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Heme Impairs Prostaglandin E_2 and TGF-β Production by Human Mononuclear Cells via Cu/Zn Superoxide Dismutase: Insight into the Pathogenesis of Severe Malaria

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In many hemolytic disorders, such as malaria, the release of free heme has been involved in the triggering of oxidative stress and tissue damage. Patients presenting with severe forms of malaria commonly have impaired regulatory responses. Although intriguing, there is scarce data about the involvement of heme on the regulation of immune responses. In this study, we investigated the relation of free heme and the suppression of anti-inflammatory mediators such as PGE_2 and TGF-β in human vivax malaria. Patients with severe disease presented higher hemolysis and higher plasma concentrations of Cu/Zn superoxide dismutase (SOD-1) and lower concentrations of PGE_2 and TGF-β than those with mild disease. In addition, there was a positive correlation between SOD-1 concentrations and plasma levels of TNF-α. During antimalaria treatment, the concentrations of plasma SOD-1 reduced whereas PGE_2 and TGF-β increased in the individuals severely ill. Using an in vitro model with human mononuclear cells, we demonstrated that the heme effect on the impairment of the production of PGE_2 and TGF-β partially involves heme binding to CD14 and depends on the production of SOD-1. Aside from furthering the current knowledge about the pathogenesis of vivax malaria, the present results may represent a general mechanism for hemolytic diseases and could be useful for future studies of therapeutic approaches. ***The Journal of Immunology*****, 2010, 185: 1196–1204.**

Severe malaria is a highly lethal condition and a major health threat in many tropical countries. Multiple factors have been implicated in the pathogenesis of the severe complications of this condition, such as uncontrolled cytokine production (1, 2), hemolysis (3), and erythropoiesis suppression (4). Severe malaria was firstly described as originating from *Plasmodium falciparum* infection (5), but severe cases, including those with lethal outcomes, have also been observed from *Plasmodium vivax* infections (6–8). One of the major factors thought to be involved in sustaining systemic inflammation is the release of free heme, as a consequence of hemolysis inherent to the life cycle of *Plasmodium* within RBCs (9). Recently, heme has been implicated in the pathogenesis of severe forms of malaria in mice (10, 11). Under homeostasis, the heme released from hemoproteins such as cell-free hemoglobin (Hb) is scavenged by plasma proteins such as hemopexin or albumin as well as by lipoproteins (12). However, these proteins can be depleted during severe hemolytic conditions, such as associated with *Plasmodium* infection (13). This leads to the accumulation of free Hb tetramers in the plasma (14), which dissociate spontaneously into dimers. In the presence of reactive oxygen species (ROS) or other free radicals, cell-free Hb dimers are readily oxidized into methemoglobin, releasing their heme prosthetic groups (12). As a consequence, in malaria and other hemolytic disorders, the concentrations of heme can reach levels of up to 50 μM in the bloodstream (15), which can trigger an intense oxidative burst and unspecific tissue damage (11). Moreover, a crystal form of heme molecules produced by *Plasmodium sp.*, and referred to as hemozoin, also acts as a proinflammatory agonist and thus could be associated with the development of severe forms of malaria (16–18). Hemozoin inhibits PGE_2 production in both mice (19) and humans (20, 21), and there is an inverse relationship between PGE_2 and blood mononuclear cell cyclooxygenase-2 with disease severity in children with *P. falciparum* malaria (22). Until now there is no clear description of the effect of free heme on the PGE_2 production.

During malaria infection, superoxide anions are thought to be the main form of ROS produced (23). In this context, the antioxidant enzyme Cu/Zn superoxide dismutase (SOD-1) is activated and may display an important role in the pathological oxidative injury. Notwithstanding, SOD-1 has been linked to an increased inflammatory activity by amplifying TNF-α production on macrophages (24). In addition, overexpression of SOD-1 increases NF-κB–related rapid responses, such as immune response and antiapoptosis factors (25). Therefore, studies have correlated SOD-1 activity with

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Abbreviations used in this paper: 7-AAD, 7-aminoactinomycin D; A, asymptomatic; ALT, alanine aminotransferase; CoPPIX, cobalt protoporphyrin IX; CRP, C-reactive protein; DETC, diethyldithiocarbamate; Hb, hemoglobin; HO-1, heme oxygenase-1; M, mild; NAC, *N*-acetyl-L-cysteine; NI, noninfected individual; PPIX, protoporphyrin IX; ROS, reactive oxygen species; S, severe; siRNA, small interfering RNA; SnPPIX, Tin protoporphyrin IX; SOD-1, Cu/Zn superoxide dismutase.

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tissue damage (26, 27). More recently, SOD-1 has been found to play a deleterious role in protozoan infectious diseases such as leishmaniasis (28). Mice overexpressing SOD-1 develop oxidative injury associated with an increased vulnerability to *Plasmodium berghei* (26). Recent investigations from our group also indicate that SOD-1 is a surrogate marker of severe vivax malaria with a better predictive power than TNF-α (29). Although intriguing, the specific link between the free heme release from Hb, expression of SOD-1, and the severity of malaria has never been addressed. In this study, we investigate the relation of free heme and the suppression of anti-inflammatory mediators such as PGE_2 and TGF-β in human vivax malaria. We demonstrate that the heme effect on the impairment of regulatory responses partially involves heme binding to CD14 and depends on the production of SOD-1. Aside from furthering the current knowledge about the pathogenesis of vivax malaria, the present results may represent a general mechanism for hemolytic diseases and could be useful for future studies of therapeutic approaches.

Materials and Methods

Reagents

The RPMI 1640 medium and the L-glutamine, penicillin, and streptomycin were obtained from Invitrogen (Carlsbad, CA). Nutridoma-SP was obtained from Roche (Indianapolis, IN). The SOD-1 Protein ELISA kit was purchased from Calbiochem (San Diego, CA). TGF-β1 (DuoSet kit) was from R&D Systems (Minneapolis, MN). The SOD-1 activity assay and PGE_2 enzyme-linked immunoassay Kits were obtained from Cayman Chemical (Ann Arbor, MI). The superoxide dismutase inhibitor (diethyldithiocarbamate [DETC]), *N*-acetyl-L-cysteine (NAC), $FeSO_4$, endotoxin-free delipidated BSA, LPS, and apotransferrin were purchased from Sigma-Aldrich (St. Louis, MO). Heme and protoporphyrin IX (PPIX) were obtained from Frontier Scientific (Logan, UT). Porphyrins were dissolved in 0.1 N NaOH, diluted in RPMI 1640, and filtered. Stock solutions from porphyrins and heme were prepared in the dark immediately before being used to avoid free radical generation. Heme used contained <0.01 endotoxin units (<1 pg) in 200 μM heme. Purified bovine liver catalase (35 U/mg) was from Boehringer (Mannheim, Germany). 7-Aminoactinomycin D (7-AAD) and Annexin V-labeled Ab were purchased by BD Biosciences (San Jose, CA). The anti-CD14 Ab (3C10) was provided by Dr. D. Golenbock (University of Massachusetts, Amherst, MA) and Dr. R. Gazzinelli (Centro de Pesquisas René Rachou, Fundação Oswaldo Cruz, Belo Horizonte, Brazil).

Field study design and sampling

Plasma samples were obtained from a previous survey (8), which was part of an effort from our group to study predictive factors of human *P. vivax* malaria severity. The survey studied individuals living in Buritis, Rondônia, in the Brazilian Amazon, during 2006 and 2007. Active and passive malaria case detections were performed. Moreover, patients admitted to the Buritis Municipal Hospital with clinical signs of mild or severe malaria (5) were also asked to participate in the study. All individuals from 15 to 70 y, of both sexes, who had been living in the endemic area for >6 mo, were invited to be included in the study. The malaria diagnosis was performed using microscopy and confirmed by nested PCR. Exclusion criteria were as follows: documented or strong clinical suspicion of viral hepatitis (A, B, C, and D), chronic alcoholism, HIV type 1 infection, yellow fever, leptospirosis, cancer and chronic degenerative diseases, sickle cell trait, and the use of hepatotoxic or immunosuppressant drugs. Two individuals presenting *Plasmodium malariae* infection and 16 with *P. falciparum* infection were identified and excluded from the study. The separation of plasma and RBCs was performed within 5 min after phlebotomy in specialized laboratory facilities built inside the diagnostic centers and the municipal hospital exclusively for this study.

After obtaining the parasitological diagnosis, all vivax malaria positive cases were followed for 30 d. Infected individuals who remained without any presumptive malaria symptoms were considered asymptomatic; patients presenting clinical or laboratory signs of complicated malaria (5) were considered severe cases, whereas those who were symptomatic without any complication were mild cases. In hospitalized participants presenting with severe disease, two plasma samples were obtained: one at the hospital admission and the other 7 d after malaria treatment initiation. The sample was then composed of noninfected volunteers (n = 90) and individuals with different clinical presentations of vivax malaria: asymptomatic (n = 60), mild (n = 50), and severe infection (n = 19). The detailed clinical description of the participants together with the outcomes has been already addressed by our group (8). A summary of the baseline characteristics of the participants is illustrated in Table I. All the malaria cases were treated by the health-care professionals, according to the standardized protocols from Fundação Nacional da Saúde (Ministério da Saúde, Brazil). Written informed consent was obtained from all participants prior to enrolling in the study. The project was approved by the Institutional Review Board from the Faculdade de Medicina, Faculdade São Lucas (Porto Velho, Brazil), where the field study was performed.

Cell culture

RBC-free PMBCs were isolated from healthy donors through Ficoll gradient centrifugation, treatment with Ammonium-Chloride-Potassium lysis buffer, and cultivated (10^6 cells/well) in serum-free RPMI 1640 supplemented with 1% Nutridoma-SP, 2 mM L-glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin. Cells were treated with heme (3, 10, or 30 μM) for 6, 24, and 48 h and/or with $FeSO_4$ (30 μM), PPIX (50 μM), Sn PPIX (SnPPIX; 50 μM), Co PPIX (CoPPIX; 50 μM), albumin (100 μg/ml), apotransferrin (100 μg/ml), DETC (2 mM), NAC (20 mM), or catalase (1 KU/ml) for 48 h. In some assays, cells were preincubated for 1 h with anti-CD14 Ab or IgG isotype controls (10 μg/ml). All of the conditions were also run in the presence or absence of polymixin B (New Bedford Laboratories, Bedford, OH), with no significant differences between the values obtained, except for those stimulated with LPS (data not shown). In some experiments, a possible toxicity induced by heme was assessed by flow cytometry analysis of cells stained with 7-AAD and Annexin V (Pacific orange)-labeled Ab after 6, 24,

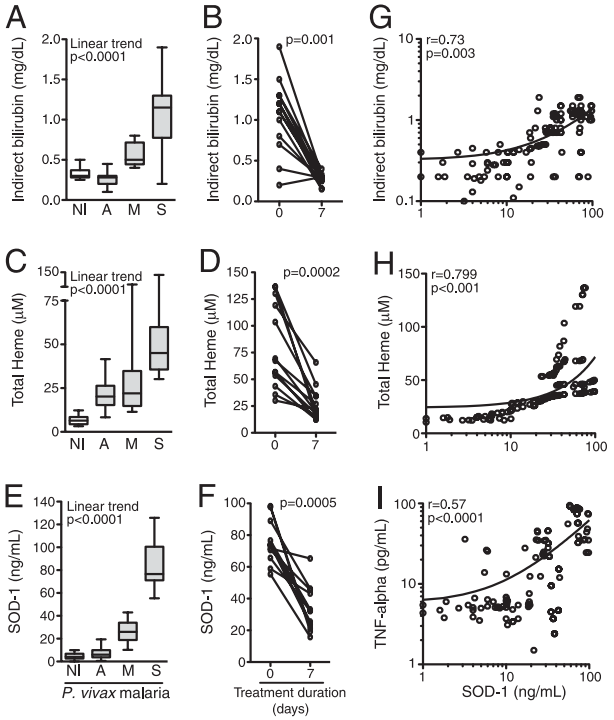

FIGURE 1. Hemolysis and SOD-1 correlations during *P. vivax* infection. Plasma concentrations of indirect bilirubin (*A*), total heme (*C*), and SOD-1 protein (*E*) were measured in noninfected individuals (NIs; n = 90) and in those with asymptomatic (A; n = 60), mild (M; n = 50), or severe (S; n = 19) *P. vivax* infection. Boxes represent medians and interquartile ranges, whereas whiskers represent maximum and minimum values. These data were analyzed using Kruskal-Wallis tests with linear trend posttests. In severe cases, indirect bilirubin (*B*), total heme (*D*), and SOD-1 (*F*) were also measured at the seventh day of the antimalarial treatment (Wilcoxon signed-rank paired tests were used to evaluate significant differences). *G–I* indicate correlations between the SOD-1 protein and indirect bilirubin, total heme, and TNF-α plasma concentrations, respectively, in malaria-infected patients (Spearman's test indicated significant correlations). The p values are shown for each graph.

and 48 h of stimulation with 3, 10, or 30 μM free heme using a LSR II cytometer (BD Biosciences).

Total heme measurement

Total heme in plasma samples was estimated by a colorimetric determination at 400 nm using the QuantiChrom Heme Assay Kit (BioAssay Systems, Hayward, CA), according to the manufacturer's protocol.

ELISA

SOD-1 protein levels and SOD activity were measured according to the manufacturer's protocols. PGE_2 levels were estimated in plasma samples or culture supernatants by enzyme-linked immunoassay, according to the manufacturer's instructions. After acidification to activate latent TGF-β followed by neutralization, total TGF-β1 was measured in the plasma or culture supernatants using ELISA, according to the manufacturer's instructions. In some experiments, the concentration of heme oxygenase-1 (HO-1) was estimated in cell lysates using the Human HO-1 ELISA kit from Assay Designs (Ann Arbor, MI), according to the manufacturer's protocol.

Small interfering RNA-mediated inhibition of SOD-1

SOD-1 and control small interfering RNAs (siRNAs) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Transfection was performed according to the manufacturer's instructions.

Statistical analysis

Kruskal-Wallis tests with Dunn's test for multiple or selected pair comparisons or a linear trend analysis were used to compare SOD-1, total heme, indirect bilirubin, PGE_2, HO-1, and TGF-β1 levels. Mann-Whitney tests were used to verify differences between mild and severe malaria. The Wilcoxon matched pairs test was performed to estimate statistical significance before and during the antimalarial treatment. Correlations were performed using a Spearman's test. The p values ≤ 0.05 were considered to be statistically significant.

Results

Hemolysis and inflammation during vivax malaria

It is widely know that severe falciparum malaria is linked to oxidative stress (23), inflammation (2, 30, 31) and high levels of hemolysis (3). To determine whether the same is true for vivax malaria, we measured plasma levels of indirect bilirubin and SOD-1 protein in patients with different clinical presentations of *P. vivax* infection. Disease severity was directly linked to higher levels of hemolysis, as indicated by increased plasma concentrations of indirect bilirubin ($p < 0.0001$ for linear trend) (Fig. 1*A*, Table I). As expected, antimalarial drug treatment led to reduced concentrations of the indirect bilirubin in those individuals with severe infection ($p = 0.001$) (Fig. 1*B*). Total heme plasma concentrations presented strong linear trend according to the degree of the disease severity ($p < 0.0001$ for linear trend; Fig. 1*C*), with a significant reduction during treatment in those severely ill ($p = 0.0002$; Fig. 1*D*). Moreover, total heme levels positively correlated with plasma levels of indirect bilirubin (Spearman $r = 0.758$; $p = 0.001$). Plasma concentrations of SOD-1 protein also displayed a positive linear trend with the severity of the infection ($p < 0.0001$ for the linear trend) (Fig. 1*E*). Considering only the severe cases of the disease, the concentrations of this antioxidant enzyme were consistently reduced during the antimalarial therapy to amounts equivalent to those of the asymptomatic infection ($p = 0.0005$) (Fig. 1*F*). In addition, there was a positive correlation between the concentrations of SOD-1 protein and indirect bilirubin in all of the symptomatic patients infected with *P. vivax* (Spearman $r = 0.73$; $p = 0.003$) (Fig. 1*G*). SOD-1 was also positively correlated with plasma concentrations of total

Table I. *Baseline characteristics of the participants*

Variables	*P. vivax* Malaria			
	Noninfected ($n = 90$)	Asymptomatic ($n = 60$)	Mild ($n = 50$)	Severe ($n = 19$)
Male, no. (%)	39 (43.3)	30 (50.0)	22 (44.0)	10 (52.6)
Age (yr)[a]				
Median	38.0	42.0	33.0	22.0
Interquartile range	25.0–51.0	32.0–48.2	26.7–48.0	16.0–35.0
Previous malaria episodes[a]				
Median	14.0	16.0	8.0	3.5
Interquartile range	10.0–18.0	13.0–20.0	1.0–12	2.0–7.5
Years resident in the area[a]				
Median	11.4	12.5	7.4	3.0
Interquartile range	3.2–12.8	4.2–14.6	0.5–9.2	0.5–5.4
Parasitemia (parasites/μl)[a]				
Median	0	73[b]	4,798	49,358
Interquartile range	0	54.0–85.0	2,934–7,483	32,796–54,244
Hb (g/dl)[a]				
Median	13.2	11.5	8.9	6.4
Interquartile range	9.2–14.5	9.5–14.2	7.3–12.6	5.8–7.4
Serum creatinine (mg/dl)[a]				
Median	0.85	0.9	1.1	1.7
Interquartile range	0.7–1.2	0.7–1.2	0.7–1.3	1.42–2.45
ALT (U/l)[a]				
Median	42.35	40	58.3	238.4
Interquartile range	37.28–53.58	23.25–65.78	43.6–87.5	105.5–364.6
Total bilirubin (mg/dl)[a]				
Median	0.35	0.4	0.8	2.1
Interquartile range	0.3–0.4	0.3–0.62	0.7–2.05	1.15–3.1
Indirect bilirubin (mg/dl)[a]				
Median	0.3	0.28	0.5	1.1
Interquartile range	0.28–0.37	0.2–0.3	0.45–0.72	0.6–1.3

Ordinal variables were compared using the Kruskal Wallis test with Dunn's multiple comparisons. The prevalence of male gender was compared between the groups using χ^2 test.

[a]Differences were significant between groups ($p < 0.05$).

[b]Six of 60 individuals with asymptomatic *P. vivax* infection were negative for malaria infection by light microscopy but were positive for *P. vivax* infection by nested PCR.

ALT, alanine aminotransferase; CRP, C-reactive protein.

heme (Spearman $r = 0.799$; $p < 0.0001$) (Fig. 1*H*) and TNF-α (Spearman $r = 0.57$; $p < 0.0001$) (Fig. 1*I*).

We further assessed the plasma concentrations of PGE_2 and TGF-β, two anti-inflammatory molecules associated with *P. falciparum* infection (19, 22, 32, 33). Both PGE_2 and TGF-β plasma concentrations were elevated during *P. vivax* infection (Fig. 2*A*, 2*B*). Moreover, individuals with severe cases of the disease presented lower amounts of these mediators compared with those presenting mild infections (Fig. 2*A*, 2*B*). Both PGE_2/TNF-α (Fig. 2*C*) and TGF-β/TNF-α (Fig. 2*D*) ratios presented a decreased linear trend according to the disease severity.

Levels of PGE_2 and TGF-β increased during the antimalarial treatment ($p = 0.004$ and $p = 0.003$, respectively) (Fig. 2*E*, 2*F*), resulting in increased PGE_2/TNF-α and TGF-β/TNF-α ratios to levels similar to those of noncomplicated infections ($p = 0.01$ and $p = 0.003$, respectively) (Fig. 2*G*, 2*H*). Platelets are a rich source of latent TGF-β (34), and little is known about its ability to generate active TGF-β. In those individuals infected with *P. vivax*, the platelet count was lower than in those who are noninfected ($p = 0.027$) (Fig. 2*I*). In addition, severely ill patients presented lower platelet count compared with those with uncomplicated infection ($p = 0.042$) (Fig. 2*I*). SOD-1 is present in RBCs, and the elevated levels of this enzyme could be a result of the increased degree of hemolysis seen during malaria. As expected, individuals with severe disease also displayed lower RBC count than those with mild or asymptomatic malaria ($p = 0.038$) (Fig. 2*J*). Within the severely ill patients, plasma concentrations of PGE_2 were negatively correlated those of SOD-1 (Spearman $r = -0.68$, $p = 0.003$) (Fig. 2*I*), with similar patterns being observed with regard to TGF-β and SOD-1 (Spearman $r = -0.48$; $p = 0.051$) (Fig. 2*J*). This indicates that during vivax malaria, patients with severe disease present high hemolysis linked to higher SOD-1 levels. It also suggests that, compared with what is seen in uncomplicated infection, these events are correlated with impaired systemic release of both PGE_2 and TGF-β.

The interference of the anti-inflammatory responses can sustain proinflammatory responses in elevated activity, which can ultimately result in the systemic collapse observed in severe cases of malaria.

Heme triggers SOD-1 and impairs PGE_2 and TGF-β production in PBMC from healthy individuals

We hypothesized that free heme release from cell-free Hb acts in a pro-oxidant manner to induce the expression of SOD-1 favoring systemic inflammation. To test this hypothesis, we cultured fresh PBMCs from healthy volunteers residing in a nonendemic malaria region with increasing doses of free heme (3, 10, and 30 μM) and assessed the levels of SOD-1 protein, SOD activity, PGE_2, and TGF-β in the supernatants. Within the concentrations used in our experiments, heme was not very toxic for the cells, because the number of live cells did not significantly differ from unstimulated cells (Fig. 3*A*). Moreover, free heme induced the secretion of SOD-1 in the supernatants, an effect that was dose dependent ($p < 0.05$ at 24 and 48 h compared with unstimulated cells) (Fig. 3*B*). Following a similar trend, the SOD activity was also induced by heme ($p < 0.05$ at 6 and 48 h compared with unstimulated cells) (Fig. 3*C*). In addition, unstimulated cells presented an increased production of both PGE_2 (Fig. 3*D*) and TGF-β (Fig. 3*E*) over time, whereas those cultured with 30 μM heme did not ($p < 0.05$ at 48 h poststimulation). The next step was to verify whether this finding could be reproduced by a component of the heme molecule (LPS-free $FeSO_4$ or PPIX) or by an iron-carrier protein (LPS-free albumin or apotransferrin). After 48 h of stimulation, only heme was capable of increasing SOD-1 protein concentrations ($p < 0.05$ compared with unstimulated cells) (Fig. 4*A*) and reducing both PGE_2 and TGF-β release in the supernatants ($p < 0.05$ for each comparison with unstimulated cells) (Fig. 4*B*, 4*C*). Therefore, these outcomes

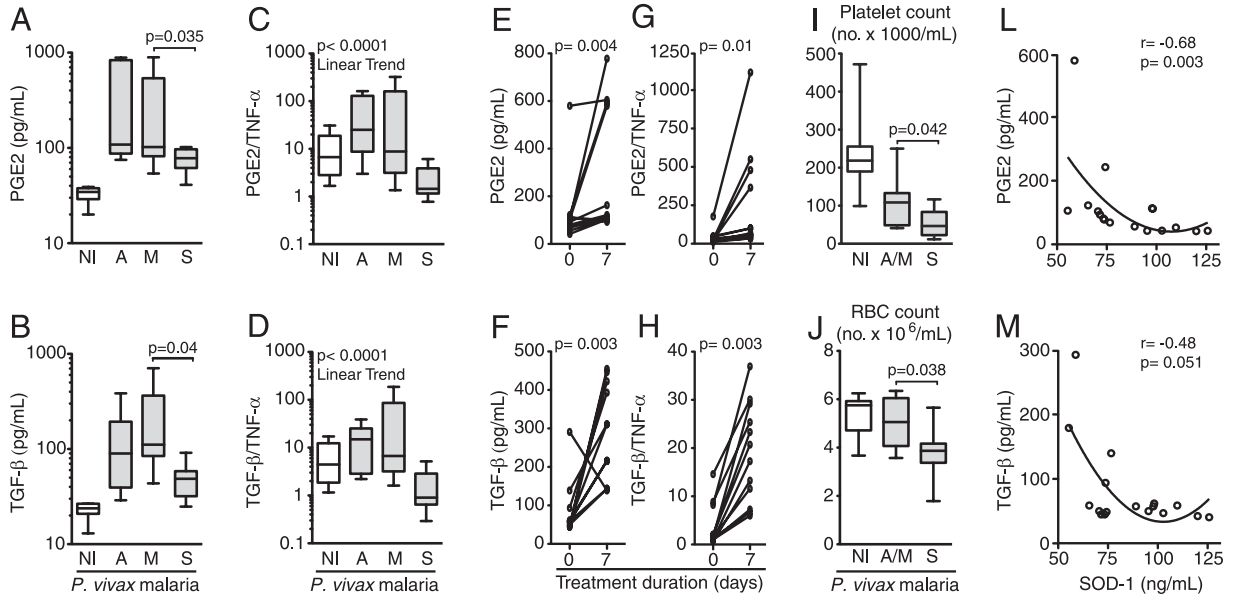

FIGURE 2. Unregulated anti-inflammatory responses during acute clinical attack of malaria. Plasma concentrations of PGE_2 (*A*) and TGF-β (*B*) were measured in uninfected individuals (NIs; $n = 90$) and in those with asymptomatic (A; $n = 60$), mild (M; $n = 50$), or severe (S; $n = 19$) *P. vivax* infection. *C* and *D* show PGE_2/TNF-α and TGF-β/TNF-α ratios, respectively. In severe cases, PGE_2 (*E*), TGF-β (*F*), PGE_2/TNF-α (*G*), and TGF-β/TNF-α (*H*) were also measured at the seventh day of the antimalarial treatment (Wilcoxon signed-rank paired tests were used to evaluate significant differences). Platelet counts (*I*) and RBC count (*J*) were estimated in the individuals. Boxes represent medians and interquartile ranges, and whiskers represent maximum and minimum values. Correlations between plasma concentrations of SOD-1 and PGE_2 (*L*) or TGF-β (*M*) were performed on patients with severe malaria at hospitalization using a Spearman's test (with a nonlinear curve fit). The *p* values are plotted in each graph. Data were analyzed using a Kruskal-Wallis test and a linear trend posttest. The *p* values are shown in each graph.

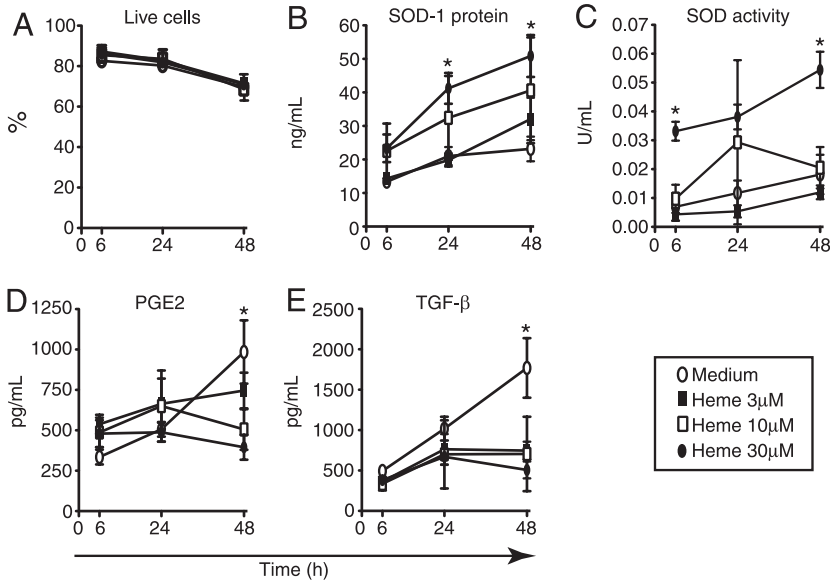

FIGURE 3. Heme triggers SOD-1 and impairs PGE_2 and TGF-β production. PBMCs (10^6/well) from seven healthy volunteers from a nonendemic malaria area were cultured with different doses of free heme (3, 10, and 30 μM). Percentage of live cells was estimated by counting cells unstained for both 7-AAD and Annexin V using flow cytometry (*A*). SOD-1 protein (*B*), SOD-1 activity (*C*), PGE_2 (*D*), and TGF-β (*E*) were measured in the supernatants at different times after stimulation (6, 24, and 48 h). Symbols represent mean, and whiskers represent SD. Data were analyzed using a Kruskal-Wallis test with Dunn's selected pairs (the conditions were compared with those cultured with the medium alone).

point to a possible intrinsic relationship between free heme, SOD-1, PGE_2, and TGF-β, as suggested by our results of human vivax malaria cases.

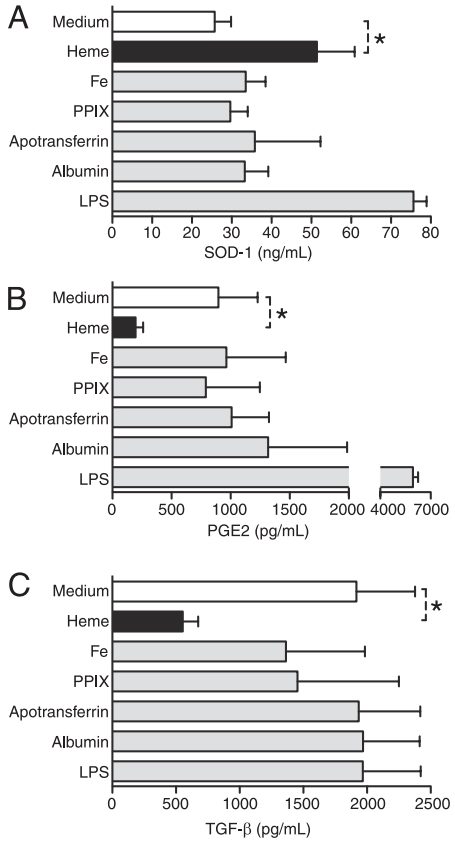

FIGURE 4. The reduction of PGE_2 and TGF-β is induced specifically by heme. PBMCs (10^6/well) from six healthy volunteers were stimulated with heme 30 μM or other stimuli such as $FeSO_4$ (30 μM), PPIX (50 μM), apotransferrin (100 μg/ml), albumin (100 μg/ml), or LPS (100 ng/ml) for 48 h, and SOD-1 protein (*A*), PGE_2 (*B*), and TGF-β (*C*) were measured in the culture supernatants. Bars and lines represent means and SD. Data were analyzed using a Kruskal-Wallis test with Dunn's selected pairs (the conditions were compared with those cultured with the medium alone). $*p < 0.05$. Other differences were considered to be significant and are described in the text.

The effect of Heme on PGE_2 and TGF-β production requires SOD-1

The idea that SOD-1 could hamper the regulatory responses to heme stimulation led us to actively interfere with SOD-1 activity and examine the resulting effects on PGE_2 and TGF-β. Interestingly, when heme-stimulated PBMCs from healthy volunteers were cultured in the presence of DETC, a cupper chelator that can inhibit SOD-1 activity (35), the inhibitory effect of heme on both PGE_2 and TGF-β release was reverted ($p = 0.01$ and $p = 0.04$, respectively, compared with cells stimulated with heme alone) (Fig. 5*A*, 5*B*). Moreover, when we treated the cells with NAC, a free radical scavenger, the concentrations of PGE_2 and TGF-β in the supernatants were reduced to a similar level as that observed with the use of heme ($p < 0.0001$ and $p = 0.004$, respectively, compared with unstimulated cells) (Fig. 5*A*, 5*B*). NAC was not capable of reducing the TGF-β concentrations in cells treated with heme plus DETC (Fig. 5*B*). In addition, when the cells were treated with exogenous catalase, the effect of heme on both PGE_2 and TGF-β was only partially reverted (data not shown). This suggests that SOD-1 might be a required source for H_2O_2 in this system and that the effect of heme on the production of PGE_2 and TGF-β is only partially mediated by the release of free radicals. Consequently, the activity of SOD-1 could play another role in this system. We then tried to test whether the heme effect on SOD-1 would be mediated by HO-1, an antioxidant enzyme that presents a major role in the pathogenesis of severe malaria (10, 36). As expected, heme induced high concentrations of HO-1 in PBMCs (Fig. 5*C*), an effect that was enhanced in the presence of CoPPIX, a HO-1 inductor, but nonaltered when cells were cocultured with SnPPIX, an inhibitor of the HO-1 activity (Fig. 5*C*). Nevertheless, neither CoPPIX nor SnPPIX interfered with SOD activity induced by heme in our model (Fig. 5*D*), suggesting that the heme effect on SOD is not directly mediated by HO-1.

To evaluate the direct role of SOD-1 on the reduction of PGE_2 and TGF-β mediated by heme, we successfully inhibited SOD-1 production using siRNA (Fig. 6*A*). siRNA-mediated SOD-1 inhibition before the addition of heme completely reverted the effect of heme on PGE_2 ($p < 0.0001$) (Fig. 6*B*) and TGF-β levels ($p < 0.0001$) (Fig. 6*C*).

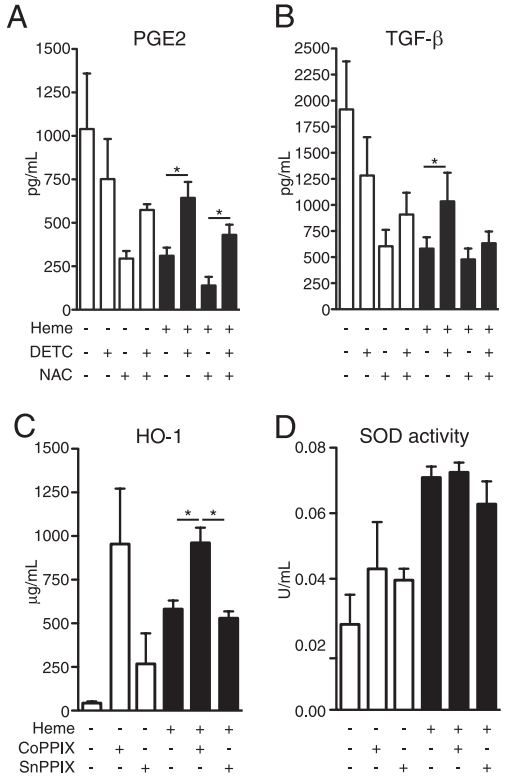

FIGURE 5. The role of SOD-1 on the release of PGE_2 and TGF-β. PBMCs (10^6/well) from six healthy volunteers were cultured in the presence of heme (30 μM) and/or DETC (2 mM) and/or NAC (20 mM) and/or CoPPIX (50 μM), and/or SnPPIX (50 μM) for 48 h as described in *Materials and Methods*. PGE_2 (*A*), TGF-β (*B*), and SOD activity (*D*) were measured in the supernatants, whereas HO-1 (*C*) protein levels were measured in cell extracts. Bars and lines represent means and SD. A Kruskal-Wallis test with Dunn's multiple comparisons or selected pairs was used to evaluate statistical significance. $*p < 0.05$. Other differences were considered to be significant and are described in the text.

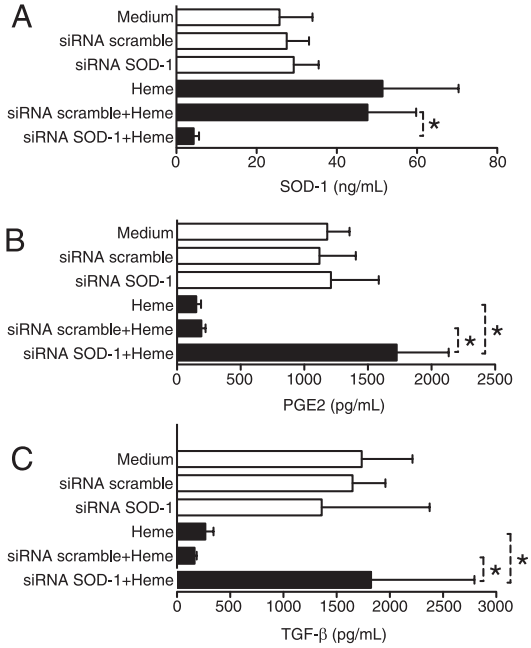

FIGURE 6. The effect of Heme on PGE_2 and TGF-β production requires SOD-1. PBMCs from six healthy volunteers (10^6/well) were cultured with heme (30μM) in the presence of siRNA for SOD-1 as described in *Materials and Methods*. After 48 h of stimulation, SOD-1 protein (*A*), PGE_2 (*B*), or TGF-β (*C*) was measured in the supernatants. Bars and lines represent means and SD. Data were analyzed using a Kruskal-Wallis test with Dunn's multiple comparisons. $*p < 0.01$.

The role of CD14 on the heme-impaired production of PGE_2 and TGF-β

Previous studies demonstrated that heme induces TNF-α production by human and murine macrophages through the activation of TLR4 and CD14 (37). In an attempt to verify whether the effect of heme on SOD-1 as well as on the release of PGE_2 and TGF-β would be mediated by CD14, we incubated fresh PBMCs from normal volunteers with anti-CD14 for 1 h prior to stimulation with heme. As expected, the neutralization of CD14 adequately reversed the effect of LPS on PGE_2 release but not the effect of TGF-β ($p < 0.0001$ and $p = 1.0$; comparing LPS plus anti-CD14 with LPS alone) (Fig. 7). In addition, the blockage of CD14 reduced SOD-1 ($p < 0.05$) (Fig. 7*A*) and increased the concentrations of PGE_2 in supernatants from cells stimulated with heme ($p < 0.05$) (Fig. 7*B*) but did not alter the levels of TGF-β ($p = 1.0$) (Fig. 7*C*). Thus, it seems that the direct effect of heme on the suppression of PGE_2 involves at least in part the binding to CD14.

Discussion

In malaria, as in diseases with release of Hb from RBCs, there is uncontrolled inflammatory imbalance and intense oxidative stress (2, 38–40). In this paper, we report that patients with severe *P. vivax* infection, similar to individuals and mice with falciparum malaria (22, 33, 41), displayed reduced plasma levels of PGE_2 and TGF-β than those with mild infections, with this reduction being inversely proportional to the bilirubin, total heme, and SOD-1 levels. Despite these similarities, one important difference can be noted between the current study and those made in falciparum malaria, which is the fact that plasma PGE_2 (22) and TGF-β (33) are lower in mild and severe falciparum malaria than in normal controls. In our study, independently of the disease severity, *P. vivax* infection was linked to an increase in the plasma concentrations of both PGE_2 and TGF-β. These data can indicate an important difference between vivax and falcipaum malaria. In this scenario, patients with *P. vivax* infection present a significant augmentation of circulating T regulatory cells producing TGF-β, and this production directly relates to the parasite load (42). This difference and other possible disparities in the degree of hemolysis and thrombocytopenia between patients with falciparum and vivax malaria need further investigation.

The association between systemic concentrations of SOD-1 and indirect bilirubin in the individuals presenting with malaria was not surprising as SOD-1 is present in RBCs (43, 44) and the severely ill individuals from our study presented lower RBC count than those with uncomplicated infection. For this reason, we tested whether human PBMCs were able to release SOD-1 in the presence of heme. Interestingly, free heme reduced both PGE_2 and TGF-β via SOD-1 production and activity in human PBMCs. These events may influence the capacity of critically ill patients to produce adequate inflammatory responses.

The possible role of SOD-1 in the pathogenesis of human malaria has only superficially been addressed before, with some degree of speculation. Patients with acute noncomplicated *P. falciparum* or *P. vivax* malaria have a lower catalase activity than noninfected individuals but a higher SOD activity (27). Reduced catalase activity, together with increased SOD activity, may result in the accumulation of H_2O_2. In the presence of divalent metals such as the iron contained within the protoporphyrin ring of heme, H_2O_2 is

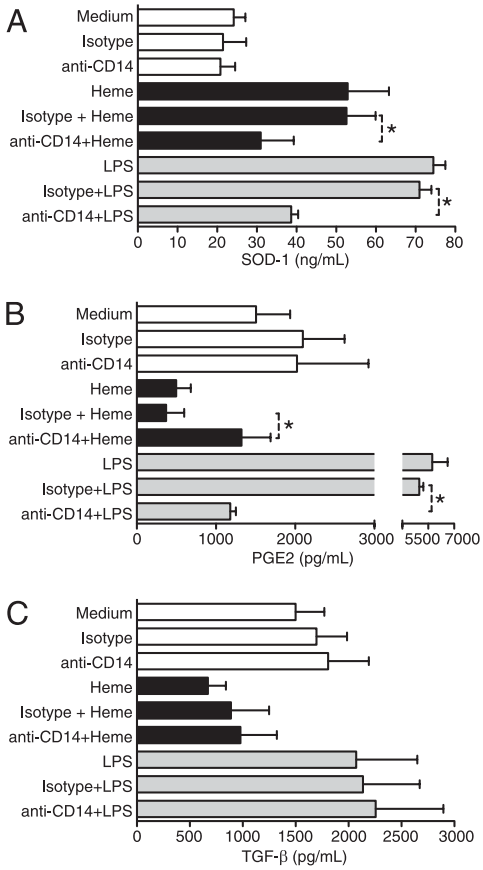

FIGURE 7. The role of CD14 on the heme-mediated effects. PBMCs from six healthy volunteers (10^6/well) were cultured with heme (30 μM) in the presence of anti-CD14 or a control isotype (10 μg/ml) as described in *Materials and Methods*. After 48 h of stimulation, SOD-1 proteins (*A*), PGE_2 (*B*), and TGF-β (*C*) were measured in the supernatants. Bars and lines represent means and SD. Data were analyzed using a Kruskal-Wallis test with Dunn's multiple comparisons. $*p < 0.05$.

rapidly converted into hydroxyl radicals, which promote tissue damage in a manner that compromises the viability of a *Plasmodium*-infected host (11). Our study showed that the direct association between hemolytic activity and SOD-1 during malaria could cause defects in the regulatory responses that could favor disease severity.

Severe malaria syndromes are heterogeneous conditions resulting from complicated infections (5). The characteristics of severe vivax malaria are controversial, because the specific pathogenesis of the clinical complications is still poorly understood. In the present paper, we used the previously indicated characteristics to define severe vivax cases (8), which are adapted from the criteria for *P. falciparum* malaria severity. Despite the heterogeneity of malaria clinical presentations, elevated hemolysis was a major symptom presented in all severe cases, documented in this paper by the measurement of indirect bilirubin and also by the estimation of total heme plasma concentrations. In addition, both heme and indirect bilirubin are elevated in experimental cerebral malaria (10), in noncerebral forms of severe malaria (11), as well as in other hemolytic disorders (45). Moreover, there is experimental evidence to suggest that heme release from Hb contributes in a critical manner to malaria-related immunopathology (10, 11, 36). During *Plasmodium* infection, the parasites metabolize free heme molecules into hemozoin. It is well known that *P. falciparum*-derived hemozoin exerts many different pathological effects on the host, such as inducing inflammation (46) and reducing cyclooxygenase-2–mediated PGE_2 production (19). The results presented in this paper suggest that general priming of inflammation could be mediated by heme itself, which causes ROS and TNF-α production in macrophages (37) and hampers PGE_2 and TGF-β secretion, similar to hemozoin. Therefore, patients with severe forms of *P. vivax* or *P. falciparum* malaria who present elevated hemolysis display two major inflammatory stimuli, free heme and hemozoin. We did not analyze possible effects of heme on the adaptive immune responses, as has been described for hemozoin (47). It is plausible to expect some degree of influence, as heme induces important oxidative stress.

To our knowledge, this is the first paper on the direct effect of heme on SOD-1, PGE_2, and TGF-β secretion by human cells. Most of the data regarding the effect of heme on oxidative stress involves the anti-oxidant enzyme HO-1 (12, 48). Interestingly, some antioxidant mechanisms of HO-1 on vascular cells require the production of extracellular SOD-1 (49, 50). In the current study, neither the HO-1 inductor CoPPIX nor the inhibitor of HO-1 activity SnPPXI interfered with SOD activity, suggesting that the heme effect on SOD is not directly mediated by HO-1. Aside from playing a protective role during oxidative stress, SOD-1 activity has been linked to several inflammatory diseases and infections, including malaria (26, 27, 51–53).

In the current study, we demonstrated that heme triggers SOD-1 release, at least in part, by binding to CD14. Heme can activate TLR4/CD14 and induce TNF-α production macrophages (37). However, ROS production induced by heme is not dependent on TLR4 (37). Our findings suggest that the consequence of ROS production is not the single mechanism responsible for the negative regulation of PGE_2 and TGF-β by heme, because this effect was reduced when we neutralized the SOD-1 activity using DETC. Thus, SOD-1 may trigger an effect that is still unknown and that could synergize with the ROS produced. This hypothesis was confirmed by the reversal of the heme effect on PGE_2 and TGF-β when PBMCs were cultured in the presence of SOD siRNA. Interestingly, LPS, a CD14 agonist, is a potent inductor of PGE_2 (54), and it induces TNF-α production via SOD-1 release as a consequence of ERK-1 phosphorylation (24). In our study, blocking CD14 led to a reversal of the heme effect on PGE_2 secretion but had no effect on TGF-β levels. Although heme and LPS bind to the same receptor, they have opposite effects on the production of PGE_2. Investigating the divergent effects of heme and LPS is beyond the scope of this paper. Unexpectedly, a CD14 blockage did not influence the effect of heme on TGF-β, indicating a diverse mechanism in its relationship with PGE_2. Heme-induced TGF-β reduction may involve ROS production rather than binding to a specific receptor. Recent studies have shown that superoxide anions increase the release of TGF-β1 and collagen from human lung fibroblasts (55). In our study, enhanced SOD-1 secretion could scavenge superoxide radicals, negatively impacting the production of TGF-β. The release of free heme during hemolysis could exert effects that are dependent on ROS generation and are also dependent on CD14 binding. SOD-1 appears to be involved in both situations.

In many infectious conditions, uncontrolled activation of the inflammatory responses is strongly associated with severe outcomes. Conversely, indiscriminate immunosuppression is linked to disseminated infection and death. Therefore, an adequate modulation of the immune responses can minimize immunopathology while limiting the infectious agent. The elevation of proinflammatory mediators is usually accompanied by the increase of anti-inflammatory factors. This balance determines the outcomes of the infections. With regard to malaria, high ratios of proinflammatory to anti-inflammatory cytokines are associated with increased symptoms (56) and disease severity (8). Intriguingly, heme hampers regulatory responses by reducing TGF-β and PGE_2 while also

inducing inflammation, which favors uncontrolled inflammation. To better understand the specific mechanism underlying this finding, a detailed description of the intracellular signaling pathways involved is urgently needed.

In a broad context, the inhibition of SOD-1 could engage the host's immune system and reinforce the regulatory responses that could ultimately diminish the severity of the disease. The SOD-1 inhibitor DETC has also been used in vivo as an adjuvant of the immune system, delaying the disease progression in HIV-infected patients (57). However, this approach would need to be cautiously evaluated before being used in humans because the consequences of interfering with highly complex systems are unknown. The insights into the pathogenesis of severe malaria reported here may present new and interesting approaches for the management of human diseases where hemolysis is an important element.

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Disclosures

The authors have no financial conflicts of interest.

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