Inhaled Nitric Oxide and Cerebral Malaria: Basis of a Strategy for Buying Time for Pharmacotherapy

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Abstract: There are approximately 225–600 million new malaria infections worldwide annually, with severe and cerebral malaria representing major causes of death internationally. The role of nitric oxide (NO) in the host response in cerebral malaria continues to be elucidated, with numerous known functions relating to the cytokine, endovascular and cellular responses to infection with *Plasmodium falciparum*. Evidence from diverse modes of inquiry suggests NO to be critical in modulating the immune response and promoting survival in patients with cerebral malaria. This line of investigation has culminated in the approval of 2 phase II randomized prospective clinical trials in Uganda studying the use of inhaled NO as adjuvant therapy in children with severe malaria. The strategy underlying both trials is to use the sytemic antiinflammatory properties of inhaled NO to "buy time" for chemical antiparasite therapy to lower the parasite load. This article reviews the nexus of malaria and NO biology with a primary focus on cerebral malaria in humans.

Key Words: inhaled nitric oxide, cerebral malaria, nitric oxide synthase, *Plasmodium falciparum*

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Plasmodium falciparum has coevolved with *Homo sapiens* and its predecessors for at least 70,000 years. Nitric oxide (NO), originally considered as solely an environmental toxin, was discovered to be a physiologically critical signaling molecule in 1988. Subsequently, NO was studied, among an enormous amount of worldwide research, as an inhaled gas with eventual approval in the United States for the treatment of term neonates with persistent pulmonary hypertension. Over the past 2 decades, a growing number of studies have examined the role of endogenous NO production in the pathophysiology of malarial disease in humans, particularly severe and cerebral malaria (CM).[1]

These efforts have generated controversy regarding the role of NO in malaria. Initial findings were interpreted to suggest that high NO levels contributed to the development of CM and severe anemia, but tended to be retrospective chemical pathology correlations. More recent work has indicated that NO is, in fact, likely to be critical in allowing survival in patients with severe and cerebral malaria. The debate has culminated in the approval of 2 phase II randomized prospective clinical trials in Uganda studying the use of inhaled NO (iNO) as adjuvant therapy in children with severe malaria. The strategy underlying both trials is to use the antiinflammatory properties of iNO in the lung and systemic circulation to "buy time" for chemical antiparasite therapy to lower the parasite load. This article reviews the nexus of malaria and NO biology with a primary focus on CM in humans.

MALARIA

There are approximately 225–600 million new malaria infections worldwide each year.[2] Severe malaria is defined by the World Health Organization as vital organ dysfunction and/or high parasite burden in the setting of demonstrated parasitemia.[3] *P. falciparum* is the organism primarily responsible for severe malaria.[4] Common perturbations in severe malaria include anemia, metabolic acidosis, hypoglycemia, respiratory failure and CM.[4] CM is typically defined as coma in the setting of parasitemia in the absence of other causes of reduced consciousness, such as other central nervous system infections, hypoglycemia, a postictal state or ongoing seizure.[4] As is true with severe malaria, definitions of CM must balance sensitivity and specificity in clinical and research contexts. In locations such as sub-Saharan Africa in which incidental parasitemia is common and the resources necessary to definitively rule out other causes of altered mental status are often limited, reliable diagnosis of CM is difficult.[5] Severe and cerebral malaria tend to occur in young children who have not yet developed effective immunity or in adult travelers from nonendemic regions. The overall fatality rate for patients with CM who seek medical attention is roughly 10–40%[4] and remains roughly 9% among children who receive the most effective and rapidly acting therapy, intravenous artesunate.[6] Children who survive CM have an approximately 1 in 10 chance of suffering long-term neurologic sequelae[4] and a 1 in 4 chance of persistent cognitive impairment.[7]

NITRIC OXIDE

NO is a free radical with protean effects in the human body. Initially described in the vasculature as the endothelium-derived relaxing factor, it is an important signaling molecule in diverse processes including neurotransmission, immune system and cytokine modulation, platelet inhibition, vascular homeostasis and regulation of hematopoiesis. Endogenous production occurs via 3 NO synthase (NOS) isoform enzymes: neuronal NOS (nNOS or NOS1), inducible NOS (iNOS or NOS2) and endothelial NOS (eNOS or NOS3).[8] Exogenous delivery by inhalation has been used therapeutically since the 1990s for a growing number of indications.[9] NO exists as a gas at physiologic temperature and pressure and has a half-life estimated to be under 2 ms in whole blood.[10]

L-arginine is converted into NO and L-citrulline by NOS1, NOS2 and NOS3. NOS1 and NOS3 are constitutively expressed and are calcium/calmodulin-dependent,[8] whereas NOS2 is only expressed when its transcription is activated.[8]

NO has many sites of action. Made in the endothelium, it diffuses into subjacent smooth muscle cells where it activates soluble guanylate cyclase, increases intracellular levels of cyclic

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guanosine monophosphate and, thereby, lowers the intracellular calcium concentration causing vasorelaxation. In the vascular lumen, NO inhibits platelet aggregation, also through a cyclic guanosine monophosphate–dependent mechanism, and has numerous effects on endothelial adhesion molecule expression and endothelial homeostasis. Two other mechanisms by which NO exerts intracellular effects are through activation of adenylate cyclase and inhibition of phospholipase C, both effected via adenosine diphosphate ribosylation.[11] Soluble guanylate cyclase activation by NO in bone marrow–derived cells, principally monocytes and other leukocytes, is also a critical pathway.[12] Of less importance, a minority of the NO intracellular signaling effect is due to posttranslational S-nitrosylation of cysteine thiols on target proteins.[11]

Initial use of iNO was directed at pulmonary effects rather than systemic. More recent work has demonstrated systemic effects of iNO, initially in animals, such as reducing cardiac ischemia-reperfusion injury in mice.[12] In humans, evidence of systemic effects of iNO has been seen in hepatic ischemia-reperfusion injury.[13] As in CM, low NO bioavailability in hepatic ischemia mediates hepatic ischemia-reperfusion injury via cytokine disregulation, increased leukocyte adhesion and decreased endothelial function with increased microcirculatory tone.[13] Among patients undergoing orthotopic liver transplantation, the use of iNO at 80 ppm was associated with more rapid restoration of liver function posttransplant, decreased hospital length of stay and a 75% reduction in hepatocellular apoptosis.[13]

Catabolism of NO takes place through multiple pathways. NO reacts with oxyhemoglobin to produce methemoglobin and nitrates, and also reacts with the superoxide anion to create peroxynitrite.[11] Additionally, NO is degraded enzymatically by peroxidases.[11] Nitrates and nitrites, typically denoted NOx, are frequently measured as the breakdown products of NO.

Numerous challenges exist in the reliable and physiologically relevant study of NO. Because of its short half-life and propensity for diffusion, NO tends to exert effects near the site of production. It is difficult, however, to measure NO levels directly at the site of action in vivo. Additionally, nitrate and nitrite levels in plasma or other body fluids, such as cerebrospinal fluid are influenced not only by NO production, but also by diet, renal function and the array of catabolic pathways available to NO. Debate continues regarding the relevance of transport in vivo of NO and its potential for distant effects, including transport bound to hemoglobin or thiol residues.[14]

Multiple pathways lead to low NO bioavailability in malaria infection, including hemolysis with release of NO-scavenging cell-free oxyhemoglobin and arginase,[15] as well as inflammatory production of superoxide, which reacts with NO to produce peroxynitrite. Anstey et al[16] demonstrated an inverse relationship between disease severity and monocyte NOS2 activity in the presence of high levels of interleukin (IL)-10. Concurrently, however, tumor necrosis factor (TNF) and interferon-gamma induce NOS2 mRNA expression in bone marrow–derived cells.[17]

PATHOPHYSIOLOGY OF CEREBRAL MALARIA

The mechanisms by which *P. falciparum* infection leads to altered mental status and coma are not completely understood, although both disruption of microcirculatory flow and the host immune response appear to be central processes. Although initial attention was drawn to parasite sequestration within the cerebral vasculature and the resulting microcirculatory flow reduction, this explanation is no longer considered sufficient. Now under investigation are the consequences of the exuberant cytokine response.

SEQUESTRATION

The term sequestration describes the tendency of parasitized erythrocytes to adhere to the endothelium as well as to other parasitized erythrocytes (agglutination), uninfected erythrocytes (rosetting) or platelets (platelet-mediated clumping), a finding that is particular to *falciparum* malaria among the human malaria species.[1] The coma of CM can resolve, with permanent neurologic sequelae seen in only 10% of pediatric patients who survive and persistent measurable cognitive deficits seen in one quarter,[4,7,18] which are lower rates than would be expected after ischemic injury to the brain. Additionally, the extent and location of sequestration found on autopsy does not correspond with high fidelity to the severity of clinical malaria.[19]

CYTOKINE RESPONSE

Recent study of the pathophysiology of CM has focused on the cytokine and inflammatory response to the parasite and the interaction between the immune system and the endothelium. The balance between proinflammatory signals (TNF, interferon-gamma, IL-1 and IL-12) and immunomodulatory signals (IL-10, NO) is of particular importance.[20] Disease severity correlates with TNF and IL-1 concentrations independent of degree of parasitemia.[21] Furthermore, patients with a TNF promoter mutation associated with elevated TNF activity are at increased risk for mortality or serious neurologic sequelae.[22] NO is known to inhibit cytochrome C oxidation via competition with oxygen and could theoretically inhibit the neuronal respiratory cycle in the setting of low partial pressure of oxygen.[23] Angiopoietin-1 (Ang-1) depletion and Angiopoietin-2 (Ang-2) elevation have been investigated as diagnostic and prognostic markers in severe and cerebral malaria. Ang-1 and Ang-2 regulate endothelial activation. Ang-1 is produced by endothelial cells constitutively and appears to maintain endothelial homeostasis via activation of the Tie-2 receptor.[24] Ang-1 levels fall during inflammation and endothelial activation, as is seen in sepsis or severe *falciparum* malaria.[24] Ang-2 is stored in endothelial Weibel–Palade bodies from where it is released, displaces Ang-1 from the Tie-2 receptor and propagates endothelial activation, including through adhesion molecule expression and sensitization of the endothelium to TNF.[24] Weibel–Palade bodies exocytosis is inhibited by NO, limiting the release of Ang-2.[25] Additionally, erythrocyte cytoadherence is reduced in vitro by NO via inhibition of intercellular adhesion molecule 1 and vascular cell adhesion molecule 1 expression.[26]

INTRACRANIAL HYPERTENSION

Some authors have additionally posited a central role of increased intracranial pressure in the pathophysiology of mental status changes and death in CM. Possible mechanisms of intracranial hypertension development include increased intracranial blood volume, decreased adenosine triphosphate availability leading to adenosine triphosphate–dependent ion pump failure and extracellular fluid shift and blood–brain barrier breakdown.[1]

Intracranial hypertension is undoubtedly responsible for some patients' neurologic deficits and deaths, and in fact papilledema and other findings of intracranial hypertension are significantly associated with poor outcome.[27] Evidence has not supported a critical effect of elevated intracranial pressure in all patients, however. Cerebral edema is commonly seen on autopsy, although studies have failed to show evidence of herniation regularly in patients with CM who have died.[28] Similarly, opening pressure on lumbar puncture and low cerebral perfusion pressure have not been shown to be associated with mortality.[29]

NITRIC OXIDE IN CEREBRAL MALARIA

Accumulating evidence suggests a protective role for NO in severe and cerebral malaria attributed to NO's modulating the inflammatory response.

HYPOARGININEMIA

L-arginine concentrations fall in the settings of inflammation, hemolysis and malaria infection, and L-arginine levels vary inversely with malaria disease severity. It is hypothesized that the low bioavailability of arginine for NO synthesis contributes to pathogenesis. L-arginine levels are low in patients with CM, moderately low in patients with uncomplicated malaria and normal in healthy controls.[30] Hypoargininemia caused by arginase release from hemolyzed erythrocytes is significantly associated with fatal outcome in patients with CM.[30] Exogenous L-arginine rescues reactive hyperemia-peripheral artery tonometry, a noninvasive measurement of vascular NO production, in patients with severe malaria.[31] Asymmetric dimethylarginine levels are higher in patients with severe malaria than in those with moderately severe malaria and each micromolar increase of this endogenous inhibitor of NOS conversion of L-arginine to NO is associated with an 18-fold increase in mortality.[32]

MURINE CEREBRAL MALARIA

Mice provide a reliable, although imperfect, model of CM.[4] Mice deficient in NOS2 or NOS3 have equivalent courses of CM as do controls when infected with *Plasmodium berghei* ANKA, and delivery of an exogenous NO donor to NOS-deficient mice prevents CM and reduces the inflammatory response.[33] Similarly, CM has been reported in a NOS2-deficient murine model with pharmaceutical NOS antagonism, suggesting that NO is not necessary for CM.[34] Exogenous NO as compared with saline placebo in *Plasmodium berghei* ANKA–infected mice leads to improved pial blood flow, fewer hemorrhagic foci and less leukocyte adherence.[35] Most recently, iNO was shown to reduce cerebral erythrocyte sequestration, endothelial activation and systemic inflammation as well as improve survival in *Plasmodium berghei* ANKA–infected mice.[36] Taken together, these findings demonstrate that NO is not necessary for the development of CM and that restoring NO bioavailability reduces the inflammatory response and improves clinical status.

POPULATION AND GENETIC STUDIES

A number of early studies found a positive correlation between NOx in the blood, cerebrospinal fluid or urine and disease severity, yet many of these studies did not control for dietary intake or renal function or compare results with healthy controls within the same population.[37–39] Later studies have demonstrated lower levels of NOS2 activity, NO and NOx in children with severe malaria compared with those with mild malaria or asymptomatic parasitemia, suggesting that higher NOS2 activity and NO production protect children against severe malaria.[16,40,41] Although it does appear that people in malaria-endemic regions have higher NOS2 expression at baseline and that low NOS2 levels may be associated with severe malaria, the differences in and weaknesses of methodology preclude definitive conclusions as to whether NOS2 expression is correlated with disease severity.

Genetic studies have investigated whether haplotypes resulting in higher or lower levels of NO production lead to variable susceptibility to severe forms of malaria. The gene for NOS2 is NOS2A. Attention has been focused on single nucleotide polymorphisms (SNPs) in NOS2A, G954C and C1173T, as well as a microsatellite pentanucleotide repeat, CCTTT(n).[42] Additional SNPs and promoter site mutations continue to be identified.

The NOS2 promoter SNP at site G954C in the promoter region was first discovered in 1998 and found to be associated with less severe malaria. This polymorphism was originally described as the Lambarene mutation at site 969, but was subsequently referred to as the 954 mutation by the same group.[43] This SNP is more prevalent among malaria-exposed populations than among controls.[44] Ex vivo studies demonstrated a 7-fold higher NOS2 activity level in patients with this mutation,[43] and in vivo studies demonstrated higher NO production in response to 15N-arginine infusion.[45] G954C has also been shown to be protective against infection with malaria[46] and associated with high NOx levels and lower symptomology in malaria infection.[47] Not all studies have corroborated these findings, however.[47–49] An additional NOS2 SNP, C1173T, is associated with less symptomatic malaria and increased fasting urine and plasma NOx.[50]

Large diversity in the CCTTT(n) repeat number is observed, and although shorter alleles were initially associated with worse malaria symptoms and CM death, more recent findings demonstrate increased disease severity with greater allele length[51,52] or no correlation between length and disease severity,[42,50] suggesting a complex relationship between the microsatellite repeat and disease outcome.

NO SYNTHASE 1 AND NO SYNTHASE 3

NOS1 and NOS3 have received less attention than NOS2 because they are constitutively expressed, although some evidence supports cytokine recruitment of NOS1 in viral encephalitis.[53] Yet, although significant enrollment of these enzymes in *P. falciparum* infection has not yet been demonstrated, variation in activity may be relevant. Two NOS1 SNPs are associated with altered NOS1 expression, G84A and C276T. The former is located in the promoter region of NOS1 and is associated with decreased basal activity[54] and higher risk for CM.[55] NOS3 mutations are correlated with protection against CM as well as plasma NOx levels.[56,57]

Collectively, these findings provide strong biochemical evidence for a protective role of NO in CM via immune response modulation as well as robust, although not invariably positive, epidemiologic and genetic support for this effect. Coupled with this elucidation of the relationship between NO and *Plasmodium* infection is an increasing availability of iNO therapy worldwide. Although engineering challenges remain in the reliable production and transportation of NO to resource-limited settings, iNO can be produced cheaply via electrical and chemical mechanisms, and methods of production and delivery suited to the developing world are under active investigation.[58,59]

CONCLUSION

Millennia of evolutionary pressure exerted by malaria have helped to shape the human organism. The role of NO in the host's response to infection has become a focus of intense investigation over the past 20 years as the interactions between NO, malaria, the immune system and the endothelium become increasingly understood. The weight of evidence suggests a protective role of NO produced by inducible NOS in severe and cerebral malaria. Demonstration of NO's immunomodulatory role as well as measurement of NOx levels, NOS promoter mutations and disease severity have provided compelling evidence that NO is essential in preventing or moderating the severe manifestations of *P. falciparum* infection. After more than 70,000 years of hominid–*Plasmodium* coevolution, the time has come to test directly whether inhaled NO can give infected children the time they need to survive cerebral malaria.

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ERRATUM

Trends in Drug Resistance Prevalence in HIV-1–infected Children in Madrid: 1993 to 2010 Analysis: ERRATUM

In the article that appeared on page e213 of volume 31, issue 11, the figure legends were incorrect for Figures 1 and 2. The figures and legends are reprinted below correctly.

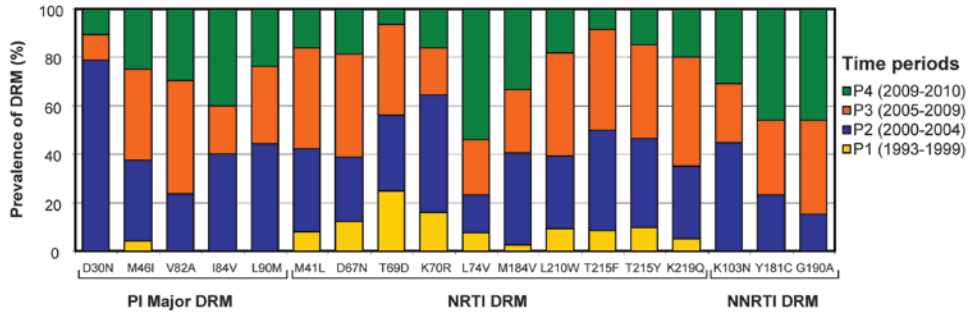

FIGURE 1. Prevalence of DRM positions for each drug class represented over 5% in the selected population.

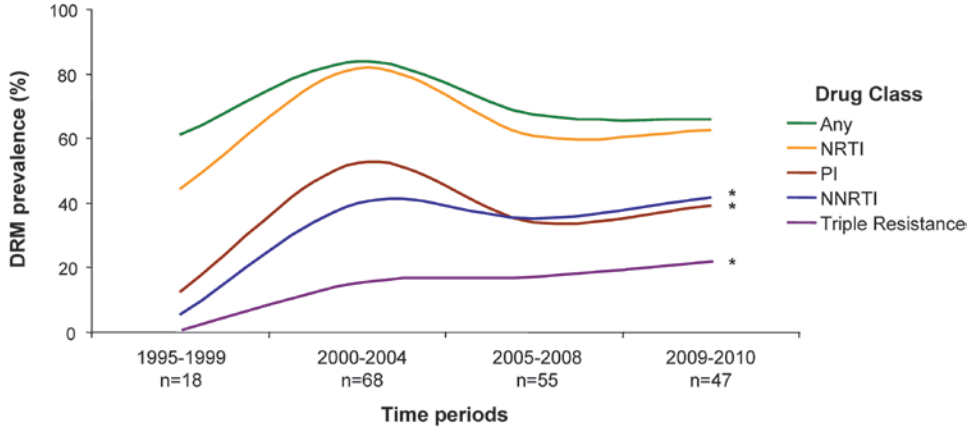

FIGURE 2. Temporal trends of DRM prevalence according to drug class. PI indicates protease inhibitors; RT, reverse transcriptase; NRTI, nucleoside reverse transcriptase inhibitors; NNRTI, nonnucleoside reverse transcriptase inhibitors. Asterisk indicates statistical differences between P1 (1993–1999) and P4 (2008–2010).

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de Mulder M, Yebra G, Navas A, et al. Trends in Drug Resistance Prevalence in HIV-1–infected Children in Madrid: 1993 to 2010 *Analysis. Pediatr Infect Dis* 2012;31:e213–Je221