inert metHb, allowing endogenous NO to play its normal homeostatic role. MetHb might activate the heme-oxygenase pathway, which is anti-inflammatory[40]. The NO donor at 1.5hours after administration also transiently converted RBC oxyHb to metHb (~10% increase). The contribution of metHb in RBCs to pathogenesis is unclear but levels below 20% are not toxic. RBC metHb levels in Tanzanian children with *P. falciparum* malaria are infection mediated, increasing from undetectable in uninfected individuals to a mean of 6%[41]. The increased RBC metHb levels may be due to impairment of metHb reductase system during malaria[42].

The second possibility is that the NO donor has direct effects on sGC-dependent and independent pathways. The observation of blood pressure reduction during the circulation lifetime of the NO donor indicates that NO is signaling within the vasculature: the exogenous NO diffuses and targets sGC in smooth muscle cells, resulting in vasodilation. The direct NO signaling effects of DPTA/NO are likely to be limited to the short period (~3 hour) of NO release. However, the effects appear to be longer lasting because basal cGMP levels (4 hours after treatment) were restored in the brains of NO donor treated animals, and the baseline MAP before administration of the NO donor did not decrease as occurred with saline-injected controls. The third possibility is that the NO pool is restored by NO treatment; the NO pool would continue to generate NO after the NO donor has been cleared or the NO gas turned off.

The causes of low NO bioavailability and its likely pathogenic effects are summarized in Figure 4. Because of NO's pleiotropic functions, administration of NO donor may function via additional pathways not identified in this study. Because more than

20% of patients with severe falciparum malaria treated effectively with anti-parasite drugs still succumb to their disease[43], our results suggest that restoring NO bioavailability may represent an important component of effective adjunct therapy to rescue patients from severe falciparum malaria. Further, Rivers' group[44] has reported that patients in septic shock may benefit from exogenous NO, suggesting that the current concept of macrophage-derived NO being harmful may need to be revisited in other diseases as well.

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## Methods

### Infection and treatment of mice

*Plasmodium berghei* ANKA was stored as a frozen stabilate and this stabilate was injected into source mice to generate an inocula for the experiments. BALB/c, and C57BL/6 mice as well as $eNOS^{-/-}$ or $iNOS^{-/-}$ mice on a C57BL/6 background were obtained from Jackson Laboratories (Bar Harbor, ME) or bred at La Jolla Bioengineering Institute from stocks purchased from Jackson Laboratories. All animals were housed in microisolator cages and provided food and water ad libitum.

To initiate the infection, experimental mice between 6 and 10 weeks of age were injected i.p. with $1x10^6$ *P. berghei* ANKA parasitized erythrocytes obtained from a source mouse. Parasitemia was assessed by counting the number of parasitized erythrocytes among 200 and 1,000 erythrocytes in Giemsa-stained thin blood films (Thomas Scientific, Swedesboro, NJ). The Institutional Animal Use Committees of La Jolla Bioengineering Institute approved all procedures. *P. berghei*-infection in mice causes experimental cerebral malaria, wherein animals exhibit obtundation, and impaired responses to neurological tests. In our hands virtually all C57BL/6 infected mice succumb on day 6, or 7 of infection[15].

*Assessment of disease severity***.** Each of the mice was evaluated for clinical disease by summing its gripping reflex and righting reflex each on a scale of 0-5 with 0 unimpaired and 5 having no reflex. Mice with a clinical score >7 are moribund.

*Treatment with NO donor*. Groups of 5 mice were injected twice daily with 1mg of DPTA/NO (Axxora, San Diego, CA) i.p or saline. Ten mg of the DPTA/NO solid was dissolved in 100µL of a cold solution of NaOH at pH=13 and was kept on ice to prevent premature release of NO. Immediately prior to injection, this stock was diluted with saline to a final concentration of 10mg/mL and 100µL (1mg) were injected i.p. into each

animal.

*Treatment with NO gas*: Groups (n=5) mice infected with PbA were placed daily for 12 hours in a custom plexiglass chamber with food and water ad libitum. The air/NO gas mixture (40±5ppm measured by Sievers 280i Chemiluminescence Nitric Oxide Analyzer and recorded on a custom Labview interface) was prepared by diluting 99.0% grade NO gas (Airgas, San Diego, CA) with air and this mixture was exchanged 10 times every minute.

*Assessment of blood pressure.* The mouse was anesthetized with an i.p. injection of ketamine plus xylazine (Western Medical, San Diego, CA). During sterile surgery, the catheter tip (0.4mm in diameter. 5-8 cm in length) was inserted into the carotid artery. The Data Systems International (DSI, Arden, MN) transmitter was secured in a subcutaneous pocket in the dorsal neck region. The receiver for the transmitter was located under the animal's cage and data was collected with Dataquest A.R.T. system. The MAP is slightly higher than reported with DSI telemetry devices[45], and may be either an offset value or stress caused by injection and turning on the instrument.

*Assessment of vascular permeability.* Vascular leak during PbA was assessed via Evans Blue technique described previously[46]. Briefly, under brief isofluorane anesthesia (Western Medical), 100 µl of Evans blue in PBS (Sigma, Saint Louis, MO; 2%) injected i.v. and allowed to circulate for 10 min. The animal was then anesthetized with ketamine plus xylazine (Western Medical). The heart was then exposed by dissection and 20mL of PBS was perfused into the right atrium, followed by 10mL PBS into the left atrium. The brain, and lung were dissected and placed in 1 ml of N,N-dimethyl formamide (Sigma) for 48 h to extract the Evans blue dye. Dye concentration was determined by measuring absorbance at 630nm using a µQuant spectrophotometer (Biotek, Winooski, VT), followed by normalization to tissue mass.

*Assessment of levels of cGMP, inflammation and coagulation factors***.** cGMP lev-

els in brain tissue and blood were assessed using a cGMP enzyme-linked immunoassay (Biotrak, GE Health Sciences, Piscataway, NJ) following kit instructions. The inflammatory cytokines (IL-1, -2, -6, -12p70, TNF, and IFNγ), proinflammatory biomarker (sCD40 and sVCAM-1), and anti-inflammatory cytokine (IL-4 and-10), coagulation factors (fVII, tissue factor, thrombopoietin, von Willebrand Factor, and fibrinogen) and protease assay (MMP-9 and TIMP-1) were assessed in a multiplexed bead based assay as described[28]. Under brief isofluorane anesthesia, blood was drawn via retro-orbital bleed and centrifuged at 500 x g for 20 min at 4ºC to pellet blood cells. Plasma samples were frozen and kept at -80ºC prior to analysis. The animal was then euthanized and the brain was dissected and immediately frozen in liquid nitrogen and kept at -80ºC prior to analysis. The frozen tissue was homogenized in ice cold 5% TCA solution. The homogenate was centrifuged and the supernatant was washed 4 times with 5 volumes of water saturated ether. The aqueous phase was then frozen and lyophilized. Samples were then dissolved in Assay Buffer and acetylated immediately prior to analysis. Plasma samples were diluted 1:10 with Assay Buffer and acetylated for analysis.

*NO donor pharmacokinetics***.** (Z)-1-[2-(2-Aminoethyl)-N-(2-ammonioethyl)amino]diazen-1-ium-1,2-diolate (DETA/NO; Axxora) has the longer in vitro half-life (>20 hours), but our pharmacokinetic experiments indicate that it has a short 2hour circulation half-life (data not shown); thus, only a fraction of the potential NO is released in vivo. We therefore selected DPTA/NO (Axxora), which has a half-life of 3 hours at 37°C[47] closely matching its circulatory half-life. Ten mice were injected i.p. with 2 mg of DPTA/NO as described previously. Pairs of animals were anesthetized and 500µL blood samples were obtained via retro-orbital plexus at 30min, 1 hour, 2 hours, 3 hours, and 4 hours after injection of DPTA/NO. Two mice were not injected with DPTA/NO and served as background controls. The blood samples were immediately placed on ice and were centrifuged at 500 x g for 20 min at 4ºC to pellet blood cells. The resultant plasma

(200μL) was then centrifuged for 40 min at 4ºC and 14,000xg in a Microcon 10kD centrifugal concentrator (Millipore, Billerica, MA) to separate the plasma proteins from the low-molecular weight DPTA/NO. The flow-through was analyzed spectrophotometrically at 252nm for DPTA/NO ($\varepsilon$=7640 $M^{-1}cm^{-1}$) using a μQuant Spectrophotometer (BioTek). The in vivo half-life of DPTA/NO results from two processes: (1) the decomposition of the NO donor to yield two NO molecules and one DPTA molecule and (2) the clearance of the DPTA/NO from the blood. We assume that both of these processes are governed by first order kinetics because both the in vivo and in vitro profiles of DPTA/NO are first order.

*Measurement of plasma hemoglobin levels***.** Blood was obtained into EDTA tubes (Becton Dickinson, San Diego, CA) from isofluorane anesthetized C57BL/6 mice via retro-orbital plexus. Blood samples were centrifuged at 500 x g for 20 min to pellet blood cells. Plasma samples (100μL) were analyzed for hemoglobin using the Winterbourn spectrophotometric method[48] on a μQuant Spectrophotometer (BioTek) or Cary 300 Spectrophotometer (Varian, Walnut Creek, CA). Methemoglobin levels were confirmed using full spectrum analysis and tracking the blue shift of the 414nm oxyHb peak.

**NO stock preparation**

PBS solution (pH=7.4, Invitrogen, Carlsbad, CA) was degassed under vacuum overnight and placed in-line with a 2 M NaOH solution (before) and a 1 M $KMnO_4$ solution (after). Pre-purified 4.8 Grade (99.998% pure) nitrogen was bubbled through the system for 30 min, followed by UHP NO (99.5% pure) (Airgas, San Diego, CA) for 5 min[49]. A saturated solution of NO has a concentration of 1.8mM at room temperature and 1 atm[49] which we verified using the amperimetric inno-T NO system (Innovative Instruments, Tampa Bay, FL). The NO probe was calibrated using two methods: the acid/iodide nitrite conversion, per manufacturers instructions, and the hemoglobin NO assay[49].

**Determination of plasma NO quenching**

A 1:30 dilution (~60μM) of the NO stock solution was prepared in degassed PBS within an anaerobic chamber filled with 4.8 grade nitrogen gas (<0.1% oxygen). Two mL of degassed PBS was placed in a reaction vessel, which was water-jacketed and maintained at 37ºC, as well as capped to eliminate the gas headspace. An amino-700 (Innovative Instruments) amperimetric NO electrode was inserted into the reaction chamber, followed by an injection of 10μL of the diluted NO stock, yielding a 200-600nM peak NO solution. The peak and decay of the NO solution were monitored using the inno-T NO measurment system (Innovative Instruments Inc.). NO quenching was assessed by determining the drop in NO concentration following the injection of 5μL of plasma. A standard curve was prepared using hemoglobin standards of known concentration. The Hb standards were generated using oxyHb purified by anion exchange chromatography and analyzed by microgel electrophoresis with fluorescence detection (>99% pure on Agilent Bioanalyzer 2100 Protein 200, Palo Alto, CA). Hemoglobin standard concentrations were also verified using the Winterbourn spectrophotometric assay (>99% oxyheme). The protein concentrations provided by the Bioanalyzer were similar to the values obtained from the Winterbourn spectrophotometric assay. We observed a linear correlation ($R^2$=0.999) between oxyheme levels and NO quenched by injection of 5μl of oxyhemoglobin standards (supplemental data). Given the dilution (1:400) of hemoglobin stock into the NO solution, our theoretical ratio is 2.5 nM of NO quenched per μM of stock oxyheme; this compares well with the calculated slope of the linear fit to our data of 2.43 nM/μM.

**Inactivation of plasma hemoglobin**

Plasma samples were treated with Hb inactivating potassium ferricyanide (1mg) plus potassium cyanide (1mg) or control KCl (1mg) for 15min, and then the salts were

removed by gel filtration. All chemicals were purchased from Sigma. The ferricyanide/ cyanide treatment chemically inactivates Hb by forming cyanomethemoglobin, preventing NO binding to the heme moiety of Hb. NO quenching was assessed before and after inactivation treatment.

**Arginine assessment**

Plasma samples (25µl) containing EDTA were added to an equal volume of NG–Methyl-L-Arginine internal standard (2.5 µM NMMA) and diluted to 1ml in water. All chemicals were purchased from Sigma. Eight point standards in the range of 1.25 to 150 µM were used for arginine and 0.05 to 6 µM for its derivatives Homoarginine, NG, NG –Dimethylarginine, NG, NG′-Dimethyl-L-Arginine and NG–Methyl-L-Arginine (NMMA; Sigma). Samples were extracted by solid phase and then derivatized with O-Phthaldialdehyde plus 2-Mercaptoethanol (Sigma) during injection. Arginine and its derivatives in each sample or standard were simultaneously separated on a Luna C18 analytical column (Phenomenex, Torrence, CA) using HPLC with fluorescence detection (Hewlitt Packard 1100 HPLC with 1046 programmable detector, Palo Alto, CA).

**Nitrite assessment**

Blood was collected via retro-orbital bleed in the presence of 2mM ethylenediaminetetraacetic acid (EDTA, Sigma) and 10mM N-ethylmaleimide (NEM, Sigma) and was kept on ice. Samples were centrifuged at 800×g and 4°C for 10 min. Erythrocytes were lysed in water containing EDTA (2mM) and NEM (10mM) (1:4 v/v) and the lysate was kept in the dark and on ice prior to analysis. Plasma and lysates were treated with methanol (1:4 v/v) to precipitate proteins, followed by centrifugation at 10,000×g and 4°C for 10 min. Samples were analyzed via HPLC (ENO-20 instrument, EiCom, Japan).

**Statistical analysis**

Analysis of variance (ANOVA) with the Statview™ program (SAS Institute) with Fischer post-hoc test was performed to statistically compare all measurements with a *P* value cut-off of 0.05. The mean and standard deviation of the results are reported in text and figures. The survival times are compared with non-parametric Logrank test with a *P* value cut-off of 0.05.

**Figure 6.1. Low rather than elevated NO bioavailability contributes to the genesis of ECM.** Cumulative survival (A) and time course of mean±SD *P. berghei* parasitemia (B) were assessed in groups (n=10) of eNOS- and iNOS-deficient mice and (n=5) C57BL/6 controls infected with *P. berghei*. Cumulative survival (C), time course of mean±SD *P. berghei* parasitemia (D), and mean±SD clinical score (gripping+righting reflex; E) in groups (n=5) of *P. berghei*-infected C57BL/6 mice treated twice daily with 1mg DPTA/NO or with saline. Dashed line represents the clinical score where animals are considered moribund and euthanized and the dotted line represents the clinical score of uninfected mice. Plasma concentration of DPTA/NO (F) followed over 4 hours in time (n=3) mice following i.p. injection of 2mg DPTA/NO. Superimposed on plasma concentration is the percentage decline in mean arterial blood pressure in a representative (n=4) uninfected mouse over 4 hours after injection of 1mg DPTA/NO (F). Mean ± SE levels of brain (G) and plasma (H) cGMP in uninfected (n=5) mice, and saline-injected (n=5), and DPTA/NO-injected mice (n=4) on day 6 of PbA infection. One NO donor treated mouse was an outlier; plasma from these groups were also analyzed by microsphere fluorescence assay (Figure 2B-D; supplemental data). Parasitemia was similar in both groups of mice on day 4 and 6 of ECM (saline: 20.9±3.3; NO donor: 21.1±3.6%, $P$=0.6), but only saline-injected mice were clinically ill (saline: 5.4±2.1; NO donor: 0.0±0.0; $P$=0.008). The means ± SD are reported in B, D, and E. The means ± SE are reported in G, H because replicate measurements were made for each mouse. **: $P$<0.005 for the comparison between NO donor-injected mice and saline controls.

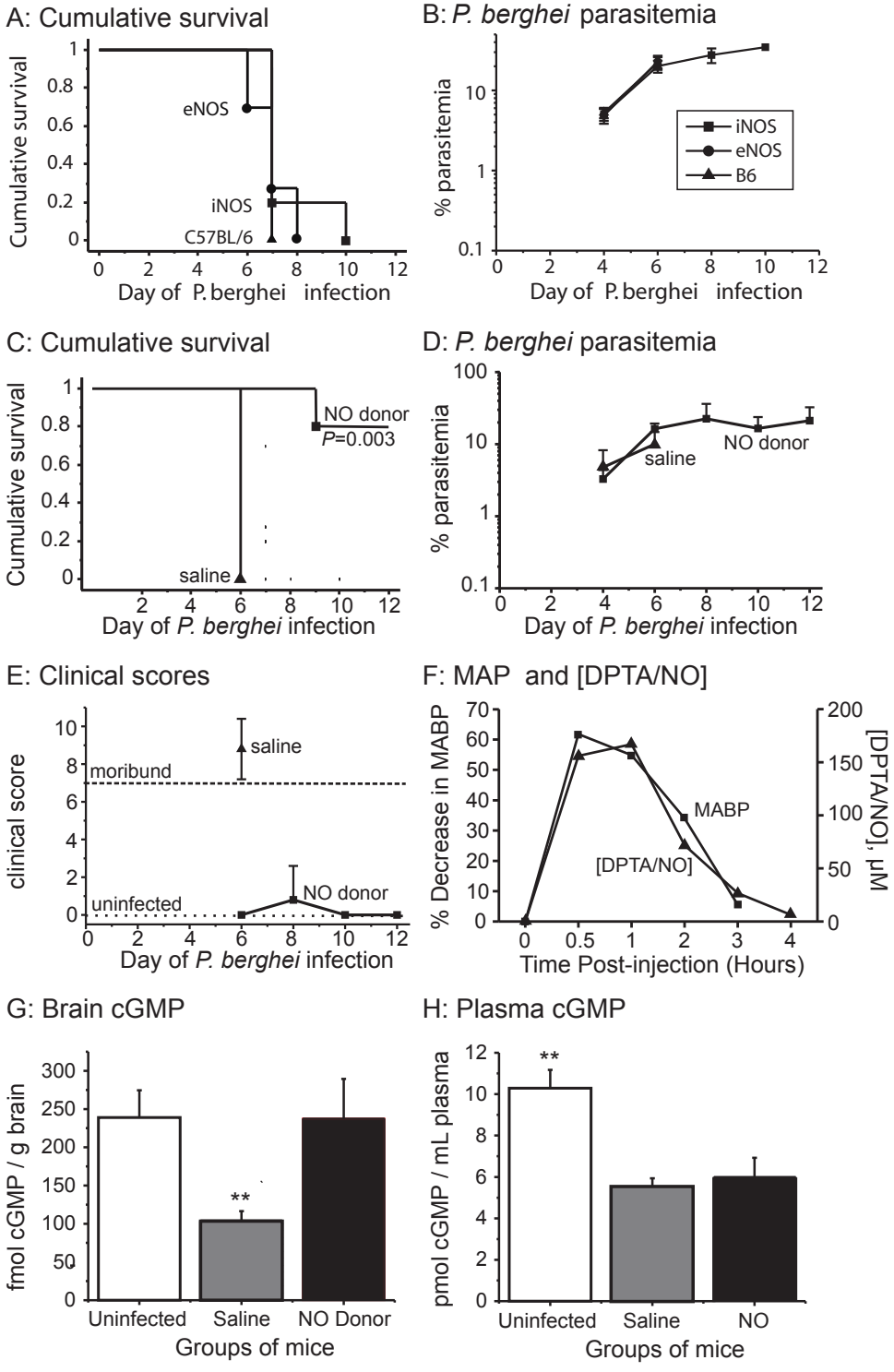

A: Cumulative survival
Cumulative survival
eNOS
iNOS
C57BL/6
Day of P. berghei infection
B: P. berghei parasitemia
% parasitemia
iNOS
eNOS
B6
Day of P. berghei infection
C: Cumulative survival
Cumulative survival
NO donor
P=0.003
saline
Day of P. berghei infection
D: P. berghei parasitemia
% parasitemia
saline
NO donor
Day of P. berghei infection
E: Clinical scores
clinical score
saline
moribund
uninfected
NO donor
Day of P. berghei infection
F: MAP and [DPTA/NO]
% Decrease in MABP
[DPTA/NO], uM
MABP
[DPTA/NO]
Time Post-injection (Hours)
G: Brain cGMP
fmol cGMP / g brain
**
Uninfected
Saline
NO Donor
Groups of mice
H: Plasma cGMP
pmol cGMP / mL plasma
**
Uninfected
Saline
NO
Groups of mice

**Figure 6.2. Administration of DPTA/NO donor inhibits the development of vascular leak in brain and hypotension during ECM, and decreases production of MMP-9 and certain inflammatory biomarkers in blood.** Groups (n=5) of mice were injected with $1x10^6$ PbA-infected RBCs and then treated with 1mg DPTA/NO or saline twice daily. All NO donor treated mice were clinically indistinguishable on day 6 of ECM from uninfected controls, whereas saline controls were moribund. The vascular leak (A) was assessed by measuring the amounts of Evans Blue dye extrusion into tissue. The levels of IL-18 (B), sCD40 (C), and MMP-9 (D) in the plasma of groups of mice in Figure 1G and H with one outlier mouse in NO donor group was analyzed by microsphere fluorescence assay. Mean arterial blood pressure was measured daily by a telemetric pressure transducer implanted in the carotid artery of PbA-infected mice (n=4) and the baseline MAP was measured each morning prior to injection of either NO donor or saline (E). A 3-4 hour transient decline in MAP was observed only in NO donor group and then the MAP returned to baseline. No clot was observed upon autopsy around the transducer of any of the mice in this experiment. *: $P<0.05$; **: $P<0.005$ for the comparison between NO donor-injected mice and saline controls. The means ± SE are reported in A-E because replicate measurements were made for each mouse.

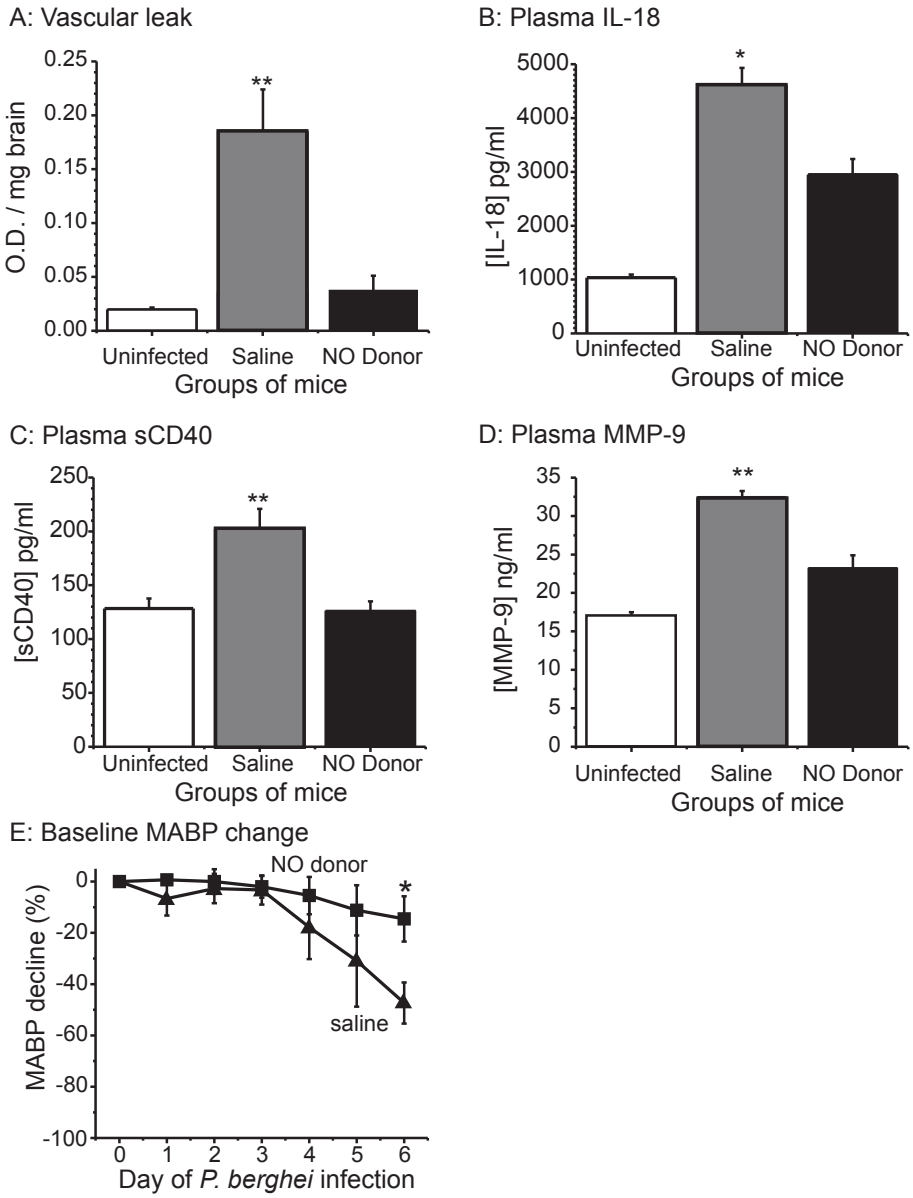
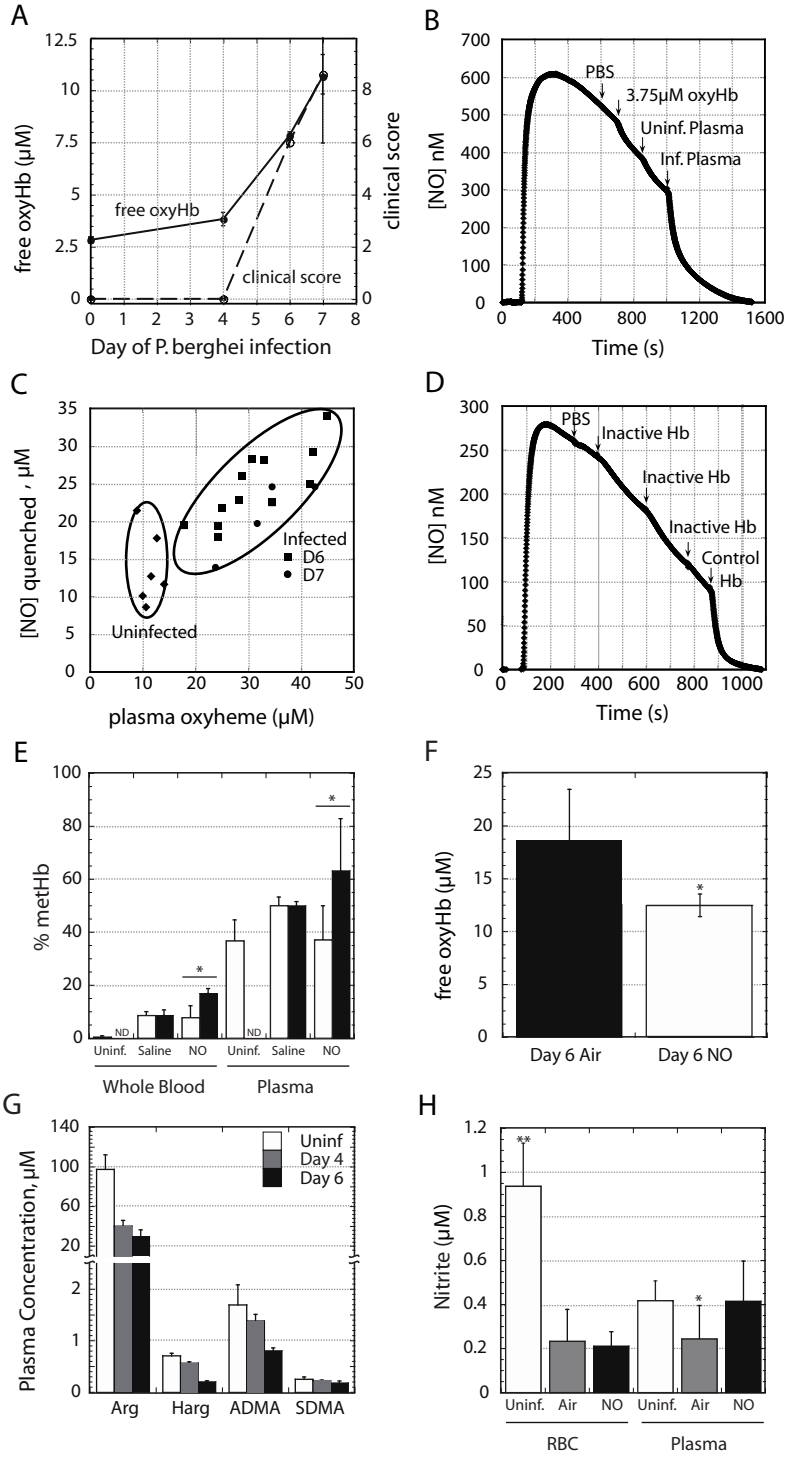

A: Vascular leak
O.D. / mg brain
0.25
0.20
0.15
0.10
0.05
0.00
**
Uninfected
Saline
NO Donor
Groups of mice
B: Plasma IL-18
[IL-18] pg/ml
5000
4000
3000
2000
1000
0
*
Uninfected
Saline
NO Donor
Groups of mice
C: Plasma sCD40
[sCD40] pg/ml
250
200
150
100
50
0
**
Uninfected
Saline
NO Donor
Groups of mice
D: Plasma MMP-9
[MMP-9] ng/ml
35
30
25
20
15
10
5
0
**
Uninfected
Saline
NO Donor
Groups of mice
E: Baseline MABP change
MABP decline (%)
0
-20
-40
-60
-80
-100
NO donor
saline
*
0
1
2
3
4
5
6
Day of P. berghei infection

**Figure 6.3**. **Free Hb in plasma and hypoargininemia contribute to low NO bioavailability during ECM.** A group of 21 (4 mice on day 4, 13 day 6, and 4 day 7) infected mice were analyzed for their levels of plasma hemoglobin on each day of infection as well as their clinical scores (A). The level of NO quenching assessed by the NO amperemeter was assessed for oxyHb standards prepared by anion exchange chromatography and verified by microgel electrophoresis with fluorescence detection and spectrophotometric analysis (supplemental data). Time course of NO levels in the reaction chamber within the anaerobic chamber; PBS: injection of degassed phosphate buffered saline; uninf: uninfected; inf: infected (B). Scatter plot of plasma NO scavenging versus plasma oxyheme concentration for plasma samples from uninfected controls (n=6) and *P. berghei* infected animals on day 6 and 7 of infection (C). Note, there are 4 heme moieities in each Hb molecule. One mouse with high free Hb (80μM) is not included in the scatterplot. Representative trace illustrating that inactivating plasma hemoglobin measured in E with cyanide/ferricyanide treatment abrogated the NO quenching ability of the sample ("Inactivated Hb") whereas KCl treatment had no detectable effect ("Control Hb"; D). The % metHb of total Hb in plasma and whole blood of groups of mice treated with NO donor or saline on day 6 of ECM (E). Control values (open bar) are prior to injection of DPTA/NO or saline and filled bars are 1.5hours after injection, which corresponds to peak production of NO (E). The levels of free oxyHb in groups (n=5) of NO gas treated and air controls on day 6 of PbA infection (F). The level of free Hb in a group (n=6) of ECM-resistant BALB/c mice on day 6 of ECM was 9.4±3μM. The amount of arginine and derivatives of arginine (G) in plasma of groups (n=5) of C57BL/6 on day 4 and 6 of infection. The clinical scores in C57BL/6 on day 6 of PbA infection were: 8.2±1.5. The parasitemia was 4.2±1.0% on day 4 and 14.5±2.6% on day 6 of infection. The amount of nitrite (H) in RBC and in plasma of groups (n=5) of NO gas-treated C57BL/6 mice and air controls on day 6 of infection. The clinical scores and parasitemia respectively were: NO gas-treated C57BL/6: 0±0 and 17.4±3.1%, and in air controls: 4.8±2.8 and 22.3±11.6%, ($P$=0.02 and $P$=0.4). ECM-resistant (n=3) BALB/c mice nitrite levels were: 0.57±0.08μM with clinical scores: 2.3±0.6 and parasitemia of 31.3±7.1%.


A
free oxyHb (μM)
clinical score
free oxyHb
clinical score
Day of P. berghei infection
B
[NO] nM
PBS
3.75μM oxyHb
Uninf. Plasma
Inf. Plasma
Time (s)
C
[NO] quenched , μM
Infected
D6
D7
Uninfected
plasma oxyheme (μM)
D
[NO] nM
PBS
Inactive Hb
Inactive Hb
Inactive Hb
Control
Hb
Time (s)
E
% metHb
ND
Uninf.
Saline
NO
Uninf.
Saline
NO
Whole Blood
Plasma
F
free oxyHb (μM)
Day 6 Air
Day 6 NO
G
Plasma Concentration, μM
Uninf
Day 4
Day 6
Arg
Harg
ADMA
SDMA
H
Nitrite (μM)
Uninf.
Air
NO
Uninf.
Air
NO
RBC
Plasma

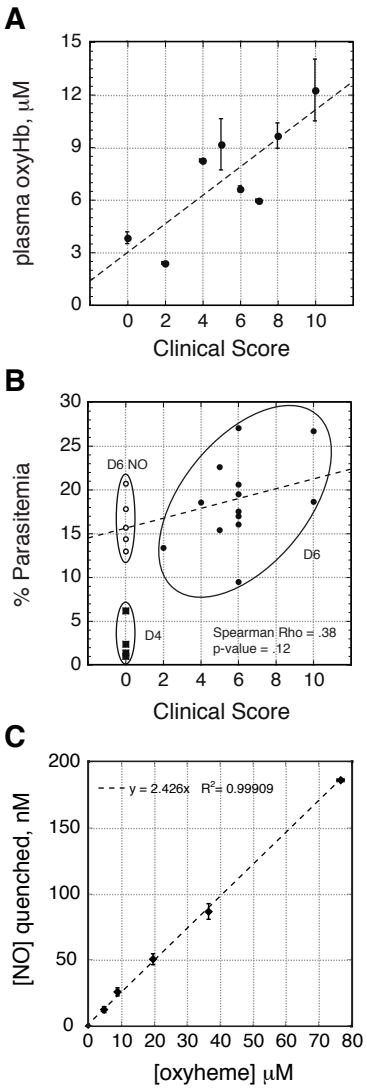
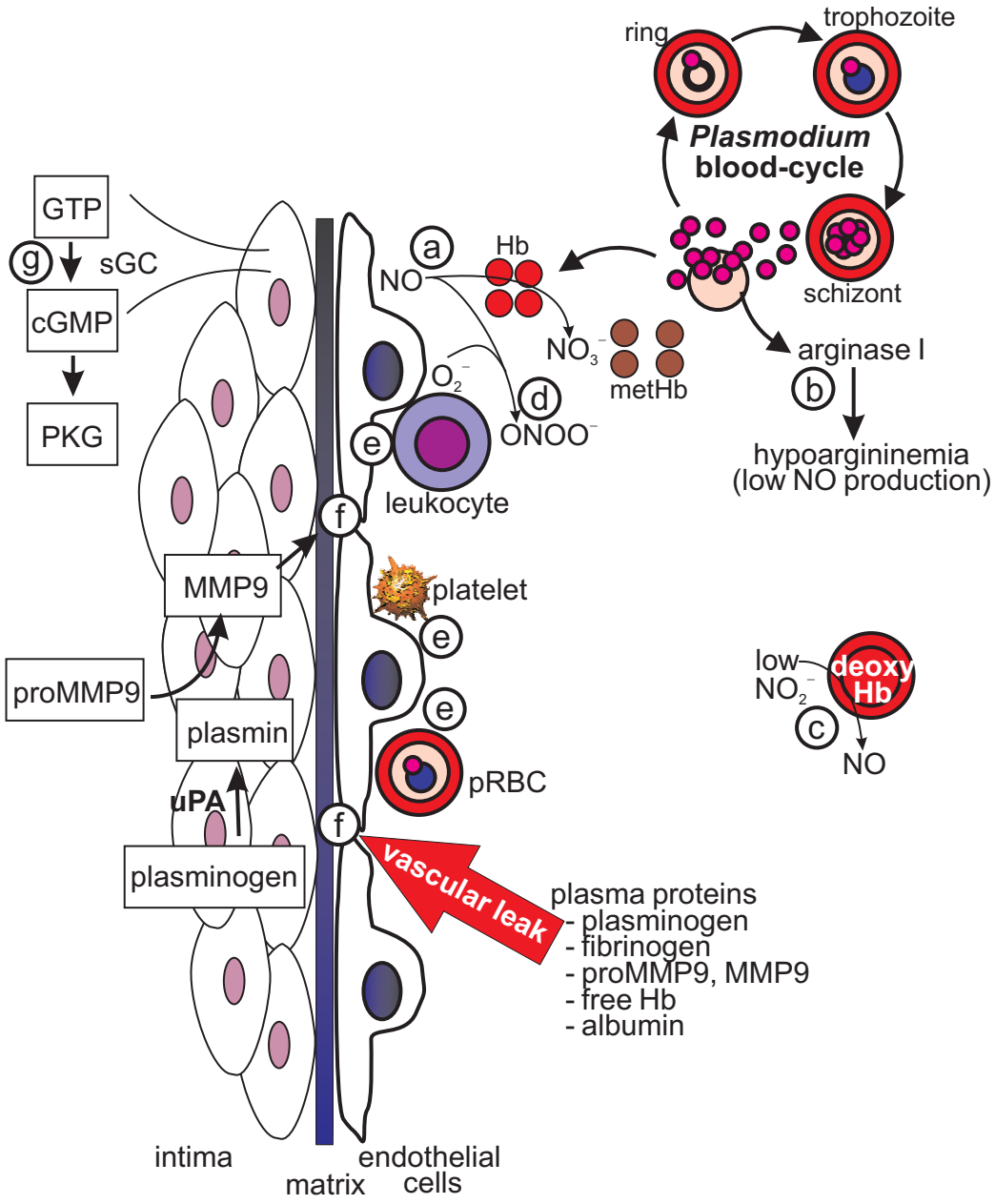



**Figure 6.3b. Supplemental Figure.** Free oxyHb levels of mice on day 4, 6, and 9 of PbA infection and their clinical score (A). The % PbA parasitemia on day 4, and 6 as well as in NO donor treated animals (B). Standard curve using purified oxyHb standards and the amount of NO quenched (C). Hb is reported as heme to obtain a 1:1 ratio.

**Figure 6.4: Model of how improving NO bioavailability through NO donor administration protects against the development of ECM.** As a consequence of parasite replication and non-specific lysis of uninfected RBCs, free Hb is released and scavenges NO produced by endothelial cells (a) and released RBC arginase I causes hypoargininemia and decreased NO production (b). Low nitrite levels result in minimal conversion by deoxyHb (c). Superoxide rapidly reacts with NO in a diffusion limited reaction to yield peroxynitrite (d). Clark's group and we (unpublished)[50] have observed that administration of pegylated-superoxide dismutase plus catalase significantly ($P<0.05$) protects against the development of ECM, suggesting superoxide too may limit NO bioavailability. Serirom et al.[38] report that decreased NO production by endothelial cells leads to increased endothelial cell adhesion molecules and *P. falciparum* adhesion. Thus, low NO bioavailability is likely to exacerbate the sequestration of pRBC to the activated endothelium, via adherence receptors P-selectin, ICAM-1, and CD36[20] (e). Subsequently, platelets activated during the course of ECM also adhere (e) via P-selectin, αIIbβ3, and ICAM-1[51,52]. Leukocyte adhesion (e) in the brain during ECM is ICAM-1 independent[53,54]. Once firmly attached, adherent cells (pRBC, platelets, leukocytes, and combinations thereof) may signal to promote the initial disruption of endothelial tight junctions. The lower levels of NO may also contribute to increased vascular permeability (f). The low NO bioavailability (a-d), prevents NO from signaling to smooth muscle cells to promote cGMP production and maintain vascular tone (g). The lack of bioavailable NO also results in an increase in activated intravascular MMP-9 produced via tPA (not shown), which may act to further damage the BBB. The BBB having been compromised is now permeable to many plasma proteins including plasminogen, tryptophan, free Hb, MMP9, and proMMP9. The tryptophan is processed via kynurenine pathway to excitatory and neurotoxic metabolites[55]. Once in the extravascular space, plasminogen can be cleaved by uPA to plasmin, which cleaves pro-MMP-9 to its active form, and MMP9 then cleaves the extracellular matrix further damaging the BBB (f). uPA and uPAR-deficient mice are protected from the genesis of ECM[56].

Protective effects: NO donor converts oxyHb to metHb (a), restoring NO homeostasis, and signaling through sGC (g). The increased concentration of fibrinogen elicited by the NO donor may inhibit both parasitized erythrocyte and platelet adhesion by disrupting these cells' interaction with ICAM-1 (e). This is supported by the observation that NO in vitro inhibits the expression of endothelial cell adhesion molecules and consequently the adhesion of *P. falciparum* parasitized RBCs under flow[38]. Cell adhesion molecules, such as ICAM1, P-selectin, and CD36) are important for the genesis of ECM[19,51,53,54,57]. Restoring NO homeostasis, and signaling and cell adhesion prevents disruption of BBB and less MMP9 is produced within the blood and interstitial fluids, thereby maintaining vessel function (f).


ring
trophozoite
Plasmodium
blood-cycle
schizont
GTP
g
sGC
cGMP
PKG
a
NO
Hb
NO3−
metHb
O2−
d
ONOO−
e
leukocyte
arginase I
b
hypoargininemia
(low NO production)
f
MMP9
platelet
e
proMMP9
plasmin
e
pRBC
uPA
f
plasminogen
vascular leak
plasma proteins
- plasminogen
- fibrinogen
- proMMP9, MMP9
- free Hb
- albumin
low
NO2−
deoxy
Hb
c
NO
intima
matrix
endothelial
cells

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# Chapter 7: Dynamics of hemoglobin release into plasma during experimental cerebral malaria.

**Abstract**

Free hemoglobin (Hb) is released into the blood when *Plasmodium* parasites rupture red blood cells (RBCs) to release progeny. Elevated levels of free Hb have been implicated in pathogenesis of malaria and other hemolytic disorders, due to quenching of nitric oxide (NO). Contrary to other disorders, free Hb levels during malaria are likely to be highly dynamic, because the parasites replicate in a synchronized fashion. We modeled free Hb levels using a one-compartment model of the blood, taking into account the parasite replication cycle, as well as Hb clearance. Our modeling predicts that Hb is released into the blood as a bolus spread over a few hours, during the time that the parasite completes its replication cycle, and is then cleared rapidly. We confirmed the modeling results experimentally in the *P. berghei* ANKA (PbA) experimental severe malaria (ESM) model in C57Bl/6 mice. On day-6 of infection, free Hb levels peaked in the middle of the night and proceeded to decay rapidly. These findings indicate that free Hb levels during malaria are dynamic, which is an important consideration in developing NO bioavailability restoring adjunct therapies.

**Introduction**

Recent research has focused on the role of cell-free hemoglobin (Hb) in the pathogenesis of human disease [1]. Cell-free Hb is approximately 600-fold more efficient at scavenging NO than Hb encapsulated in red blood cells (RBCs) [2] and its role as an NO scavenger has been implicated in both sickle cell disease [3], as well as malaria [4]. Further, cell-free Hb is also cytotoxic, due to its redox activity and ability to generate radicals [5].

During malaria, it is an unavoidable consequence of *Plasmodium* infection that

Hb is released into the plasma during parasite replication. Every 24-72 hours, depending on species, *Plasmodium* parasites undergo schizogony and rupture their host RBCs, releasing progeny merozoites, which infect subsequent RBCs. As a result, elevated levels of cell-free Hb have been reported in human patients infected with *P. falciparum* [6-8] and in the *P. berghei* ANKA (PbA) experimental severe malaria (ESM) mouse model [9]. The elevated cell-free Hb levels during ESM have been implicated in NO scavenging and restoration of NO bioavailability protected mice from developing disease [9]. This finding suggests that NO treatment may be able to function as adjunct therapy, which is important in light of the fact that over 20% of patients with severe falciparum malaria still die, even with effective anti-parasitic treatment [10]. Because Hb is released into the plasma periodically during malaria, when the *Plasmodium* parasite replicates, we predict that Hb levels are very dynamic. In order to maximize the efficacy of NO restoration therapy during malaria, the dynamics of cell-free hemoglobin must be well understood, such that the dosage and timing of treatment can be optimized.

## Methods

### Model of plasma Hb levels during ESM

We modeled the Hb dynamics in a C57BL/6 mouse infected with *P. berghei* ANKA. The blood was modeled as one compartment with Hb being released into the plasma as a result of RBC lysis due to parasite replication. Average values of mouse hematological parameters were obtained from the Mouse Phenome Database (The Jackson Laboratory, URL: http://www.jax.org/phenome). Erythrocyte rupture was assumed to be synchronous and was modeled as being Gaussian, with 80% occurring between 2AM and 6AM (CJ Janse, personal communication). Parasitemia was modeled as 5% on day-4 and 11% on day-6, based on typical values measured in out lab. Residual Hb following parasite replication was assumed to be 50% [11]. Hemoglobin clearance rates were

determined from the clearance rates of bolus Hb iv injections in dogs obtained by Monke and Yuile [12] and are in agreement with the observations of Frank et al in C57BL/6 mice [13]. Curve-fitting this data ($R^2$=.99) yielded second order clearance kinetics, with the rate constant, $k$ (units: $M^{-1}$ $hr^{-1}$), being a first order function of the dosage:

$$k = 3.78\times10^{3}\ e^{\,-1.24\times10^{3}\times heme0} \qquad (1)$$

where *heme0* is the initial value of the plasma heme concentration. During Hb release into the plasma, $k$ was computed using this expression and once Hb levels have peaked, the final value of $k$ was used. The overall expression for plasma heme was obtained via numerical quadrature using a Riemann sum. All computations were performed using Matlab (Mathworks).

**Infection and treatment of mice**

*Plasmodium berghei* ANKA was stored as a frozen stabilate and this stabilate was injected into source mice to generate an inoculum for the experiments. The C57BL/6 mice were obtained from Jackson Laboratories (Bar Harbor, ME) or bred at La Jolla Bioengineering Institute. All animals were housed in microisolator cages and provided food and water ad libitum.

To initiate the infection, experimental mice between 6 and 10 weeks of age were injected i.p. with $1\times10^{6}$ *P. berghei* ANKA parasitized erythrocytes obtained from a source mouse. Parasitemia was assessed by counting the number of parasitized erythrocytes among 200 and 1,000 erythrocytes in Giemsa-stained thin blood films. The Institutional Animal Use Committees of La Jolla Bioengineering Institute approved all procedures.

**Assessment of plasma heme**

Blood samples (~20μL) were collected during the night of day-5 to day-6 every two hours via saphenous vein bleed into microhematocrit tubes. Tubes were then centrifuged in a microhemtocrit centrifuge and plasma (4μL) was analyzed using Cary 300 spectrophotometer (Varian, Inc.) fitted with a TrayCell microcuvette (Hellma USA). Oxyheme concentration was determined using the Winterbourn method [14].

## Results

### Plasma Hb levels are dynamic during PbA infection

Our model predicts that Hb is released into the plasma over the course of ~4 hours, as a result of parasite replication, and is then rapidly cleared (Fig. 1A). As a result, cell-free Hb levels are very dynamic, with high concentrations transiently occurring every night, peaking at >150μM heme on day-6, and much lower levels being present during the day (Fig. 1B).

Analyzing blood samples every 2 hours during the early morning of day-6 of PbA infection confirmed the predictions of the model (Fig. 2). Plasma Hb levels were high in the early morning hours and decayed rapidly. The observed half-life of plasma Hb was 2.5 hrs ± .6 and the half-maximum time was 3.1 hrs ± .25.

Examining the blood smears taken at the same time as the blood samples indicates that ring forms begin to occur approximately concurrently with the peak of plasma Hb, confirming that the dynamics of plasma Hb are a result of parasite replication.

## Discussion

Our findings clarify the time-course of cell-free Hb during malaria. Rather than cell-free Hb levels being constant, or rising steadily with disease progression, Hb levels are predicted to be highly dynamic. Assuming synchronization of the *Plasmodium*

parasite life-cycle, Hb is released into the plasma rapidly during the rupture of the erythrocytes by the schizont stage and is then rapidly cleared. Overall, the experimental data confirm the predictions of our model. Peak Hb levels measured in the plasma and the day-6 time-courses (Fig. 2) were similar to the model predictions (Fig. 1B). The observed variation between animals suggests that while parasite replication is synchronous, there is intra-animal variation in the timing, even under laboratory conditions. Likewise, the observed half-life and half-maximum times being longer than the model prediction suggests that, in our hands, *P. berghei* is less synchronized than assumed. Further, the model assumes cell-free Hb is only produced due to parasite replication, but there are more complex, immune-system mediated mechanisms of RBC lysis, such as T-cell- and TNF-mediated cytotoxicity [15], which may also be important. Likewise, Hb clearance rates used were based on data obtained in healthy dogs and mice and, as a result, are dominated by renal clearance [12]. However, during malaria cell-free Hb is likely to be cleared by additional mechanisms, such as vascular leak [16], and renal failure is common in both ESM [17] and severe human malaria [18], which may result in altered Hb clearance.

An additional limitation of the model stems from the fact that the parasite digests Hb during the course of its lifecycle and converts the heme into ferric form, which is stored as hemozoin crystals [19]. As such, only a fraction of the Hb content of the RBC is available to be released into the plasma. By taking advantage of the difference in magnetic properties between Hb (ferrous iron) and hemozoin (ferric iron), Moore et al determined the Hb to hemozoin conversion to be 50% in RBCs infected with late-stage falciparum schizonts [11]. This is the value used in the model, however given that *P. berghei* has a 2-fold shorter life-cycle, as compared to *P. falciparum*, PbA-infected RBCs may have a greater amount of remaining Hb. However, mouse RBC are also approximately 50% smaller then human RBCs and contain about 50% less Hb, thus the percentage of Hb remaining may be similar.

These results have to be extrapolated with caution to the human severe malaria patients, but there are many similarities between severe falciparum malaria and the PbA ESM model [17], including sequestration, hemorrhage, and coma. Important differences remain, as *P. falciparum* has a 48-hour replication cycle and the degree of synchronicity, as well as parasite burden, can vary greatly from patient to patient [20]. However, recurrent fever and paroxysms, indicating synchronicity in parasite replication, are common in patients with severe falciparum malaria [18]. We would predict that a dramatic rise in fever, which is a clinical indicator schizogony [20], would also coincide with a transient increase in cell-free Hb due to RBC rupture. This increase in cell-free Hb would result in reduced NO bioavailability and an increase in oxidative stess and radical generation [4].

These findings have important implications for the potential use of NO as adjunct therapy, because cell-free Hb is a potent scavenger of NO. Timing NO treatments to avoid the peak Hb levels in the plasma may allow for a lower dose to be used, reducing the risk of transient hypotension and methemoglobinemia. However, it may also be therapeutic to provide NO when plasma Hb levels are high, in order to mitigate Hb toxicity and compensate for the increase in NO quenching. Alternately, it may be possible to combat the rise in cell-free Hb released during parasite replication by the administration of iron chelators during paroxysm. Existing iron chelation therapy for malaria has shown some promising, though inconclusive results [21], however the focus has been on iron chelators as anti-parasitic agents reducing iron availability to the parasite, rather than on cell-free Hb clearance. Iron chelation therapy could be developed to mitigate the coincident release of hemozoin into the blood. Although the iron moiety of hemozoin is in the ferric state (the same as methemoglobin) and has a several orders of magnitude lower affinity for NO than ferrous heme [22], hemozoin is known to induce proinflammatory events [23]. Finally, our results also suggest that anti-oxidant therapy [24] might be most effective if administered during paroxysm, in order to combat the oxidative effects

of cell-free Hb.

In conclusion, we present here modeling and experimental data suggesting that cell-free Hb levels are very dynamic during malaria. This finding is important, as it may provide an opportunity to optimize NO restoration or anti-oxidant therapy by adjusting its timing and it may lead to progress in developing effective iron chelation therapy.

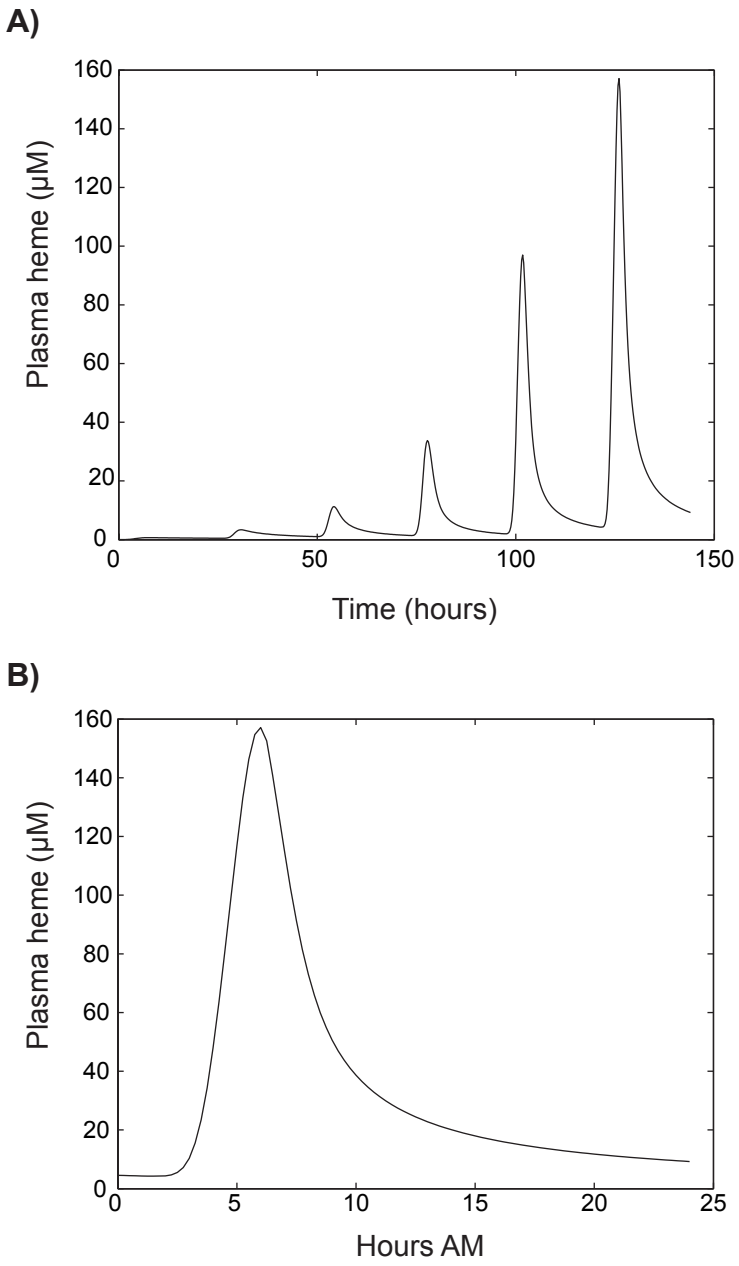


**Figure 7.1: Model predictions of Hb dynamics in plasma during ESM.** A) Time-course of plasma heme during ESM. B) Time-course of plasma heme on day-6 of ESM.

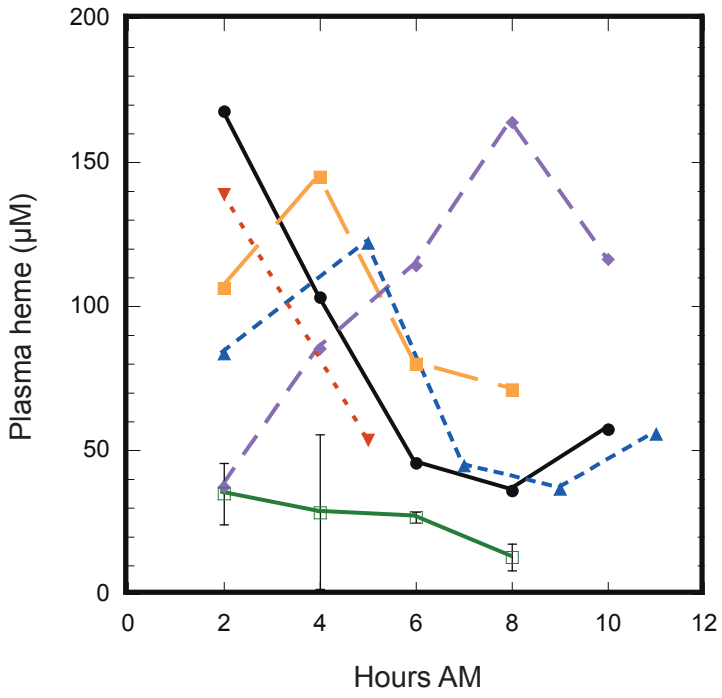


**Figure 7.2: Plasma oxyheme levels during the morning of day-6 of ESM.** Plasma oxyheme levels were tracked in 5 C57BL/6 PbA-infected mice during the morning of day-6. For comparison, bottom curve (open squares) shows time-course in uninfected controls (mean +/- SD, samples were collected during the day).

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# Chapter 8: Conclusions and future directions.

Under normal physiological conditions, hemoglobin (Hb) is able to fulfill its role as an oxygen carrying molecule in red blood cells (RBCs). Inside the RBC multiple metabolic and enzymatic pathways are dedicated to maintaining its redox state and protecting it from oxidation. Further, the simple encapsulation of Hb at high concentration within the RBC serves to minimize NO scavenging in the vasculature by a combination of intrinsic and external diffusional barriers. However, if cell-free Hb is present in the plasma, as a blood substitute or due to hemolysis, the protective mechanisms are not available. As a result, cell-free Hb can play a pathogenic role in hemolytic anemia. During malaria, Hb plays a dual role. Intracellular Hb reduces the possibility of NO, superoxide, and peroxynitrite acting upon the parasite and playing an anti-parasitic role. Meanwhile, the cell-free Hb released into the blood during parasite replication plays a pathogenic role by scavenging NO and contributing to a pro-oxidant state.

**Adjunct therapy for malaria**

The identification of cell-free Hb and low NO bioavailability as important elements in malarial pathogenesis naturally suggests the development of adjunct NO therapy focusing on ameliorating disease rather then parasite killing. Such therapies is particularly important in light of the fact that over 20% of patients with severe falciparum malaria still die, even with effective anti-parasitic treatment [1]. However, existing attempts at adjuvant therapy for severe malaria have all failed in clinical trials [2]. An additional concern is the development of drug resistance by *Plasmodium* parasites; at present the drug resistance has been reported to all existing anti-parasitic agents, other then artemisinin [3]. As a result, artemisinin combination therapy is now the preferred first-line treatment for malaria [4], however drug resistance to artemisinin is also becoming a serious concern [5].

The success of NO donor and NO gas treatments in protecting *P. berghei*-infected

mice from developing disease suggests that similar therapies may be beneficial in humans. It is important to keep in mind that with all NO therapies there exists the concern of methemoglobinemia. Malaria patients may be particularly vulnerable, because increased blood metHb levels have been observed in children with cerebral malaria [6] and mice infected with *P. yeolii* have been shown to have a compromised metHb reductase system [7]. However, the results of the modeling of Hb dynamics during malaria suggest two possible timing strategies for the optimization of NO therapy. First, NO may be adminsitered during paroxysm and peak cell-Hb release, in order to reduce NO consumption, by converting the cell-free Hb to metHb. Alternately, NO therapy may be administered several hours after paroxysm, when cell-free Hb levels are lower, in order to restore homeostatic NO signaling. Adjusting dosage and timing will be important in order to minimize the risk of methemoglobinemia, as well as the possibility of dangerous hypotension during NO therapy.

In addition to optimizing the timing of NO delivery, it will be important to consider the method of NO delivery. While the NO donor approach was successful in animal experiments, this approach faces some clinical limitations. Both sodium nitroprusside (SNP) or organic nitrates (eg. nitroglycerin) are used clinically as NO drugs to counter hypertension [8], however both of these drugs have serious limitations in the context of restoring NO bioavailability during hemolytic anemia. Sodium nitroprusside is limited by efficiency and toxicity issues: it requires enzymatic action for maximal efficiency and releases free iron and thiocyanate [9]. As a result, one would anticipate large doses would be required for sufficient intravascular activity during malaria and the iron and thiocyanate released could serve to aggravate oxidative stress and methemoglobinemia during malaria. Likewise, organic nitrates require enzymatic processes to enable their pharmacological activity and NO release [8], thus their ability to function in the vasculature to counter the effects of cell-free Hb may be limited. A new class of drugs, the

diazeniumdiolates (also known as NONOates), has shown promise [10] and includes the NO donor DPTA/NO used in the *P. berghei* animal studies. NONOates are commercially available with a range of *in vitro* decomposition and NO release rates, however they are predominantly small molecules and are rapidly cleared from the circulation. Further, the many of the decomposition products that are produced following NO release are toxic and/or carcinogenic [11], limiting these drugs to *in vitro* and short-term research studies. However, the NONOate chemistry is very flexible and new drugs are being developed to circumvent current limitations [11]. A diazeniumdiolate-derivitized albumin [12], for example, may be promising in treating malaria, as it would have a long circulating life-time and could provide sustained NO bioavailability with reduced toxicity. On the other hand, a NONOate that releases NO rapidly could be administered during paroxysm to inactivate cell-free Hb. Ultimately, a combination of new NO releasing drugs may be necessary to optimize NO as an adjunct treatment for malaria.

NO inhalation therapy may provide a simpler alternative to NO donor drugs; it is used clinically in the treatment of acute respiratory distress and has shown promise in treating pulmonary hypertension [13]. In fact, while there remain questions as to its systemic effects, NO inhalation has already shown promise in the treatment of sickle cell disease and is undergoing additional clinical trials [14, 15]. A high dose of inhaled NO (80ppm, for 1.5 hours) was therapeutic in the treatment of sickle cell anemia, by converting the cell-free Hb to metHb, thereby reducing NO consumption by plasma [14]. Similarly, in the treatment of malaria, NO inhalation could be used to deliver NO during hemolysis, in order to convert the cell-free Hb to metHb and reduce NO consumption. However, given the higher levels of cell-free Hb present during malaria as compared to sickle-cell anemia, this approach may require an even higher NO dose then that used in sickle cell anemia, increasing the risk of methemoglobinemia. Alternatively, the studies with NO inhalation during ESM suggest that administering NO while avoiding peak

cell-free Hb levels, thus restoring the homeostatic NO signaling, can also be beneficial. This approach may allow for a lower NO dosage to be used, reducing the possibility of patients developing methemoglobinemia. In order for NO inhalation to be a successful adjunct therapy for malaria, it will be necessary to determine the ideal mode of action (conversion of cell-free Hb to metHb vs. NO restoration) and optimize NO inhalation dosage and duration.

Molecules that act in similar fashion to or in synergy with NO, such as phosphodiesterase (PDE) inhibitors may also hold potential as alternatives to or amplifiers of NO therapy. This has been a very active area of research over the past 10 years, with a PubMed search for "phosphodiesterase inhibition" returning >8,000 hits, however as of yet, none of this research has been translated to malaria. Phosphodiesterases are a class of enzymes that hydrolyze cyclic GMP (cGMP) and cyclic AMP (cAMP), which are important secondary messengers [16]. The development of selective PDE inhibitors has led to new therapies for a host of vascular and inflammatory disorders, including heart failure, asthma, and erectile dysfunction [17]. Because cGMP is produced as a result of NO binding to soluble guanylate cyclase (sGC), cGMP-PDEs are candidates for inhibition as an adjunct therapy during malaria. Particularly promising may be PDE5, which is highly expressed in the lung, heart, platelets, and brain [16]. This research direction is further motivated by the success of NO therapy in *P. berghei*-infected mice and the fact that cGMP levels in the brain are reduced on day-6 of infection and restored by NO therapy. Inhibitors of cGMP-PDE (eg. sildenafil, a PDE5 inhibitor) have the potential to potentiate or amplify the effect of NO by preserving cGMP, the secondary messenger of NO. However, because cGMP-PDE inhibition still requires some bioavailable NO to bind to sGC in the first place, during malaria it may be ideally suited as a combination therapy with NO restoration therapy. Such a combination may allow for lower NO dosage to be used, particularly if timed to avoid peak hemoglobin levels, reducing the risk of methemoglo-

binemia. While cGMP-PDEs are particularly attractive in light of the NO bioavailabilty studies during malaria, other PDEs (e.g. PDE2, PDE3, PDE4, PDE7), which are cAMP specific (though PDE2 and PDE3 also have an affinity for cGMP) have also been targeted in the treatment of cardiovascular, central nervous system, and inflammatory disorders [17] and may also be potential targets in malaria. This rapidly expanding field of research has been unexplored in the context of malaria and provides great potential for the development of an adjunct therapy.

Due to the involvement of cell-free Hb, as well as the profound inflammation, in the pathogenesis of malaria a combination of therapies may be necessary. For example, iron chelation and anti-oxidant therapy could be aimed at ameliorating the adverse effects of the redox reactions of cell-free Hb [18] and delivered to coincide with paroxysm. Meanwhile, NO therapies, including both direct NO restoration via NO donor or NO gas, as well as PDE inhibition, would be administered during the day, as to avoid peak cell-free Hb levels. Finally, recent research effort has gone into derivatizing various drugs, including the PDE5 inhibitor sildenafil, in order for them to also be NO donors [9]. Such multi-functional or combination therapies may provide the best therapeutic effect, given the complex pathology of malaria, while minimizing the risks of side effects. Clearly, despite past failures [2], numerous opportunities are available for the development of a successful adjunct therapy for the treatment of severe malaria. Further, given the common vascular complications of many hemolytic disorders [19], the development of novel therapies aimed at ameliorating the toxic effects of cell-free Hb and restoring NO bioavailability is likely to have a broad impact beyond malaria.

**The role of heme oxygenese during malaria**

Based on the observations of high cell-free Hb levels in malaria patients and in the *P. berghei* mouse model, it is natural examine the role of heme oxygenase (HO), the

enzyme responsible for degrading heme into iron, carbon monoxide (CO), and biliverdin [20]. Given the toxic nature of free heme, it is not surprising that several mechanisms exist for the catabolism of heme [21]. The HO enzyme family is composed of an inducible isotype, HO-1, as well as two constitutive isotypes, HO-2 and HO-3. The HO-2 enxyme is the primary consitutive mechanism of heme degradation and is expressed in most tissues, but it is particularly abundant in the liver and the brain. HO-1, on the other hand, has a ten-times greater enzymatic activity, is constitutively expressed in the spleen, and can be induced not only by heme, but also by a myriad of other inducers, including heavy metals, oxidative stress, and cytokines. The important protective role of HO-1 in response to heme has been studied in the kidney and it appears to play an anti-inflammatory role in various inflammation models. Furthermore, it has been proposed that HO is not only important because it catabolizes toxic heme, but also because it produces CO, which can act as a NO analog. By this mechanism, HO-1 induction is suggested to be particularly important in protecting the endothelium from heme-induced injury, particularly by counter-acting the up-regulation of adhesion molecules. [20]

Given the observations that endothelial dysfunction and cell-free Hb both occur during malaria and other hemolytic disorders, HO is likely to play a role in these disorders. For example, in a transgenic mouse model of sickle cell disease it has been observed that inhibiting HO-1 serves to exacerbate the disease, suggesting that HO-1 plays a protective role in this hemolytic anemia [22]. Furthermore, it has been suggested that HO-1 may play a complex role in malaria [23], given that in addition to heme release, which induces HO-1, malaria is characterized by fever, hypoxia, and cytokine release, which can serve to repress HO-1. A recent study examining the (GT)n repeat in the promotor of the HO-1 gene suggests that the presence of the short allele (<28 repeats, corresponding to increased transcription) was associated with cerebral malaria [24]. The conflict between this observation and the apparent protective role of HO-1 in sickle cell and other inflam-

matory diseases suggests the need for mechanistic studies in malaria and other hemolytic anemia, aimed at determining the involvement of HO-1 in protection or pathogenesis. In particular it would be important to assess HO-1 expression during malaria and the role of HO-1 inhibition in pathogenesis of severe disease. Additionally, it may be important to examine the role of constitutive HO, such as HO-2, and CO in hemolytic anemia, including malaria. The presence of cell-free Hb is likely to result in increased activity of these constitutive HO enzymes, in addition to inducing HO-1. Recent evidence suggests that CO produced by HO-2 in the brain can bind to the heme moiety of nitric oxide synthase (NOS), thereby inhibiting NO production [25]. This effect could explain the potential deleterious effect of increased HO-1 expression in malaria, as the resulting increase in CO production could serve to inhibit NO production, further reducing NO bioavailability. Thus, in order to fully understand the ramifications of cell-free Hb during malaria and other hemolytic diseases, it will be important to measure CO production as an index of HO acitivty and due to its complex role as an NO analog and potential NOS inhibitor. Finally, comparing the levels of cell-free Hb, HO expression, CO production, and the effects of HO inhibitors in different hemolytic disorders may clarify whether there is a threshold above which the effects of HO become deleterious rather than beneficial. This would allow for the development of tailored iron chelation or HO inhibition therapies, which could be used to reduce or counter any pathogenic role of HO. Alternately, it may be possible to promote and/or maintain HO expression in the treatment of hemolytic disorders where it plays a beneficial role.

**Conclusion**

The study of malaria in the context of hemolytic anemia, taking into account the toxic nature of cell-free Hb and its potency as an NO scavenger, may to lead to the development of new adjunct therapies. From a clinical point of view, the greatest and/or

most rapid impact may be achieved by translating existing therapies, such as NO inhalation or PDE inhibition, to malaria. However, more research is also necessary to determine the role of heme detoxification in malarial pathogenesis, as well as the precise mode of action of NO therapies. Additionally, any progress made in the understanding of these mechanisms in the context of malaria is also likely to be applicable in the broader context of hemolytic anemia.

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