Reduced Peripheral PGE_2 Biosynthesis in *Plasmodium falciparum* Malaria Occurs through Hemozoin-Induced Suppression of Blood Mononuclear Cell Cyclooxygenase-2 Gene Expression via an Interleukin-10–Independent Mechanism

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Molecular immunologic determinants of disease severity during *Plasmodium falciparum* malaria are largely undetermined. Our recent investigations showed that peripheral blood mononuclear cell (PBMC) cyclooxygenase-2 (COX-2) gene expression and plasma prostaglandin E_2 (PGE_2) production are suppressed in children with falciparum malaria relative to healthy, malaria-exposed children with partial immunity. Furthermore, decreased COX-2/PGE_2 levels were significantly associated with increased plasma interleukin-10 (IL-10), an anti-inflammatory cytokine that inhibits the expression of COX-2 gene products. To determine the mechanism(s) responsible for COX-2–derived PGE_2 suppression, PBMCs were cultured from children with falciparum malaria. PGE_2 production was suppressed under baseline and COX-2–promoting conditions (stimulation with lipopolysaccharide [LPS] and interferon [IFN]-γ) over prolonged periods, suggesting that an in vivo–derived product(s) was responsible for reduced PGE_2 biosynthesis. Ingestion of hemozoin (malarial pigment) by PBMC was investigated as a source of COX-2/PGE_2 suppression in PBMCs from healthy, malaria-naive adults. In addition, synthetically prepared hemozoin, β-hematin, was used to investigate the effects of the core iron component of hemozoin, ferriprotoporphyrin-IX (FPIX). Physiologic concentrations of hemozoin or β-hematin suppressed LPS- and IFN-γ–induced COX-2 mRNA in a time- and dose-dependent manner, resulting in decreased COX-2 protein and PGE_2 production. Suppression of COX-2/PGE_2 by hemozoin was not due to decreased cell viability as evidenced by examination of mitochondrial bioactivity. These data illustrate that ingestion of FPIX by blood mononuclear cells is responsible for suppression of COX-2/PGE_2. Although hemozoin induced overproduction of IL-10, neutralizing IL-10 antibodies failed to restore PGE_2 production. Thus, acquisition of hemozoin by blood mononuclear cells is responsible for suppression of PGE_2 in malaria through inhibition of de novo COX-2 transcripts via molecular mechanisms independent of increased IL-10 production.

INTRODUCTION

Although the underlying pathophysiologic determinants of severe malaria are only partially defined, cytokines and effector molecules are important immunologic determinants for regulating disease susceptibility. Our recent investigations have focused on defining the role of prostaglandins as immunomodulatory factors in malaria. Eicosanoids, such as prostaglandin E_2 (PGE_2), regulate macrophage function, vascular permeability, extracellular adhesion molecules, fever, and cytokine production (1). PGE_2 formation is initiated by the actions of phospholipase A_2, which releases arachidonic acid from the membrane phospholipid bilayer in response to inflammatory stimuli (2). Free arachidonic acid is rapidly metabolized to prostaglandin H_2 (PGH_2) via the enzymatic activity of the cyclooxygenase (COX) enzymes, of which there are 2 distinct isoforms encoded by separate genes: COX-1 and COX-2 (1,3). COX-1 is constitutively expressed in most tissues and generates PGs for physiologic homeostasis, whereas COX-2 is an inducible gene that generates high levels of PGs during an inflammatory event (4). Following formation of PGH_2 by COX, PGE_2 synthase converts PGH_2 to PGE_2 (5).

Our previous studies showed that plasma levels of PGE_2 were significantly reduced in children with malaria and were significantly associated with increased plasma interleukin (IL)-10 levels (6). Furthermore, peripheral blood mononuclear cell (PBMC) COX-2 gene products were inversely related to disease severity (6). We have also shown that children with cerebral malaria (CM) have suppressed systemic levels of PGE_2 (7). Although suppression of COX-2/PGE_2 in our previous studies was associated with enhanced severity of falciparum malaria, the mechanism(s) for decreased PGE_2 synthesis remain undefined.

During the erythrocytic stage of malaria, *Plasmodia* digest host hemoglobin, releasing heme in the process. To avoid the toxic effects of heme, enzymes within the parasitiphorus vacuole catalyze the

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polymerization of heme into a nontoxic insoluble compound, hemozoin (malarial pigment) (for review, see Goldie and others [9]). Hemozoin is composed predominately of protein and a smaller fraction of carbohydrates, lipids, and nucleic acids, which are attached to the polymerized iron core structure, ferriprotoporphorin-IX (FPIX) (9). Upon rupture of infected erythrocytes, hemozoin is released into the blood stream and phagocytosed by circulating monocytes and tissue macrophages (10). Hemozoin is also acquired by monocytes/macrophages through phagocytosis of parasitized erythrocytes (11). Experiments in cultured blood mononuclear cells illustrate that hemozoin augments the release of both proinflammatory cytokines, such as tumor necrosis factor (TNF)-α (12), and anti-inflammatory cytokines, such as IL-10 (13). This appears important in the current context because TNF-α induces high levels of sustained COX-2 gene expression (14), whereas IL-10 decreases COX-2 gene expression through destabilization of COX-2 message (15). Additional effects of hemozoin on blood mononuclear cells include inhibition of oxidative burst and protein kinase C activity (16), reduction of microbicidal and antitumor capabilities (17), impaired expression of MHC class II and CD54 expression (10), and an inability of monocytes to undergo repeated phagocytosis (18).

In the present investigations, PGE_2 production was examined in cultured PBMCs from children with falciparum malaria under baseline conditions and following treatment with COX-2/PGE_2-inducing stimuli: lipopolysaccharide (LPS) and interferon (IFN)-γ. In addition, COX-2 mRNA and protein and PGE_2 formation were examined in cultured PBMCs from malaria-naive healthy donors stimulated with LPS and IFN-γ in the presence of physiologically relevant doses (10, 1.0, and 0.1 μg/mL [19]) of hemozoin or β-hematin (synthetic malarial pigment). The effect of hemozoin on IL-10 production and the ability of IL-10 to downregulate PGE_2 biosynthesis were also investigated.

MATERIALS AND METHODS

Study Participants

Children (n = 30, age 2 to 7 y) were recruited from a longitudinal prospective study at the Albert Schweitzer Hospital in Lambaréné, Gabon, in the province of Moyen Ogooue. In this hyperendemic area of malaria transmission, the primary clinical manifestations of severe childhood malaria is severe anemia and/or hyperparasitemia, with cerebral malaria occurring rarely (20). Classification of malaria was defined according to our previously published methods (6,21). Only those children with mild malaria (defined by parasitemias < 100000 parasites/μL and the absence of any signs or symptoms of severe malaria [6,20]) were included in the present study because of the limited amount of blood available from severe cases. Healthy, malaria-exposed subjects were defined as participants with a previous episode(s) of malaria and the absence of a positive thick blood film for malaria, or any other illnesses, within the last 4 wk. All blood samples were obtained prior to treatment with antimalarials. Informed consent was obtained from the parents of participating children.

Healthy, malaria-naive adult donors (n = 21) were recruited from the University of Pittsburgh, PA, USA. The study was approved by the ethics committee of the International Foundation of the Albert Schweitzer Hospital, Duke University Medical Center, and the University of Pittsburgh Investigational Review Boards.

Isolation and Culture of Peripheral Blood Mononuclear Cells

For in vitro experiments in Gabonese children (n = 30) and US adults (n = 21), venous blood (3 and 40 mL, respectively) was drawn into EDTA-containing vials. PBMCs were prepared using ficoll/Hypaque, as described earlier (22), and plated at 1×10^6 cells per mL in Dulbecco's modified Eagle medium (DMEM) containing 10% pooled human serum (heat inactivated at 56 °C for 30 min). Cells were incubated with media alone (controls) or concomitant stimulation with LPS (100 ng/mL; Alexis Corp., San Diego, CA, USA) and IFN-γ (200 U/mL; BD Pharmingen, San Diego, CA, USA). For experiments in healthy adults, cultures were incubated with varying concentrations of hemozoin (10, 1.0, and 0.1 μg/mL) or β-hematin (10, 1.0, and 0.1 μg/mL) in the presence and absence of LPS and IFN-γ stimulation. For neutralization of endogenous IL-10, cultures were stimulated with IL-10 neutralizing antibodies (1.0, 0.5, and 0.1 μg/mL).

Hemozoin and β-hematin Preparation

Crude hemozoin was isolated from in vitro cultures of *P. falciparum*–infected red blood cells (strain Pf-D6) according to our previously published methods (19). Briefly, cultured red blood cells (RBC) were treated with 2 mL saponin for 10 min, and the solution was washed in phosphate-buffered saline and spun at 14000 rpm for 15 min (4 to 7 times). The final pellet was dried, weighed, and resuspended in filter-sterilized H_2O at a concentration of 1.0 mg/mL. As a control, a parasite-free RBC lysate was prepared from uninfected RBC following the protocol outlined above for the hemozoin preparation.

β-Hematin was formed in a 4.5-M acidic acetate solution at pH 4.5 by the method of Egan and others (23). Briefly, hemin chloride (Sigma, St. Louis, MO, USA) was added to a 0.1-M solution of NaOH followed by addition of HCl and acetate. The mixture was incubated for 150 min at 60 °C without stirring, spun at 14000 rpm, washed 3 times with filter-sterilized H_2O, and dried at 60 °C under vacuum. The final pellet was weighed and resuspended at 1.0 mg/mL in filter-sterilized H_2O. Resuspended β-hematin was sonicated extensively to disperse the preparation. Under these preparation conditions, β-hematin has an identical infrared spectroscopy pattern as detergent-purified hemozoin, and β-hematin crystals appear morphologically similar to natural hemozoin crystals as evidenced by scanning electron microscopy (23).

PGE_2 and IL-10 Measurements

Concentrations of PGE_2 and IL-10 were measured in the supernatants of cultured PBMCs using a quantitative indirect sandwich enzyme immunoassay (Cayman Chemical, Ann Arbor, MI, USA) and quantitative sandwich ELISA (Pharmingen, San Diego, CA, USA), respectively, according to our previously defined methods (24). Sensitivity of detection for PGE_2 and IL-10 was greater than 7.8 pg/mL.

MTT Assay

Cell viability was determined by the MTT assay, which measures active mitochondrial conversion of MTT salt into formazan crys-

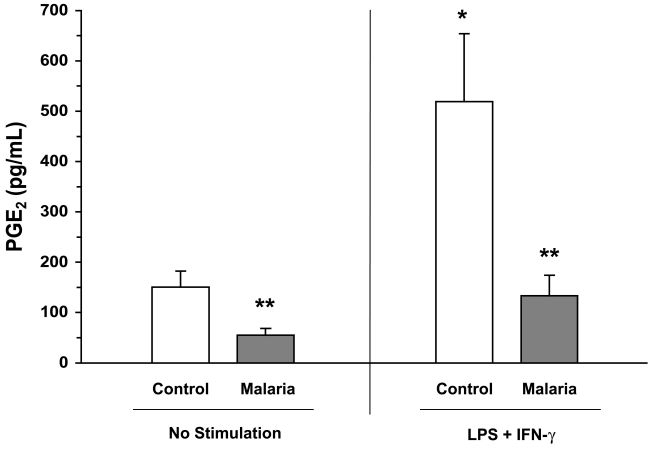

Figure 1. PGE_2 production in cultured PBMCs from children with malaria. PBMCs (1×10^6 cells/mL) from healthy malaria-exposed children (control, $n = 20$) and children with malaria (malaria, $n = 10$) were cultured in the presence and absence of LPS (100 ng/mL) and IFN-γ (200 U/mL). Supernatants were collected at 120 h for PGE_2 determination. Values are the mean ± SEM. Statistical significance was determined by the Mann-Whitney U test. *$P < 0.01$ compared with unstimulated conditions, **$P < 0.05$ compared with control.

tals. Briefly, 20 µL of MTT salt (5 mg/mL in 1X PBS [Sigma]) was added to cultured cells 5 h prior to termination of cultures. At the end of culture, supernatants were aspirated and 200 µL of dimethyl sulfoxide was added to dissolve the formazan crystals. Plates were incubated for 5 min at room temperature. Absorbance was determined at 550 nm.

Immunoblot Analyses

Cellular extracts were prepared according to our previously defined methods (25). Protein samples (30 µg) were fractionated on a 10% sodium dodecyl sulfate–polyacrylamide gel, transferred to nitrocellulose membranes, and COX-2 antigen was measured by our previously defined methods (6). Extracts of mouse macrophage cells (RAW 264.7), stimulated with LPS (*Escherichia coli* LPS 0127B8, 1.0 µg/mL) and IFN-γ (10 ng/mL) were used as a positive control. Densitometry readings for COX-2 protein expression were obtained using commercially available software (Scion Image, Scion Corp, http://www.scioncorp.com).

Quantitative Real Time Reverse Transcriptase–Polymerase Chain Reaction (RT-PCR)

Total RNA was isolated from PBMCs by the GITC method (26). Total RNA (1 µg) was reverse transcribed into cDNA, and COX-2 gene expression was analyzed by quantitative real time RT-PCR on an ABI Prism 7700 sequence detection system (Applied Biosystems, Foster City, CA, USA). cDNA (100 ng) was amplified in duplicate with COX-2 specific primers and probe as follows: forward, 5′-CCTTCCTCCTGTGCCTGATG-3′; reverse, 5′-ACAATCTCATTTGAATCAGGAAGCT-3′; probe (FAM labeled), 5′-TGCCCGACTCCCTTGGGTGTCA-3′ (sequence information obtained from Sales and others [27]). The COX-2 cDNA was amplified for 40 cycles following the parameters specified by the manufacturer (Applied Biosystems). To control for nonspecific background fluorescence, no template controls were included in triplicate. An endogenous control gene, β-actin (Ascession number NM_001101 [Applied Biosystems]), was used as a reference gene to normalize cDNA loading between samples. Data was compared using the $-\Delta\Delta C_T$ method as previously described (19).

Statistical Analyses

Supernatant concentrations of PGE_2 (pg/mL) and IL-10 (pg/mL) were measured in triplicate and at several different dilutions. COX-2 mRNA expression was measured in duplicate. Pairwise comparisons between conditions, including pairwise tests of dose-dependent effects, were performed using the Mann-Whitney U test. Multiple group comparisons for dose-dependent effects were performed using the Kruskal-Wallis test (statistical significance set at $P \leq 0.05$).

RESULTS

PGE_2 Production in Cultured PBMCs from Children with Malaria

To determine if mononuclear phagocyte production of PGE_2 is altered in children with falciparum malaria, PGE_2 synthesis was determined in culture supernatants of PBMCs from healthy, malaria-exposed children (control, $n = 20$) and children with malaria (malaria, $n = 10$). To minimize the influence of the in vivo environment, PBMCs were cultured for 120 h. PGE_2 synthesis was examined under baseline conditions (media alone) and in the presence of COX-2–promoting stimuli (LPS and IFN-γ). Under baseline conditions, PGE_2 production was lower in the malaria group compared with the control group ($P < 0.05$, Figure 1). Stimulation of PBMCs augmented PGE_2 production in the control group ($P < 0.01$) but failed to elevate PGE_2 formation in the malaria group (see Figure 1). Production of PGE_2 in stimulated PBMCs was lower in the malaria group relative to the control group ($P < 0.05$, see Figure 1). The percent of hemozoin containing monocytes in the cultured cells from children with malaria was 5.6%. In addition, the relative percentage of monocytes and lymphocytes in the 2 groups of children were equivalent (control: 7.1% monocytes and 54.3% lymphocytes, and malaria group: 7.9% monocytes and 51.0% lymphocytes). The relative proportion of monocytes and lymphocytes remained comparable when determined in a subset of the healthy controls ($n = 10$) and children with malaria ($n = 10$) following ficoll/Hypaque separation. These results suggest that monocyte-derived PGE_2 synthesis is significantly suppressed in children with malaria and is refractory to COX-2–promoting stimuli.

Effect of Hemozoin on PGE_2 Production

Because PBMCs from children with malaria had significantly reduced baseline and stimulated PGE_2 synthesis over a 120-h period, we postulated that an in vivo parasite-driven event induced PGE_2 suppression. As such, a crude hemozoin preparation, representing that most closely observed during a natural infection, was added to cultured PBMCs. Because these experiments required larger volumes of blood than were

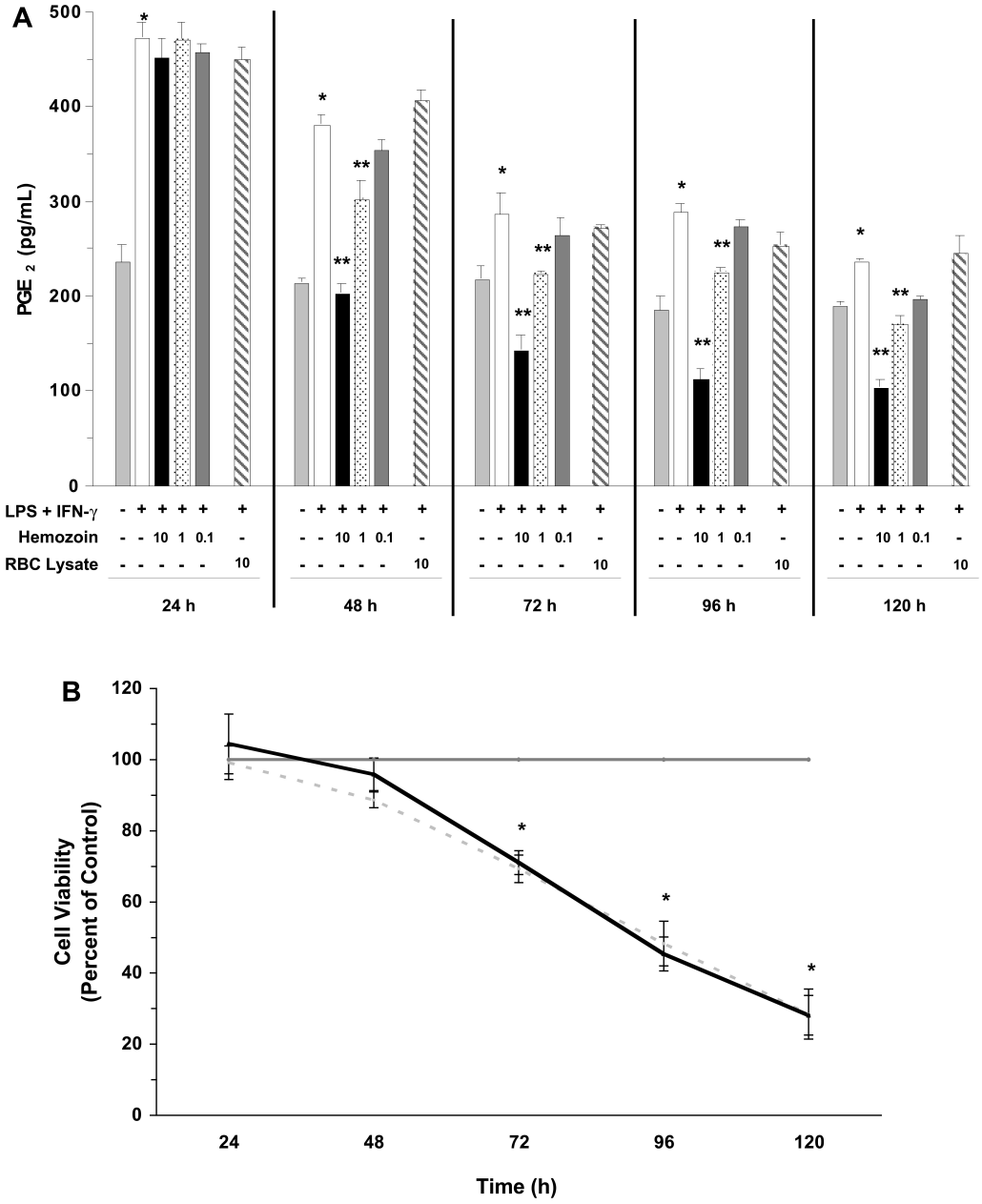

Figure 2. Temporal effect of hemozoin on PGE_2 production. A: PBMCs (1 x 10^6 cells/mL) were stimulated with media alone, LPS (100 ng/mL) and IFN-γ (200 U/mL), and LPS and IFN-γ in the presence of hemozoin (10, 1.0, and 0.1 μg/mL) or an RBC lysate (10 μg/mL). Supernatants were removed at 24, 48, 72, 96, and 120 h for PGE_2 determination. Values are the mean ± SEM and are representative of three independent experiments. Statistical significance determined by the Mann-Whitney U test. *$P < 0.05$ compared with control (media alone), **$P < 0.01$ compared with stimulated conditions. B: Temporal effect of hemozoin on cell viability. PBMCs (1 x 10^6 cells/mL) were stimulated with media alone (solid gray line), LPS (100 ng/mL) and IFN-γ (200 U/mL) (dashed gray line), and LPS and IFN-γ in the presence of hemozoin (10 μg/mL, solid black line). Cell bioactivity was determined by the MTT assay at 24, 48, 72, 96, and 120 h. Values are the mean ± SEM, $n = 3$. Statistical significance was determined by the Mann-Whitney U test. *$P < 0.05$.

available in anemic children, PBMCs were cultured from healthy, malaria-naive adult US donors, followed by stimulation with media alone, LPS and IFN-γ, and LPS and IFN-γ in the presence of physiologic concentrations of hemozoin (10, 1.0, and 0.1 μg/mL [19]). LPS- and IFN-γ stimulation increased PGE_2 levels over baseline conditions at 24 h ($P < 0.05$), with hemozoin having no effect on PGE_2 production (Figure 2A). However, at 48 h and all subsequent time points examined, a high and intermediate concentration of hemozoin reduced PGE_2 production ($P < 0.05$, Figure 2A). Treatment of cultures with an RBC lysate did not alter PGE_2 production at any of the time points examined (see Figure 2A).

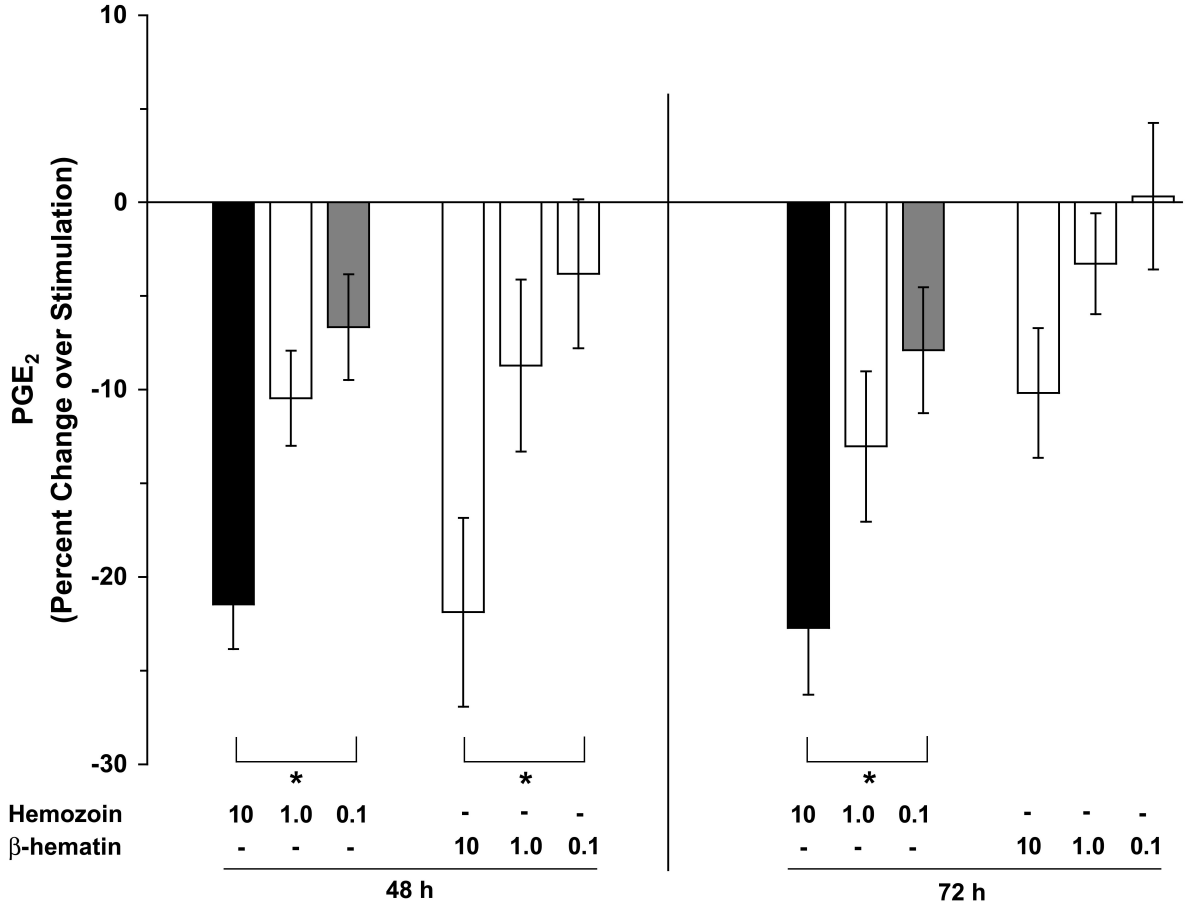

Figure 3. Effect of hemozoin and β-hematin on PGE_2 production. PBMCs (1×10^6 cells/mL) were stimulated with media alone, LPS (100 ng/mL) and IFN-γ (200 U/mL), and LPS and IFN-γ in the presence of hemozoin (10, 1.0, and 0.1 μg/mL) or β-hematin (10, 1.0, and 0.1 μg/mL). Supernatants were removed at 48 and 72 h for PGE_2 determination. Values are the mean ± SEM, n = 18. Data are expressed as percent change over LPS and IFN-γ stimulation. Statistical significance was determined by the Kruskal-Wallis test. *P < 0.05.

Because suppression of PGE_2 by hemozoin could result from hemozoin-induced cell death, the temporal profile of cell viability was examined using the MTT assay. LPS and IFN-γ stimulation decreased cell viability at 72, 96, and 120 h (P < 0.05), with the addition of hemozoin having no effect on cell viability (Figure 2B). Thus hemozoin significantly suppresses PGE_2 through mechanisms that are independent of altering cell viability.

Because PGE_2 production varied in different individuals, we investigated the effect of hemozoin on a larger cohort of malaria-naive healthy adults (n = 18) at 48 and 72 h, the times at which the greatest reductions in PGE_2 were observed (see Figure 2A). Cultures were also stimulated with β-hematin to determine the effects of the core malarial pigment compound, FPIX, on PGE_2 synthesis in the absence of host- and parasite-derived products. PBMCs were stimulated with LPS and IFN-γ in the presence of hemozoin or β-hematin, and data were expressed as percent change in PGE_2 production in hemozoin-treated cells relative to cells stimulated with LPS and IFN-γ. The addition of both hemozoin and β-hematin to PBMCs caused a dose-dependent decrease in PGE_2 production (Figure 3), suggesting that FPIX is responsible for suppression of PBMC-derived PGE_2 production.

Effect of Hemozoin and β-hematin on COX-2 Gene Products

Because inflammatory-derived PGE_2 is dependent on de novo COX-2 mRNA and protein, we hypothesized that hemozoin suppresses PGE_2 through blockade of COX-2 gene expression. As such, COX-2 protein expression was determined by immunoblot analysis, using a COX-2–specific monoclonal antibody in stimulated cells, in the presence and absence of hemozoin or β-hematin. Stimulation of cells increased COX-2 protein levels at 48 h (Figure 4A to 4D), with the addition of hemozoin or β-hematin causing a dose-dependent reduction in COX-2 protein (see Figure 4A to 4D).

To establish whether hemozoin caused a decrease in COX-2 transcripts, COX-2 message was examined using quantitative real time RT-PCR. At 48 h in culture, LPS- and IFN-γ–stimulated PBMCs produced higher levels of COX-2 mRNA (P < 0.01, Figure 5). The addition of a high and intermediate concentration of hemozoin or β-hematin reduced COX-2 transcript formation (P < 0.01, see Figure 5).

To determine the kinetics by which COX-2 transcripts are suppressed by hemozoin, the time-dependent induction of COX-2 mRNA was examined. LPS and IFN-γ stimulation increased COX-2 mRNA by 8 h, which peaked at 24 h (P < 0.01, Figure 6). The addi-

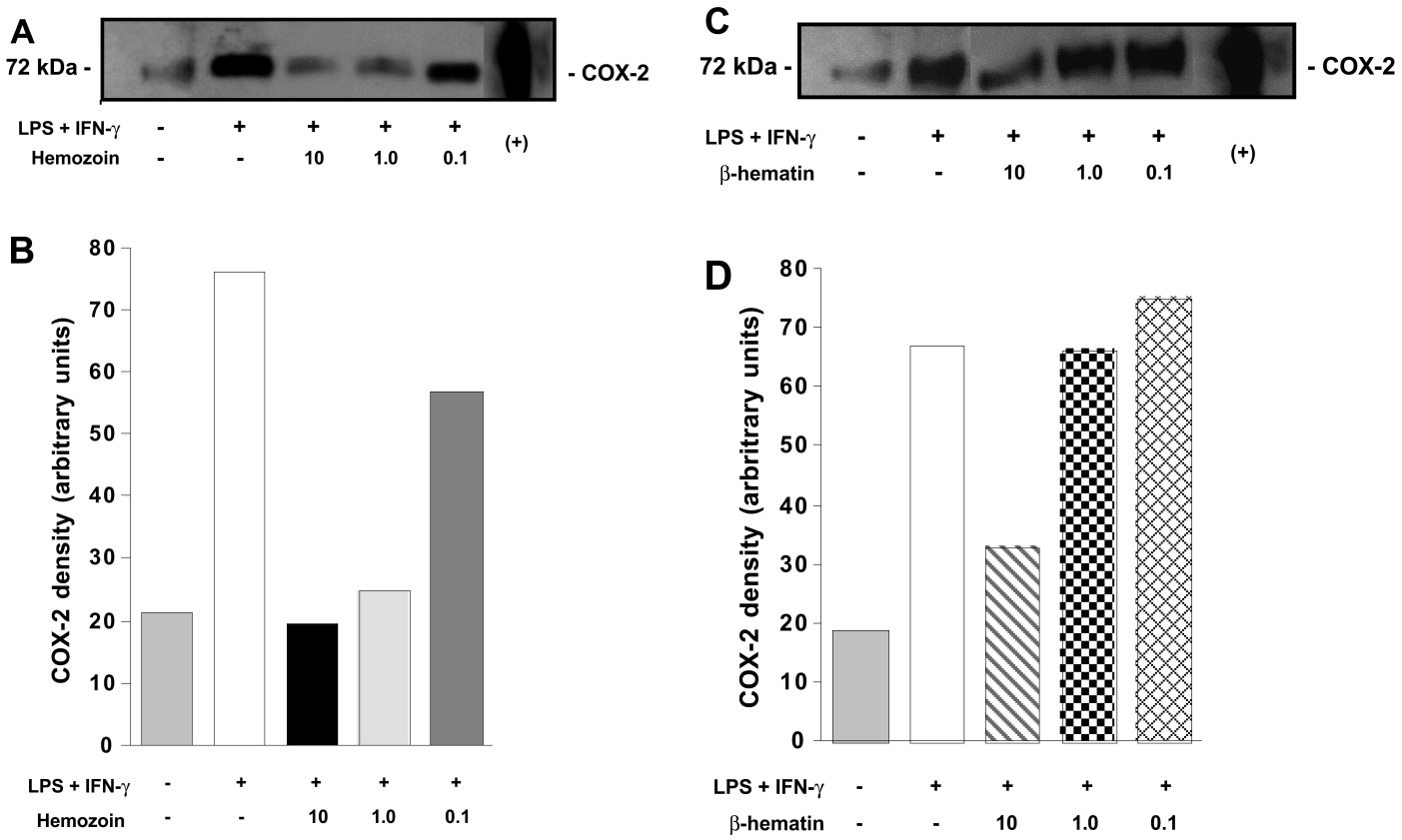

Figure 4. Effect of hemozoin and β-hematin on COX-2 protein expression. PBMCs (1×10^6 cells/mL) were stimulated with media alone, LPS (100 ng/mL) and IFN-γ (200 U/mL), and LPS and IFN-γ in the presence of hemozoin (10, 1.0, and 0.1 μg/mL) or β-hematin (10, 1.0, and 0.1 μg/mL). A,C: Cells were harvested at 48 h for COX-2 protein determination by immunoblot analysis. Shown is a representative immunoblot of 4 independent experiments in different individuals for cells treated with (A) hemozoin or (C) β-hematin. As a positive control (+), extracts of mouse macrophage cells (RAW 264.7), stimulated with lipopolysaccharide (*E. coli* LPS 0127B8, 1.0 μg/mL) and IFN-γ (10 ng/mL) were included. B,D: COX-2 antigen expression was quantified using commercially available software (Scion Image, Scion Corporation) in cells treated with (B) hemozoin or (D) β-hematin and expressed as relative arbitrary units.

tion of hemozoin decreased COX-2 transcript formation at 24 and 48 h ($P < 0.01$, see Figure 6). Taken together, results presented here demonstrate that decreased PGE_2 production occurs in hemozoin-treated cells through reductions in COX-2 transcripts and protein.

Effect of Endogenous IL-10 on Hemozoin-Induced PGE_2 Suppression

Anti-inflammatory cytokines, such as IL-10, suppress de novo COX-2 transcription in monocytes (15). Moreover, we have previously shown that decreased PGE_2 production in children with falciparum malaria was significantly associated with increased plasma IL-10 production (6). We, therefore, investigated whether hemozoin-induced suppression of COX-2/PGE_2 was a consequence of increased IL-10 production. IL-10 was determined in PBMCs stimulated with media alone, LPS and IFN-γ, and LPS and IFN-γ in the presence of hemozoin. At 48 h, stimulation increased IL-10 production ($P < 0.05$), which was further augmented by the addition of hemozoin ($P < 0.05$, Figure 7A).

Because addition of hemozoin to PBMCs increased IL-10 production and suppressed COX-2–derived PGE_2 formation, we determined whether neutralizing antibodies to IL-10 could restore hemozoin-induced reductions in PGE_2. As shown in Figure 7B, addition of IL-10 neutralizing antibodies failed to restore PGE_2 production. Measurement of IL-10 confirmed that endogenously produced IL-10 was dose-dependently reduced in culture supernatants with increasing concentrations of IL-10 neutralizing antibodies (see Figure 7C). Determination of IL-10 transcripts by real-time RT-PCR showed that the addition of IL-10–neutralizing antibodies had no effect on IL-10 transcript levels (data not shown). In addition, the addition of isotype-matched control antibodies had no effect on IL-10 levels (data not shown). Thus, suppression of COX-2–derived PGE_2 occurs through an IL-10–independent mechanism.

DISCUSSION

Our previous studies demonstrated that plasma bicyclo-PGE_2 and ex vivo blood mononuclear cell COX-2 levels are suppressed in children with malaria (6). In the present study, we extend those findings by showing that cultured PBMCs from children with malaria have reduced baseline and stimulated PGE_2 production.

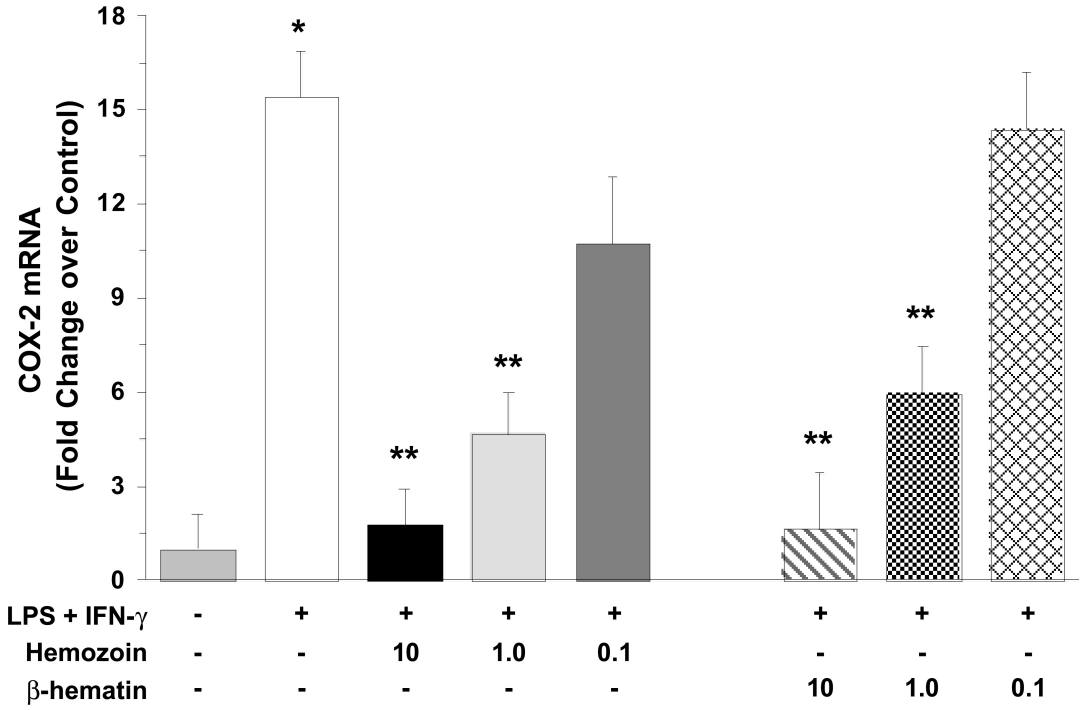

Figure 5. Effect of hemozoin on COX-2 mRNA expression. COX-2 mRNA was quantified for 4 individuals by real time RT-PCR. PBMCs (1×10^6 cells/mL) were stimulated with media alone, LPS (100 ng/mL) and IFN-γ (200 U/mL), and LPS and IFN-γ in the presence of hemozoin (10, 1.0, and 0.1 μg/mL) or β-hematin (10, 1.0, and 0.1 μg/mL). Cells were collected at 48 h for COX-2 mRNA determination. Values are the mean ± SEM, $n = 4$. Statistical significance was determined by the Mann-Whitney U test. *$P < 0.01$ compared with control (media alone), **$P < 0.01$ compared with stimulated conditions.

Because cells were cultured for 120 h to avoid influence of host-derived in vivo regulatory factors, we postulated that suppression of PGE_2 was due to a phagocytosed parasitic product(s). Because hemozoin can persist in mononuclear cells for prolonged periods (28), the effect of hemozoin on PGE_2 was examined. A crude hemozoin isolate was used because this preparation mimics the hemozoin moiety acquired during a natural infection. Consistent with studies illustrating that phagocytosis of hemozoin by monocytic cells occurs within 24 h with the biologic effects occurring over prolonged periods (29,30), hemozoin significantly decreased PGE_2 synthesis in PBMCs from 48 h onward.

The molecular mechanism responsible for hemozoin-induced PGE_2 suppression does not occur through reduced cell viability, because the addition of hemozoin to stimulated PBMCs did not alter mitochondrial bioactivity (that is, MTT assay) at the time points selected for the mechanism-based studies (48 and 72 h). It is important to note that after 72 h in culture, hemozoin reduced cell viability, which was further reduced by LPS and IFN-γ treatment. Thus, it was difficult to interpret experimental results in which cells were pretreated with hemozoin followed by stimulation. Although, results presented here clearly illustrate that hemozoin suppresses COX-2–derived PGE_2 biosythesis, it appears that the natural acquisition of hemozoin in the cytokine-activated in vivo milieu differs slightly from that offered by the in vitro environment. This rationale is supported by the fact that hemozoin reduced PGE_2 biosynthesis by 15% to 53% in cells from healthy donors versus the 70% reduction observed in children with malaria. The apparent discrepancy between in vitro hemozoin-induced reductions in PGE_2 and naturally acquired hemozoin in mononuclear cells from children with malaria may be due to additional host- and/or parasite-derived factors independent of hemozoin. We are currently investigating the effects of additional host immune factors and parasitic products that may alter PGE_2 synthesis during acute malaria.

While we consistently observed PGE_2 suppression in PBMCs stimulated with hemozoin, there was substantial variation in PGE_2 production in different individuals. To address this variation, data were expressed as percent reduction in PGE_2 relative to stimulated conditions in a larger number of individuals ($n = 18$) at time-points when the cell viability was greatest (that is, 48 and 72 h). Expression of data in this manner "normalized" individual variation and demonstrated a dose-dependent suppressive effect of hemozoin on PGE_2 biosynthesis. These experiments further demonstrated that β-hematin dose-dependently reduced PGE_2 production. Because β-hematin is a synthetically prepared compound of FPIX, which lacks host- and parasite-derived proteins and lipids, and is structurally identical to purified (detergent-treated) hemozoin (31,32), it appears that FPIX is responsible for suppression of PGE_2.

Our previous results showed that ex vivo PBMC COX-2 protein and mRNA were decreased in children with malaria (6). This, along with our previous data showing that de novo induction of COX-2 message is required for high levels of sustained PGE_2 production (25), prompted us to investigate hemozoin-induced changes in COX-2 expression. Hemozoin and β-hematin dose-dependently suppressed COX-2 mRNA and protein, indicating

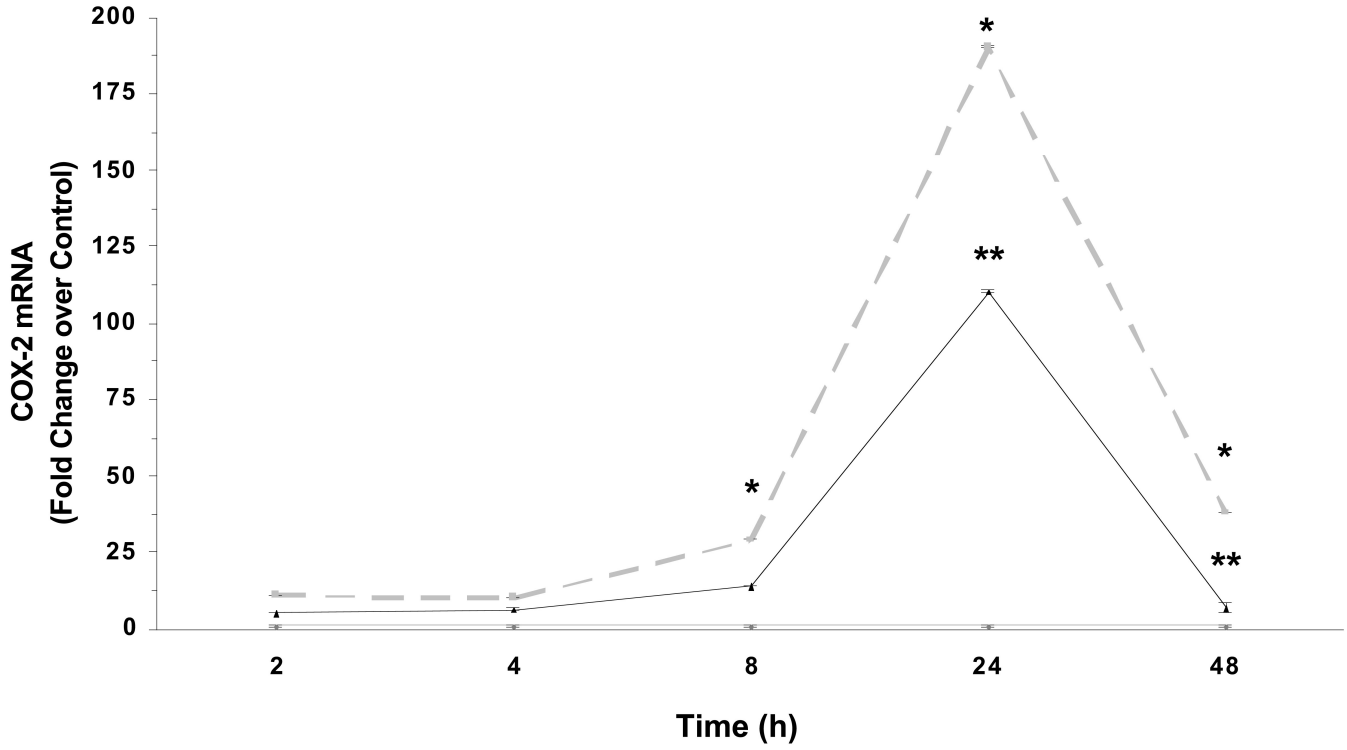

Figure 6. Temporal effect of hemozoin on COX-2 transcripts. PBMCs (1 x 10^6 cells/mL) were stimulated with media alone (solid gray line), LPS (100 ng/mL) and IFN-γ (200 U/mL) (dashed gray line), and LPS and IFN-γ in the presence of hemozoin (10 μg/mL, (solid black line). Cells were collected at 2, 4, 8, 24, and 48 h for COX-2 mRNA determination. Values are the mean ± SEM and are representative of three independent experiments. Statistical significance was determined by the Mann-Whitney U test. *P < 0.01 compared with control (media alone); **P < 0.01 compared with stimulated conditions.

that PGE_2 suppression is caused by reduced levels of COX-2 gene products. Results from kinetic experiments illustrated that hemozoin inhibits de novo COX-2 message at 8 and 24 h. Thus, reductions in COX-2 protein and PGE_2 formation at 48 h are consistent with decreased COX-2 transcripts at earlier time points.

Previous studies in individuals with CM showed increased COX-2 protein in Durck's granulomas within the central nervous system (33). However, our results in plasma and PBMCs from children with malaria (6), and here in hemozoin-treated PBMCs, illustrate that COX-2/PGE_2 is suppressed in circulating blood mononuclear cells and plasma. Our recent results in Tanzanian children with CM also show that peripheral PGE_2 levels are decreased during acute disease, with the most profound reductions in PGE_2 being associated with increased disease sequeale (7). We have further shown that placental monocytes from women with malaria during pregnancy have decreased PGE_2 formation; there was a dose-dependent reduction in PGE_2 with increasing amounts of naturally acquired hemozoin (24). Recent studies in a murine model of CM also showed that COX-2 transcripts were significantly increased within the brain, but nonsignificantly decreased in PBMCs (34). Furthermore, infection of mice with *P. berghei* K173, a parasite strain causing anemia in the absence of CM, significantly decreased peripheral COX-2 transcripts (34). Thus, during acute malaria, COX-2/PGE_2 regulation within the brain differs from COX-2/PGE_2 regulation in the local tissue compartments and in circulating blood monocytes.

Our previous studies showed that decreased plasma PGE_2 levels were significantly associated with increased plasma IL-10 concentrations (6). This is consistent with data illustrating that IL-10 decreases PGE_2 through suppression and/or destabilization of COX-2 transcripts (15). As such, we determined if hemozoin-induced PGE_2 suppression occurred through increased IL-10 production. Hemozoin augmented LPS- and IFN-γ–induced IL-10 production, however hemozoin-induced IL-10 production does not appear responsible for PGE_2 suppression because IL-10 neutralizing antibodies failed to restore PGE_2. Thus, the molecular mechanism(s) by which hemozoin suppresses COX-2/PGE_2 remains unclear. Previous studies show that activation of the protein kinase C (PKC) signaling pathway is essential for COX-2 induction in human intestinal myofibroblasts (35) and that inhibition of PKC attenuates IL-1β induction of COX-2/PGE_2 in human pulmonary epithelial cells (36). This appears important for results presented here because previous studies found that hemozoin suppresses PKC activity in cultured monocytes (16). We are currently determining if reduced levels of COX-2 in malaria occur through inhibition of PKC signal transduction pathways.

Although decreased systemic PGE_2 levels are associated with increasing disease severity, it is unclear how suppressed peripheral PGE_2 promotes enhanced malaria pathogenesis. Potential pathogenic effects may occur through suppression of erythropoiesis because previous studies demonstrate that PGE_2 enhances burst-forming unit erythroid formation (37), suggesting that suppression

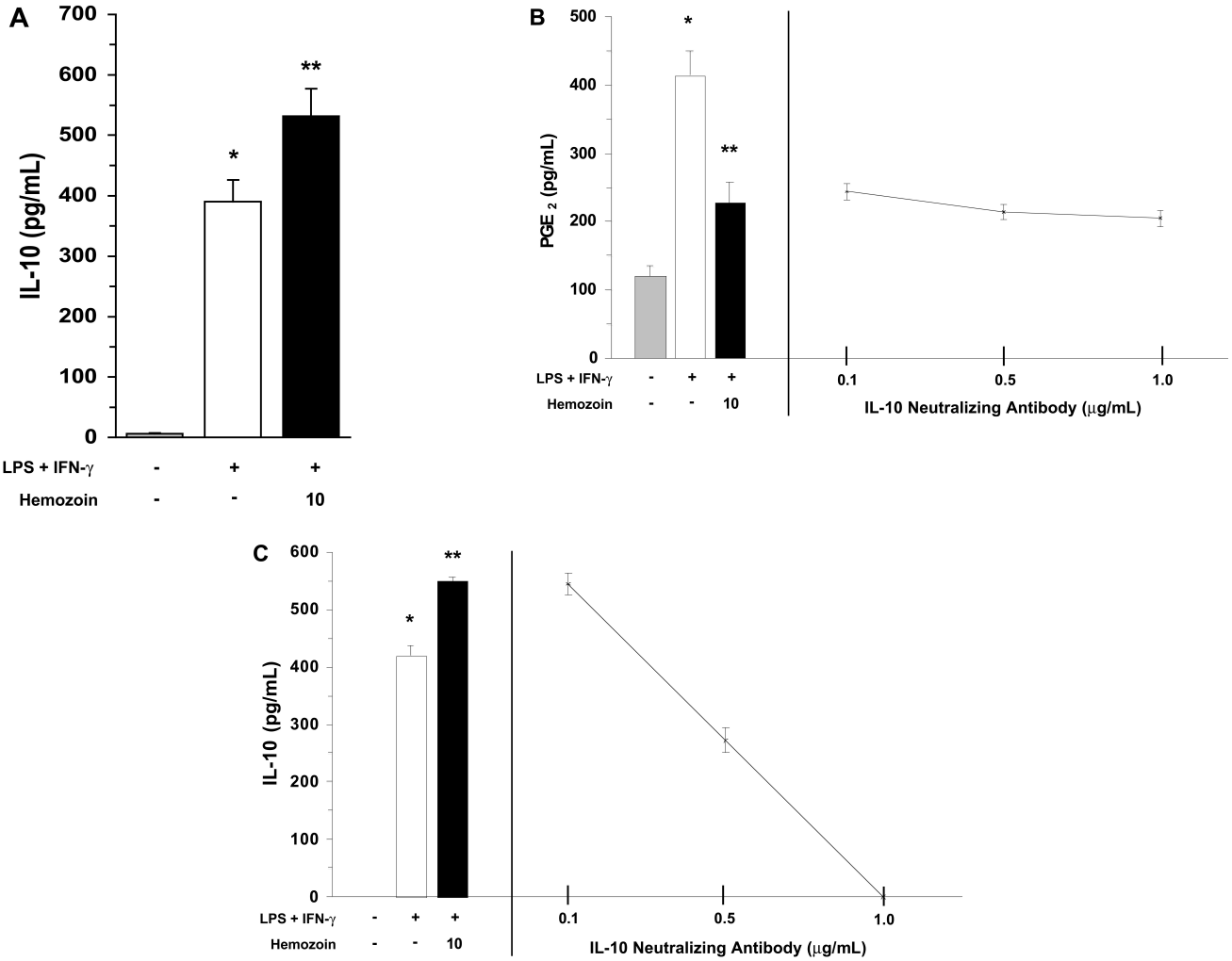

Figure 7. Effect of endogenous IL-10 on hemozoin-induced PGE_2 suppression. A: PBMCs (1 x 10^6 cells/mL) were stimulated with media alone, LPS (100 ng/mL) and IFN-γ (200 U/mL), and LPS and IFN-γ in the presence of hemozoin (10 μg/mL), and supernatants were obtained at 48 h for IL-10 determination. Values are the mean ± SEM for 10 individuals, *n* = 4/condition for each individual. Statistical significance was determined by the Mann-Whitney U test. **P* < 0.01 compared with control (media alone); ***P* < 0.05 compared with stimulated conditions. B,C: PBMCs (1 x 10^6 cells/mL) were stimulated with media alone, LPS (100 ng/mL) and IFN-γ (200 U/mL), and LPS and IFN-γ in the presence of hemozoin (10 μg/mL, solid black line), and were treated with varying concentrations of an IL-10 neutralizing antibody (1.0, 0.5, and 0.1 μg/mL). Supernatants were obtained at 48 h for (B) PGE_2 and (C) IL-10 determination. Values are the mean ± SEM, *n* = 5. Statistical significance was determined by the Mann-Whitney U test. **P* < 0.01 compared with control (media alone); ***P* < 0.05 compared to stimulated conditions.

of systemic PGE_2 by hemozoin may reduce formation of new RBC. Additional mechanisms by which decreased systemic PGE_2 could promote increased pathogenesis may occur through overexpression of the pro-inflammatory response. For example, PGE_2 is important for resolution of the late-phase immune response through promotion of T_H2-derived cytokine production (for review, see Phipps and others [38]). PGE_2 also regulates the pro-inflammatory response by suppressing de novo TNF-α transcription (39) and by modulating the secretion of soluble TNF-α receptors (40). PGE_2 may also alter malaria pathogenesis through modulation of lymphocytic activity. Previous studies in cultured human mononuclear cells from individuals with acute falciparum malaria showed that the non-selective COX inhibitor, indomethacin, reversed malarial antigen-induced suppression of lymphoproliferation (41). However, levels of COX-2/PGE_2 were not determined in these studies (41). Subsequent studies showed that exogenous addition of PGE_2 failed to suppress T-cell proliferation (42). The apparent discrepancy in these results may be related to the fact that indomethacin alters a number of cellular functions in a COX-independent fashion (for review, see Tegeder and others [43]). We are currently investigating the effects of COX-2/PGE_2 suppression on malaria pathogenesis in children with severe malarial anemia.

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The study was approved by the ethics committee of the International Foundation of the Albert Schweitzer Hospital, Duke University Medical Center Investigational Review Board, and the University of Pittsburgh Investigational Review Board, and informed consent was obtained from the participants or the parents of participating children.

There is no conflict of interest for any of the authors of the manuscript due to commercial or other affiliations.

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