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Low nitric oxide bioavailability contributes to the genesis of experimental cerebral malaria

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The role of nitric oxide (NO) in the genesis of cerebral malaria is controversial. Most investigators propose that the unfortunate consequence of the high concentrations of NO produced to kill the parasite is the development of cerebral malaria. Here we have tested this high NO bioavailability hypothesis in the setting of experimental cerebral malaria (ECM), but find instead that low NO bioavailability contributes to the genesis of ECM. Specifically, mice deficient in vascular NO synthase showed parasitemia and mortality similar to that observed in control mice. Exogenous NO did not affect parasitemia but provided marked protection against ECM; in fact, mice treated with exogenous NO were clinically indistinguishable from uninfected mice at a stage when control infected mice were moribund. Administration of exogenous NO restored NO-mediated signaling in the brain, decreased proinflammatory biomarkers in the blood, and markedly reduced vascular leak and petechial hemorrhage into the brain. Low NO bioavailability in the vasculature during ECM was caused in part by an increase in NO-scavenging free hemoglobin in the blood, by hypoargininemia, and by low blood and erythrocyte nitrite concentrations. Exogenous NO inactivated NO-scavenging free hemoglobin in the plasma and restored nitrite to concentrations observed in uninfected mice. We therefore conclude that low rather than high NO bioavailability contributes to the genesis of ECM.

Most investigators in the malaria field propose that NO produced by immune cells controls *Plasmodium* replication[1,2], but that an unfortunate side-effect of increased NO production is development of the disease[3]. However, experimental evidence supporting this high NO bioavailability is lacking. Key predictions of the high NO bioavailability hypothesis are, first, that the lack of vascular NO synthase (NOS) should ameliorate ECM; second, that exogenous NO administration should exacerbate ECM; and third, that the *in vivo* footprints of NO bioavailability should increase. To test these predictions, we used mice infected with *Plasmodium berghei* ANKA (PbA), a well-recognized model of human cerebral malaria in which, similar to humans, mice show neurological dysfunction, acute respiratory distress with lactic acidosis, and nephritis[4,5]. The advantages and disadvantages of this mouse model using a species of *Plasmodium* that is distinct from *P. falciparum* have been reviewed elsewhere[5]. The pathogenic mechanisms leading to human cerebral malaria are controversial but are likely to involve a complex interaction including sequestration of parasitized erythrocytes in the brain, inflammation, and dysregulation of the hemostasis system[6]. Indeed, ECM mice show a marked proinflammatory response, profound thrombocytopenia, hypotension, vascular leak and petechial hemorrhaging in the brain[5] — impairments that are observed in humans with cerebral malaria.

There was no significant difference in survival or parasitemia between mice deficient in inducible NOS (iNOS) or endothelial NOS (eNOS) and C57BL/6 controls ($n = 10$ per group; **Fig. 1a**,**b**), indicating that an increase in vascular NO production is not required for ECM. To determine the effect of high NO, we injected PbA-infected mice twice daily with an NO donor (1 mg of dipropylenetriamine NONOate; DPTA/NO) or saline vehicle. Because the parasite completes its life cycle every 24 h, it was continually replicating during the experiment. However, parasitemia in NO donor–injected mice was similar to that in saline-injected controls (**Fig. 1c**), indicating that NO had no effect on parasite replication or viability. All five saline-injected mice were moribund on day 6 after infection, whereas only one of the five NO donor–treated mice was moribund on day 9 after infection (**Fig. 1d**). The difference between the two groups was marked: on day 6 of infection, the NO donor–treated mice were clinically indistinguishable from uninfected control mice, whereas the saline-treated group were moribund (**Fig. 1e**). Similar results were obtained in a replicate experiment and in two experiments in which mice were treated with NO gas (40 ± 5 ppm, data not shown).

The maximum concentration of DPTA/NO in plasma was achieved after 1 h, and the compound was cleared within 4 h of injection. The decay of the compound was well described by a single exponential function (τ = half-time of decay = 1.6 ± 0.3 h). From the first-order kinetics of decomposition and clearance, we estimate that a 1-mg injection of DPTA/NO results in a cumulative production of 100 μM NO. The pharmacokinetics of DPTA/NO correlated well with the kinetics of the 61% decline in mean arterial blood pressure (MAP) elicited by DPTA/NO injection (**Fig. 1f**). Although this dose caused a marked decline in MAP, none of mice treated showed any clinical signs or

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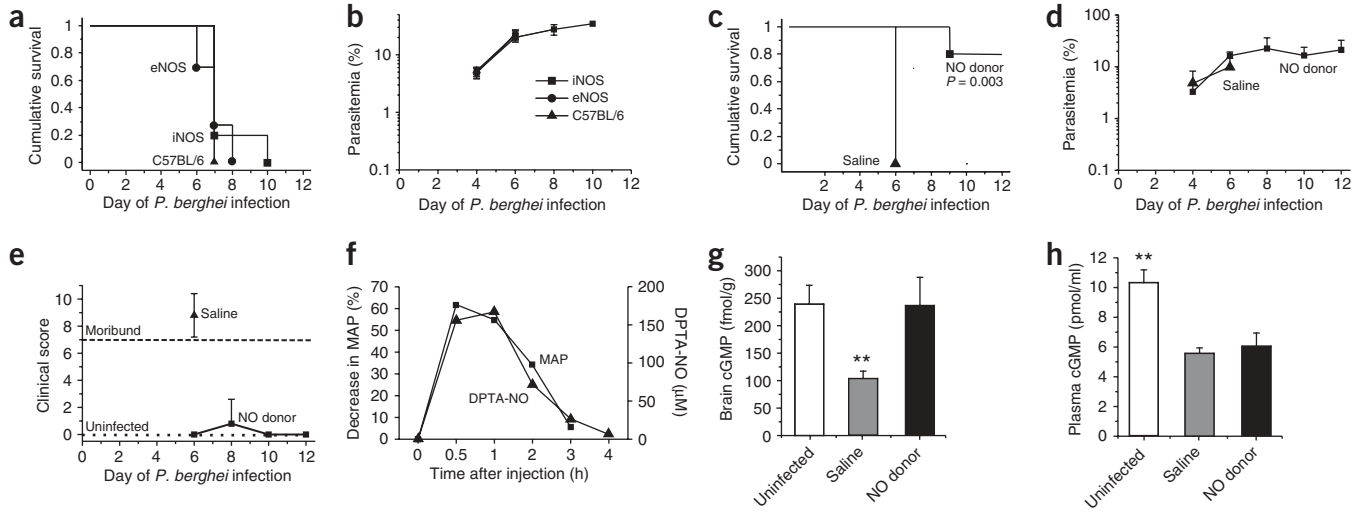

Figure 1 Low rather than increased NO bioavailability contributes to ECM. (**a**,**b**) Cumulative survival (**a**) and time course of *P. berghei* parasitemia (**b**) were assessed in eNOS- or iNOS-deficient mice ($n = 10$ per group) and C57BL/6 controls ($n = 5$) infected with *P. berghei*. (**c**–**e**) Cumulative survival (**c**), time course of *P. berghei* parasitemia (**d**) and clinical score (gripping plus righting reflex; (**e**) in *P. berghei*–infected C57BL/6 mice treated twice daily with either 1 mg of DPTA/NO or saline ($n = 5$ per group). Dashed line represents the clinical score when mice are considered moribund and killed; dotted line represents the clinical score of uninfected mice. (**f**) Plasma concentration of DPTA/NO over 4 h after i.p. injection of 2 mg of DPTA/NO in mice ($n = 3$). Superimposed on plasma concentration is the percentage decline in MAP in a representative uninfected mouse over 4 h after injection of 1 mg of DPTA/NO ($n = 4$). (**g**,**h**) Brain (**g**) and plasma (**h**) cGMP in uninfected ($n = 5$), saline-injected ($n = 5$) and DPTA/NO-injected ($n = 4$) mice on day 6 of PbA infection. One NO donor–treated mouse was an outlier; plasma from these mice was also analyzed by microsphere fluorescence assay (**Fig. 2b–d** and **Supplementary Table 1**). Parasitemia was similar in both groups of mice on days 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; $P = 0.008$). Data are the mean ± s.d. (**b**,**d**,**e**) or the mean ± s.e.m. (**g**,**h**). $^{**}P < 0.005$ for indicated group versus both other groups. Error bars in **h** represent s.e.m.

appeared in cardiac distress, and no mice died as a result of the treatment.

To determine whether NO-dependent signaling through soluble guanylate cyclase (sGC) was decreased during ECM and whether NO donor administration restored cGMP levels, we used enzyme-linked immunoassay to assess the concentration of cGMP in brain and plasma on day 6 of ECM in mice 4 h after injection with NO donor or saline ($n = 5$), a time point 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 had significantly less cGMP in the brain as compared with uninfected controls or NO donor–treated mice with no clinical signs of ECM ($P < 0.05$; **Fig. 1g**). Plasma cGMP was significantly lower both in ECM-susceptible saline-injected mice and in ECM-protected, NO donor–treated mice as compared with uninfected controls ($P < 0.005$; **Fig. 1h**).

We observed significantly lower extravasation of Evans blue/albumin (a measure of vascular leak) into the brains of DPTA/NO treated mice as compared with infected saline-injected controls on day 6 of ECM ($n = 5$ per group; $P < 0.005$; **Fig. 2a**). In fact, the amount of

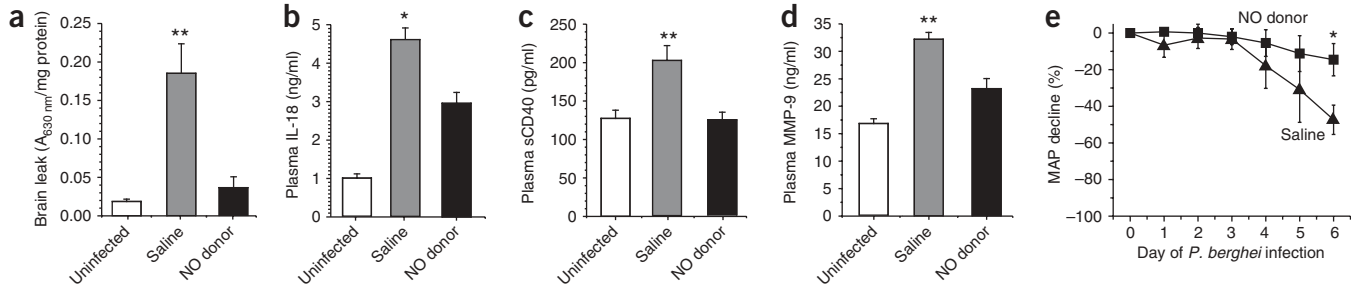

Figure 2 Administration of DPTA/NO donor inhibits vascular leak in brain and hypotension during ECM, and decreases MMP-9 and inflammatory biomarkers in blood. Mice were injected with 1×10^6 PbA-infected RBCs and then treated with 1 mg of DPTA/NO or saline twice daily ($n = 5$ per group). On day 6 of ECM, all NO donor–treated mice were clinically indistinguishable from uninfected controls, whereas saline-injected controls were moribund. (**a**) Vascular leak was assessed by measuring Evans blue dye extrusion into tissue. (**b**–**d**) Plasma IL-18 (**b**), sCD40 (**c**) and MMP-9 (**d**) in the mice in **Figure 1g,h** were analyzed by microsphere fluorescence assay. (**e**) MAP was measured daily by a telemetric pressure transducer implanted in the carotid artery of PbA-infected mice and baseline MAP was measured each morning before the injection of either NO donor or saline ($n = 4$ per group). A 3–4-h transient decline in MAP was observed only in the NO donor group and the MAP then returned to baseline. No clot was observed on autopsy around the transducer of any of the mice in this experiment. $^{*}P < 0.05$, $^{**}P < 0.005$ for NO donor–treated mice versus saline-injected controls. Data are the mean ± s.e.m. in **a**–**e** (replicate measurements were made for each mouse).

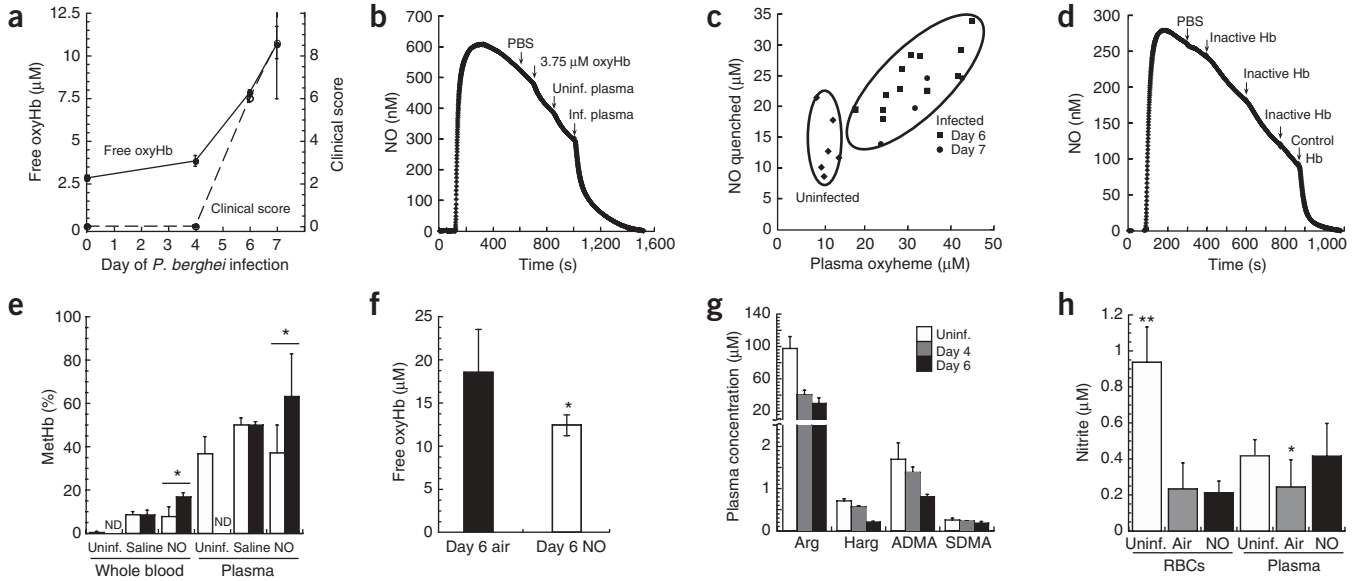

Figure 3 Free hemoglobin in plasma and hypoargininemia contribute to low NO bioavailability during ECM. (**a**) Twenty-one infected mice were analyzed (4 on day 4, 13 on day 6, and 4 on day 7) for plasma hemoglobin and assessed for clinical score. NO quenching assessed by the NO amperemeter was calibrated with oxyhemoglobin (oxyHb) standards prepared by anion exchange chromatography and verified by microgel electrophoresis with fluorescence detection and spectrophotometric analysis (**Supplementary Fig. 1**). (**b**) Time course of NO in the reaction chamber within the anaerobic chamber. PBS, injection of degassed PBS; uninf, injection of uninfected sample; inf, injection of infected sample. (**c**) Scatter plot of plasma NO-scavenging versus plasma oxyheme concentrations in plasma samples from uninfected controls and *P. berghei*–infected mice on days 6 and 7 of infection ($n = 6$ per group). Note that there are four heme moieties in each hemoglobin (Hb) molecule. One mouse with high free Hb (80 μM) is not included in the scatter plot. (**d**) Representative trace showing that inactivating plasma hemoglobin with cyanide/ferricyanide treatment abrogated the NO quenching ability of the sample ('Inactivated Hb'), whereas potassium chloride treatment had no detectable effect ('Control Hb'). (**e**) Percentage of metHb of total Hb in plasma and whole blood of groups of mice treated with NO donor or saline on day 6 of ECM. Open bars are control values before injection of DPTA/NO or saline; filled bars are values 1.5 h after injection, corresponding to the peak production of NO. ND, not done. (**f**) Free oxyHb in NO gas–treated and air controls on day 6 of PbA infection ($n = 5$ per group). $^*P < 0.04$. The free Hb in ECM-resistant BALB/c mice on day 6 of ECM was 9.4 ± 3 μM ($n = 6$). (**g**) Amount of arginine and derivatives of arginine in plasma of C57BL/6 mice on days 4 and 6 of infection ($n = 5$ per group). Harg, homoarginine. ADMA, N^G,N^G-dimethylarginine. SDMA, N^G,N^G-dimethyl-L-arginine. The clinical score in C57BL/6 mice on day 6 of PbA infection was 8.2 ± 1.5; the parasitemia was 4.2 ± 1.0% on day 4, and 14.5 ± 2.6% on day 6 of infection. (h) Nitrite in RBCs and plasma of NO gas-treated C57BL/6 mice and air controls on day 6 of infection ($n = 5$ per group). The clinical score and parasitemia were, respectively, 0 ± 0 and 17.4 ± 3.1% (NO gas–treated C57BL/6) and 4.8 ± 2.8 and 22.3 ± 11.6% (air controls; $P = 0.02$ and $P = 0.4$). $^{**}P < 0.005$ uninfected versus air or NO in RBCs. $^*P < 0.05$ air versus uninfected or NO in plasma. ECM-resistant BALB/c mice had a nitrite concentration of 0.57 ± 0.08 μM, a clinical score of 2.3 ± 0.6, and parasitemia of 31.3 ± 7.1% ($n = 3$).

Evans blue in the brain of NO donor–treated mice on day 6 of PbA was similar to that in 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, but none was detected in the NO donor–treated group or uninfected controls. The exogenous NO had no detectable effect on thrombocytopenia during ECM (data not shown).

Interleukin-18 (IL-18; **Fig. 2b**), a proinflammatory cytokine, and sCD40 (**Fig. 2c**), an inflammatory marker, were significantly decreased in plasma of NO donor–treated mice on day 6 of ECM as compared with saline-treated controls ($P < 0.005$), suggesting that the NO donor provided some amelioration of inflammation in these mice. Other cytokines were at or below the limit of detection (IL-2, IL-4, IL-5, IL-12 p70 and TNF), or were similar (IL-1β, IL-6, IL-10 and IFN-γ) in the two groups of mice on day 6 of ECM (**Supplementary Table 1** online), indicating that not all pathways were affected equally by NO donor treatment. 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 two molecules determines whether MMP-9 can affect endothelial barrier integrity. MMP-9 was significantly decreased in NO donor–treated mice on day 6 of ECM as compared with saline-injected controls ($P < 0.005$; **Fig. 2d**), whereas TIMP-1 concentrations were similar in the two groups (**Supplementary Table 1**).

The vehicle control C57BL/6 mice ($n = 4$) developed hypotension, starting on day 4, which progressively worsened on day 6 (**Fig. 2e**). By contrast, the NO donor–treated mice maintained their baseline MAP and on day 6 of PbA were significantly protected from the development of hypotension ($P = 0.03$).

Possible factors leading to decreased NO bioavailability during ECM might include an increase in NO-scavenging free hemoglobin, hypoargininemia, or low concentrations of nitrite, which is converted by deoxyhemoglobin to NO[7–10]. We found that plasma from C57BL/6 mice on day 6 of infection contained significantly higher concentrations of oxyhemoglobin as compared with day 0 ($P < 0.005$; **Fig. 3a**). The increase in free hemoglobin correlated with disease severity, but parasitemia correlated poorly when NO donor–treated mice were included because they showed parasitemia similar to that of the controls but had no disease (**Supplementary Figure 1** online). 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 as compared with plasma from an uninfected control (**Fig. 3b**). The free hemoglobin concentrations in the plasma of infected mice on day 6 or 7 of infection and uninfected controls correlated with NO quenching by plasma from these mice ($R = 0.85$,

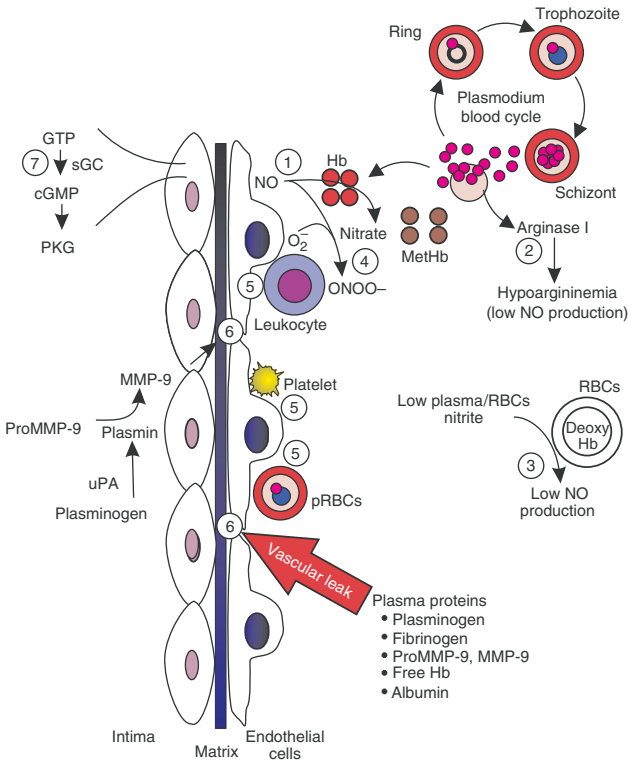

Figure 4 Improving NO bioavailability through NO donor administration protects against the development of ECM. (**1**) As a consequence of parasite replication and non-specific lysis of uninfected RBCs, free Hb is released and scavenges NO produced by endothelial cells. (**2**) Released RBC arginase I causes hypoargininemia and may decrease NO production. (**3**) Low nitrite results in minimal production of NO from nitrite by deoxyHb. (**4**) Superoxide rapidly reacts with NO in a diffusion-limited reaction to yield peroxynitrite. (**5**,**6**) Decreased NO bioavailability leads to an increase in endothelial cell-adhesion molecules[19] and consequently an increase in parasitized RBC (pRBCs), platelet and leukocyte adhesion[28,29] (**5**), and possibly vascular leak (**6**). Firmly attached, adherent cells (pRBCs, platelets, leukocytes and combinations thereof) may signal to promote the initial disruption of endothelial tight junctions. (**7**) Low NO bioavailability prevents NO from signaling to smooth muscle cells to promote cGMP production and increases intravascular MMP-9 produced through tissue plasminogen activator (tPA, not shown), which may further damage the blood-brain barrier (BBB). Once comprised, the BBB is permeable to many plasma proteins including plasminogen, tryptophan, free Hb, MMP-9 and proMMP-9. Tryptophan is processed through the kynurenine pathway to excitatory and neurotoxic metabolites[30]. Once in the extravascular space, plasminogen can be cleaved by urokinase plasminogen activator (uPA) to plasmin, which cleaves proMMP-9 to its active form; MMP-9 then cleaves the extracellular matrix further damaging the BBB. The protective effects of an NO donor are mediated by its conversion of oxyHb to metHb (**1**), restoring NO homeostasis and signaling through sGC (**7**). This restoration, together with effects on cell adhesion and decreased MMP-9 production, prevents disruption of the BBB and maintains vessel function (**6**).

$P < 0.005$); NO quenching in infected mice was significantly increased in infected mice versus controls ($P = 0.005$; **Fig. 3c**). Inactivation of free hemoglobin of infected plasma with cyanide/ferricyanide significantly reduced NO quenching of plasma, almost eliminating it ($n = 3$; $P < 0.005$; **Fig. 3d**). By contrast, there was no significant change in NO quenching caused by the control potassium chloride treatment of plasma ($n = 3$) and untreated plasma.

During NO donor treatment (1.5 h after injection with 1 mg of DPTA/NO), plasma showed a significant conversion of free oxyhemoglobin to methemoglobin in PbA-infected and uninfected groups of mice ($P < 0.05$; **Fig. 3e**). The baseline level of free methemoglobin before NO administration was higher in the infected group than in the uninfected group, indicating increased conversion of oxyhemoglobin to methemoglobin during infection. Similar increases in methemoglobulinemia are observed in Tanzanian children with *P. falciparum* malaria[11], and the increase in red blood cell (RBC) methemoglobin may be due to impairment of methemoglobin reductase system during malaria[12]. Exogenous NO further increased RBC methemoglobin ($P < 0.05$; **Fig. 3f**) as compared with infected mice, but significantly decreased free hemoglobin ($P = 0.04$). These results collectively indicate that the concentration of NO-scavenging free hemoglobin increases during ECM and is partially inactivated by exogenous NO treatment.

We found that the concentrations of arginine and its analogs declined markedly and significantly by day 4 and even further by day 6 in mice with ECM ($n = 5$ per group; $P < 0.005$; **Fig. 3g**). Concentrations of nitrite were significantly lower in erythrocytes and in plasma on day 6 of ECM as compared with uninfected controls ($P < 0.05$; **Fig. 3h**). Protective NO gas treatment during infection restored plasma but not erythrocyte nitrite levels (**Fig. 3h**).

Our data do not support the hypothesis that an increase in NO bioavailability in the vasculature contributes to the genesis of ECM. First, mice lacking iNOS or eNOS showed parasitemia similar to that of the controls and were not protected from ECM[13]. Second, exogenous NO administration did not affect parasitemia but protected against the development of ECM, which precludes NO from being pathogenic. Third, mice with ECM showed an increase in free hemoglobin and marked hypoargininemia, suggesting that vascular NO bioavailability is probably low. Last, the *in vivo* footprints of labile NO (cGMP and nitrite) were markedly decreased rather than increased as is predicted by the high NO bioavailability hypothesis. In fact, our findings indicate the converse: namely, that low NO bioavailability contributes to the genesis of ECM and that restoring NO bioavailability is protective.

The limited clinical studies in *P. falciparum* patients addressing NO bioavailability also support the concept that NO bioavailability is low in severe falciparum malaria (reviewed in ref. 7). Low nitrite levels and hypoargininemia are associated with the development of cerebral malaria in Tanzanian children[14,15]. Reported concentrations of free hemoglobin are higher in *P. falciparum* infections than in less-virulent *P. vivax* infections, and increased free hemoglobin levels in individuals infected with *P. falciparum* correlate with increased myoglobin and creatine kinase levels, two biomarkers of disease[16].

An increased level of free hemoglobin in the blood — indicative of low NO bioavailability — is not necessarily pathogenic by itself, however. Indeed, increased free hemoglobin levels are observed not only in individuals with virulent *P. falciparum* infection but also in those with hemolytic uremic syndrome, where cerebral malaria does not develop. Hemoglobin-based oxygen carriers do not show toxicity in control subjects but contribute to mortality in individuals with hemorrhagic shock[17]. Thus, we posit that low NO bioavailability exacerbates underlying pathogenic processes, as observed in sickle cell disease[18]. Indeed, it has been reported that exogenous NO downregulates endothelial cell-adhesion molecules, decreasing parasite adhesion *in vitro*[19]. Low NO bioavailability would therefore be an essential cofactor that is necessary but not sufficient for the development of cerebral malaria.

Three primary mechanisms may account for the amelioration of ECM pathogenesis by administration of an NO donor. First, the

NO donor might reduce the concentration of circulating free hemoglobin by converting it to NO-inert methemoglobin, enabling endogenous NO to fulfill its normal homeostatic role. Methemoglobin might also activate the heme oxygenase pathway, which is anti-inflammatory[20]. We found that exogenous NO also converted RBC oxyhemoglobin to methemoglobin (~10% increase), which may be problematic for implementing NO treatment as a therapy; however, a level of methemoglobulinemia below 20% does not require treatment.

Second, the NO donor might have 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 h) of NO release. The other protective effects seem to be longer lasting, however, because basal concentrations of cGMP were restored in the brains of NO donor–treated mice 4 h after treatment, and the baseline MAP before administration of the NO donor did not decrease as it did in saline-injected controls. Third, the NO pool might be restored and maintained by exogenous NO treatment; if so, the NO pool will continue to generate NO after the NO donor has been cleared.

The causes of low NO bioavailability and its likely pathogenic effects are summarized in **Figure 4**. Because of the pleiotropic functions of NO, administration of an NO donor may function through additional pathways not identified in this study. Because more than 20% of individuals with severe falciparum malaria who are treated effectively with anti-parasite drugs still succumb to the disease[21], our results suggest that restoring NO bioavailability may represent an important component of effective adjunct therapy to rescue individuals from severe falciparum malaria. In addition, it has been reported that individuals in septic shock may benefit from exogenous NO[22], suggesting that the current concept of macrophage-derived NO being harmful may need to be revisited in other diseases as well.

METHODS

Infection of mice. All protocols were approved by the La Jolla Bioengineering Institutional Animal Care and Use Committee. Infection of mice between 6 and 10 weeks of age with PbA and assessment of parasitemia is described in the **Supplementary Information** online. C57BL/6 mice, and eNOS- or iNOS-deficient mice on a C57BL/6 background were obtained from The Jackson Laboratory. Each mouse was evaluated for clinical disease by summing its gripping reflex and righting reflex each on a scale of 0–5, where 0 is unimpaired and 5 has no reflex. Mice with a clinical score of >7 are moribund.

Treatment with NO donor or NO gas. Groups of five mice were injected intraperitoneally (i.p.) twice daily with 100 µl of a 10 mg/ml solution of DPTA/NO (Axxora) or saline. Groups of mice ($n = 5$) infected with PbA were placed daily for 12 h in a custom plexiglass chamber with free access to food and water under an air and NO gas mixture (40 ± 5 ppm; measured by a Sievers 280i Chemiluminescence Nitric Oxide Analyzer and recorded on a custom Labview interface) that was exchanged ten times every minute.

cGMP, vascular permeability, inflammation and coagulation factors, and hypotension. Concentrations of cGMP in brain tissue and blood were assessed by using a cGMP enzyme-linked immunoassay (GE Health Sciences) in accordance with the manufacturer's instructions. Vascular leak during PbA was assessed with the Evans blue technique as described[23]. Inflammatory cytokines (IL-1, IL-2, IL-6, IL-12p70, TNF and IFNγ), proinflammatory biomarkers (sCD40 and sVCAM-1), anti-inflammatory cytokines (IL-4 and IL-10), and protease (MMP-9 and TIMP-1) were measured by a multiplexed bead-based assay as described[24]. During sterile surgery, the catheter tip of a telemetric pressure transducer (Data Systems International) was inserted into the carotid artery of the anesthetized mouse and the transmitter was secured in a subcutaneous pocket in the dorsal neck region. The MAP is slightly higher than that reported with DSI telemetry devices[25], and may be either an offset value or stress caused by injection and turning on the instrument.

NO donor pharmacokinetics. Ten mice were injected i.p. with 2 mg of DPTA/NO. Pairs of mice were anesthetized and 500-µl blood samples were obtained with a retro-orbital plexus at 0 min, 30 min, 1 h, 2 h, 3 h and 4 h after injection of DPTA/NO, processed into plasma (200 ml), and centrifuged for 40 min at 4 °C and 14,000*g* in a Microcon 10-kDa centrifugal concentrator (Millipore) to separate the plasma proteins from the low-molecular weight DPTA/NO. We analyzed the low molecular weight flow-through spectrophotometrically at 252 nm for DPTA/NO ($\varepsilon = 7{,}640\ M^{-1}\ cm^{-1}$) using a spectrophotometer (BioTek).

Plasma hemoglobin and NO quenching. Plasma samples (100 µl) were analyzed for hemoglobin by the Winterbourn spectrophotometric method[26] on a µQuant Spectrophotometer (BioTek) or Cary 300 Spectrophotometer (Varian). Methemoglobin concentrations were confirmed by full-spectrum analysis and by tracking the blue shift of the 414-nm oxyhemoglobin peak.

The NO stock was prepared and monitored as described[27]. Assessment of NO quenching with an NO electrode (Innovative Instruments) inserted into the reaction chamber placed in an anaerobic chamber is described at natureprotocols.com. Plasma samples were treated with hemoglobin-inactivating potassium ferricyanide (1 mg) plus potassium cyanide (1 mg) or control potassium chloride (1 mg) for 15 min, and the salts were then removed by gel filtration. All chemicals were purchased from Sigma. Plasma quenching of NO was assessed before and after inactivation treatment.

Arginine and nitrite assessment. Arginine and its derivative in plasma samples or standards were analyzed on a Luna C18 analytical column (Phenomenex) by HPLC with fluorescence detection (Hewlitt Packard 1100 HPLC with 1046 programmable detector). Nitrite concentrations in erythrocytes and plasma were analyzed by HPLC (ENO-20 instrument).

Statistical analysis. Analysis of variance (ANOVA) with the Statview program (SAS Institute) with Fischer post-hoc test was done to compare all measurements statistically with a P value cutoff of 0.05. Reported data are the mean ± s.d. unless otherwise indicated. Survival times are compared with a non-parametric log-rank test with a P value cutoff of 0.05.

Note: Supplementary information is available on the Nature Medicine website.

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COMPETING INTERESTS STATEMENT

The authors declare that they have no competing financial interests.

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