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Hemozoin Increases IFN-γ-Inducible Macrophage Nitric Oxide Generation Through Extracellular Signal-Regulated Kinase- and NF-κB-Dependent Pathways[1]

Maritza Jaramillo,* D. Channe Gowda,[‡] Danuta Radzioch,[†] and Martin Olivier[2]*[†]

NO overproduction has been suggested to contribute to the immunopathology related to malaria infection. Even though a role for some parasite molecules (e.g., GPI) in NO induction has been proposed, the direct contribution of hemozoin (HZ), another parasite metabolite, remains to be established. Therefore, we were interested to determine whether *Plasmodium falciparum (Pf)* HZ and synthetic HZ, β-hematin, alone or in combination with IFN-γ, were able to induce macrophage (Mϕ) NO synthesis. We observed that neither *Pf* HZ nor synthetic HZ led to NO generation in B10R murine Mϕ; however, they significantly increased IFN-γ-mediated inducible NO synthase (iNOS) mRNA and protein expression, and NO production. Next, by investigating the transductional mechanisms involved in this cellular regulation, we established that HZ induces extracellular signal-regulated kinase (ERK)1/2 mitogen-activated protein kinase phosphorylation as well as NF-κB binding to the iNOS promoter, and enhances the IFN-γ-dependent activation of both second messengers. Of interest, cell pretreatment with specific inhibitors against either NF-κB or the ERK1/2 pathway blocked the HZ + IFN-γ-inducible NF-κB activity and significantly reduced the HZ-dependent increase on IFN-γ-mediated iNOS and NO induction. Even though selective inhibition of the Janus kinase 2/STAT1α pathway suppressed NO synthesis in response to HZ + IFN-γ, HZ alone did not activate this signaling pathway and did not have an up-regulating effect on the IFN-γ-induced Janus kinase 2/STAT1α phosphorylation and STAT1α binding to the iNOS promoter. In conclusion, our results suggest that HZ exerts a potent synergistic effect on the IFN-γ-inducible NO generation in Mϕ via ERK- and NF-κB-dependent pathways. ***The Journal of Immunology,*** **2003, 171: 4243–4253.**

Nitric oxide, an unstable free radical gas, results from the oxidative deamination of L-arginine to produce L-citrulline through a reaction catalyzed by the enzyme NO synthase (NOS)[3] (1). Among the different isoforms of NOS, inducible NOS (iNOS) is responsible for the high output of NO synthesis in macrophages (Mϕ) (2) and it can be induced by proinflammatory cytokines, such as IFN-γ and IL-1 (3). As an intercellular messenger, NO has been implicated in diverse physiological processes (e.g., vascular homeostasis, neurotransmission, host immunity) as well as in many pathological conditions (e.g., arthritis, diabetes, cancer) (4). As a cytotoxic/cytostatic effector molecule, NO has been shown to inhibit the growth and function of diverse infectious disease agents (e.g., bacteria, fungi, protozoan parasites) mainly by inactivating some of their critical metabolic pathways (2).

In malaria, increased NO production has been reported in both laboratory models of infection (5) and human patients (6). Numerous studies regarding its role during malaria have revealed that NO can exert both beneficial and detrimental effects on the host, depending on, among other things, the timing and amounts of its production and the biological milieu in which it is released (7). As a host defender, NO has been shown to mediate in vitro killing of both hepatic and blood forms of malaria parasites in response to IFN-γ (8, 9). These results have been further supported in vivo by demonstrating that protection against parasite challenge requires IFN-γ and occurs via a NO-dependent mechanism (10). Despite the beneficial effects, excess of NO has been proposed to contribute to malarial immunosuppression (11) as well as to the development of cerebral malaria pathology, the most lethal complication of *Plasmodium falciparum (Pf)* infection (e.g., increased intracraneal pressure, reversible cerebral coma) (12).

Even though NO production and function during malaria are well-documented, the malaria-specific metabolites leading to such induction, as well as the signal transduction mechanisms underlying this process, have not yet been fully characterized. At this regard, Tachado et al. (13) reported that malarial GPI was able to induce NO release and to synergize with IFN-γ in regulating NO production in both murine Mϕ and human endothelial cells, through a mechanism depending on tyrosine kinase, protein kinase C, and NF-κB activity. However, no studies have been performed to evaluate the potential contribution of hemozoin (HZ), another important parasite metabolite, to NO overproduction during malaria.

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[3] Abbreviations used in this paper: NOS, NO synthase; iNOS, inducible NOS; Mϕ, macrophage; *Pf*, *Plasmodium falciparum*; HZ, hemozoin; sHZ, synthetic HZ; ERK, extracellular signal-regulated kinase; Poly B, polymixin B; TLR, Toll-like receptor; GAS, γ-activated site; Jak, Janus kinase; MAPK, mitogen-activated protein kinase; ROS, reactive oxygen species; IRF, IFN-γ-responsive factor; DFX, desferrioxamine.

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HZ or malarial pigment is a polymer of heme produced by the parasite during hemoglobin degradation inside the host RBC. This pigment is released along with merozoites as the erythrocyte bursts and is avidly phagocytosed by circulating monocytes, neutrophils, and resident Mϕ both in vitro and in vivo (reviewed by Arese in Ref. 14). Formerly, HZ was considered only as a waste product of the parasite's metabolism; however, several lines of evidence strongly suggest that it could play an important role in malaria pathophysiology related to cytokine overproduction and parasite cytoadherence. In vitro studies reported that HZ induces the release of pyrogenic cytokines (TNF-α, macrophage inflammatory proteins-1α and -1β) in murine Mϕ and human monocytes (15) as well as adhesion molecule expression and IL-6 production in human endothelial cells (16). These data were further supported by those of Biswas et al. (17) who recently described the presence of IgM Abs against *Pf* HZ among complicated malaria patients, which had inhibitory effects on TNF-α and IL-1β production by monocytes.

In the present study, we demonstrate that both native *Pf* HZ and synthetic HZ (sHZ), β-hematin, which is structurally identical to the native pigment (18), potently enhance IFN-γ-mediated iNOS mRNA and protein expression leading to a significant increase on NO production. Analysis of the signal transduction mechanisms involved in this process revealed that *Pf* HZ and sHZ exert their up-regulating effect by inducing the activation of the extracellular signal-regulated kinases (ERK) 1 and 2 pathway and the binding of NF-κB to the murine iNOS promoter. Taken together, our data suggest that HZ could play an important role on the malaria pathology related to NO overproduction.

Materials and Methods

Materials

Hemin chloride, polymixin B (Poly B) sulfate, LPS (*Escherichia coli*, serotype 0111:B4), and desferrioxamine mesylate salt were purchased from Sigma-Aldrich (St. Louis, MO). Recombinant murine 2×10^5 U/ml IFN-γ was obtained from Life Technologies (Rockville, MD). Isotopes [α-^{32}P]dCTP (3000 Ci/mmol) and [γ-^{32}P]dATP (3000 Ci/mmol) were purchased from ICN Pharmaceuticals (Montreal, Québec, Canada). Specific inhibitors AG 490 and caffeic acid phenethyl ester (CAPE) were obtained from Biomol Research Laboratories (Plymouth Meeting, PA). Apigenin and PD 98059 were obtained from Calbiochem (San Diego, CA).

Cell and culture conditions

The Mϕ cell line B10R is derived from the bone marrow of B10A.Bcgr (B10R) mice (19) and the Mϕ cell line Toll-like receptor (TLR)4 DEL is derived from the bone marrow of LPS-unresponsive mice of the strain C57BL/10ScCr (deletion mutant for *Tlr4* gene) (20), according to the previously described method (21). Cells were maintained in DMEM (Life Technologies) supplemented with 10% heat-inactivated FBS (HyClone Laboratories, Logan, UT) plus 100 μg/ml streptomycin and 2 mM L-glutamine at 37°C and 5% CO_2.

Cell viability assays

(3-(4,5-Dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt) (MTS) assays (22) for cell viability were performed and indicated no cytotoxic or cytostatic effect from the various specific inhibitors (AG 490, PD 98059, apigenin, and CAPE) at the concentrations used (data not shown). Briefly, B10R Mϕ were seeded in 96-well plates (3×10^4 cells/well) and were stimulated overnight with increasing doses of the various inhibitors (100 μl). Then, cells were incubated with 10 μl of a 20:1 solution of 2 mg/ml MTS, 3 mM phenazine methosulfate for 1 h and OD_{492} was read. MTS and phenazine methosulfate were kindly provided by Dr M. Tremblay (Université Laval, Québec, Canada).

NO generation

Mϕ were seeded in 24-well plates (5×10^5 cells/well) and cultured in the presence or absence of specific inhibitors for 1 h before *Pf* HZ or sHZ ± IFN-γ (100 U/ml) stimulation for 24 h. Then, NO generation was evaluated by measuring the accumulation of nitrite in the culture medium (Griess reaction) as we previously described (23).

Northern blot analysis

Expression of iNOS mRNA was evaluated by Northern blot as we reported previously (23). Briefly, following stimulation, cells were washed twice with PBS and total RNA was extracted with TRIzol reagent (Life Technologies). Then, 10 μg of RNA were loaded on 1% agarose gels, transferred onto Hybond-N filter paper, and hybridized with random primer-labeled cDNA probes. Equal RNA loading was confirmed by hybridization with a GAPDH cDNA probe. All washes were performed under stringent conditions and transcripts were visualized by autoradiography. Laser densitometry was performed by using an α Imager 2000 digital imaging and analysis system (Alpha Innotech, San Leandro, CA). A 2.3-kb fragment of the putative Mϕ *iNOS* gene was kindly provided by Dr S. Snyder (Johns Hopkins University, Baltimore, MD). The GAPDH probe was generated as previously described (24).

Preparation of nuclear extracts

Cell stimulation was terminated by the addition of ice-cold PBS and nuclear extracts were prepared according to the microscale preparation protocol (25). In brief, sedimented cells were resuspended in 400 μl of cold buffer A (10 mM HEPES, pH 7.9, 10 mM KCl, 1.0 mM DTT, and 0.5 mM PMSF). After 15 min on ice, 25 μl of 10% igepal (v/v) (Sigma-Aldrich) were added, and the lysate was vortexed for 10 s and centrifuged for 30 s at $12,000 \times g$. The supernatant was discarded and the cell pellet was resuspended in 100 μl of cold buffer B (20 mM HEPES, pH 7.9, 0.4 M NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, and 1 mM PMSF). Cells were then rocked vigorously at 4°C for 15 min. Cellular debris were removed by centrifugation at $12,000 \times g$ for 5 min at 4°C, and the supernatant was stored at −80°C until used.

EMSA

EMSA was performed with 6 μg of nuclear extract. Protein concentrations were determined using the commercial BCA Protein Assay Reagent (Pierce, Rockford, IL). As we previously described (26), nuclear extracts were incubated for 20 min at room temperature in 1.0 μl of binding buffer (100 mM HEPES, pH 7.9, 40% glycerol, 10% Ficoll, 250 mM KCl, 10 mM DTT, 5 mM EDTA, 250 mM NaCl), 2 μg of poly(dI-dC), and 10 μg of nuclease-free BSA (fraction V) (Sigma-Aldrich) containing 1.0 ng of radio-labeled dsDNA oligonucleotide. dsDNA (100 ng) was end-labeled by using [γ-^{32}P]dATP and T4 polynucleotide kinase (New England Biolabs, Beverly, MA). This mixture was incubated for 20 min at room temperature and the reaction was stopped using 5 μl of 0.2 M EDTA. The labeled oligonucleotide was extracted with phenol/chloroform and passed through a G-50 spin column. The dsDNA oligonucleotides, used either as probes or as competitors, containing NF-κB or STAT (γ-activated site (GAS)) binding sites present in the murine iNOS promoter were synthesized in our laboratory as follows: NF-κB/iNOS, 5′-GGATAAGCTTGGGGGATTTTCCCTCT-3′ (27); GAS/iNOS, 5′-CTTTTCCCCTAACAC-3′ (28). The nonspecific probe Oct-2A 5′-GGAGTATCCAGCTCCGTAGCATGCAAATCCTCTGG-3′ was used to confirm the specificity of the DNA/nuclear protein reaction and was kindly provided by Dr M. Tremblay (Université Laval). DNA-protein complexes were resolved from free-labeled DNA by electrophoresis in native 4% (w/v) polyacrylamide gels containing 50 mM Tris-HCl, pH 8.5, 200 mM glycine, and 1 mM EDTA. The gels were subsequently dried and autoradiographed. Cold competitor assays were conducted by adding a 100-fold molar excess of homologous unlabeled oligonucleotides of the various labeled dsDNA probes. Supershift assays were performed by preincubation of nuclear extracts with 2 μg of polyclonal Abs against p65 (Rel A), p50, or STAT1α obtained from Santa Cruz Biotechnology (Santa Cruz, CA), in the presence of all components of the binding reaction described above for 30 min at 4°C.

Western blotting

Cells were collected following stimulation, lysed in cold buffer containing 20 mM Tris-HCl, pH 8.0, 0.14 M NaCl, 10% glycerol (v/v), 1% igepal (v/v), 25 μM nitrophenyl guanidinobenzoate, 10 μM sodium fluoride, 1 mM sodium orthovanadate, 25 μg/ml leupeptin and aprotinin. The lysates (20 μg/lane) were subjected to SDS-PAGE and the separated proteins transferred onto a polyvinylidene difluoride membrane (Millipore, Bedford, MA), as we previously described (29). After a 1 h blocking period in TBST containing 5% milk, the membranes were incubated with one of the following Abs: anti-iNOS was obtained from Cedarlane Laboratories (Hornby, Ontario, Canada), anti-phospho-Janus kinase (Jak)2 (Tyr^{1007}, Tyr^{1008}) and Jak2 were obtained from Upstate Biotechnology (Lake Placid, NY); phospho-ERK1/2 (Thr^{202}/Tyr^{204}) and ERK1/2 were obtained from New England Biolabs; anti-STAT1α p91 and anti-actin were obtained from Santa Cruz Biotechnology (Santa Cruz, CA); anti-phospho-STAT1α

(Tyr^{701}) and anti-phospho-STAT1α (Ser^{727}) were kindly provided by Dr. D. Frank (Harvard Medical School, Boston, MA). Proteins were then detected with an anti-mouse or anti-rabbit HRP-conjugated goat Ab (Affinipure; Jackson ImmunoResearch Laboratories, West Grove, PA) and subsequent visualization by ECL (ECL Western blotting detection system; Amersham, Arlington Heights, IL).

Pf *HZ extraction*

Parasites were released from RBC by mild saponin lysis, washed, and purified on a cushion of 5% BSA to remove RBC debris. Next, parasites were lyophilized and extracted with chloroform-methanol (2:1 v/v) to remove lipids, and with chloroform-methanol-water (10:10:3 v/v) to remove GPIs. After drying, the residue was resuspended in 100 mM Tris-HCl, 1 mM $CaCl_2$, pH 7.5, and digested with pronase to remove proteins and protein-linked GPIs. The insoluble residue was extracted with 50 mM sodium phosphate, pH 7.2, 4 M guanidine hydrochloride, 0.5% Triton X-100, stirred overnight at 4°C to remove nucleic acids. The residue (insoluble pigment, HZ) was recovered by centrifugation and washed three times with water, once with 80% 1-propanol, and dried. HZ was resuspended in PBS-endotoxin free (Life Technologies) at a final concentration of 2.5 mg/ml and kept at −20°C.

β-hematin (sHZ) preparation

The method for β-hematin synthesis described by Egan et al. (30) was adapted for the present study. Briefly, 45 mg of hemin chloride (Sigma-Aldrich) were solubilized in 4.5 ml of 1 N NaOH and neutralized with 450 μl of 1 N HCl. Then, 10.2 ml of 1 M sodium acetate, pH 4.8, were added and the suspension was stirred with a magnet for 2–3 h at 60°C. Following addition of a 1/100 volume of 10% SDS and 14,000 × *g* centrifugation for 15 min, the pellet was sonicated at the lowest setting in 100 mM sodium bicarbonate, pH 9.0, 0.5% SDS and again centrifuged. The pellet was then washed three to four times in SDS 2% and then in water to wash out SDS. The pigment was dried at 37°C overnight, resuspended in PBS-endotoxin free (Life Technologies) at a final concentration of 2.5 mg/ml and kept at −20°C.

Heme quantitation

Total heme content was determined, as described by Sullivan et al. (31), by depolymerizing heme polymer in 1 ml of 20 mM NaOH/2% SDS, incubating the suspension at room temperature for 2 h, and then reading the OD_{400} (Beckman DGB UV/visible spectrophotometer). Twenty micrograms of sHZ equals 26 nmol heme content and 25 μg of *Pf* HZ equals 29 nmol heme content.

Statistical analysis

Statistically significant differences were determined by using the ANOVA (ANOVA) module of SAS software (version 6.07; SAS Institute, Cary, NC) and the Fisher least significant difference test. Values of $p < 0.05$ were deemed statistically significant. All data are presented as mean ± SEM.

Results

Pf *HZ and sHZ increase IFN-γ-inducible NO generation in murine Mϕ*

We initially sought to determine whether *Pf* HZ and sHZ were sufficient to induce NO formation in Mϕ. In addition, given that IFN-γ plays a key role on NO-dependent host defense against malaria infection (10), we also investigated whether *Pf* HZ and sHZ had the capacity to modulate Mϕ NO synthesis in response to IFN-γ. To this end, murine B10R Mϕ were stimulated with increasing 10–50 μg/ml doses of either *Pf* HZ or sHZ and/or 100 U/ml IFN-γ for 24 h, and nitrite production was monitored by the Griess reaction. As shown in Fig. 1, neither *Pf* HZ nor sHZ led to NO generation; however, they significantly increased IFN-γ-mediated NO production in a dose-dependent manner, reaching at maximal *Pf* HZ or sHZ concentrations up to a 3-fold increase over cells treated with IFN-γ alone. In addition, kinetic analysis revealed that the observed synergy occurred regardless of the sequence of stimulation (IFN-γ followed by HZ or vice versa) as well as of the time of HZ treatment, 8, 4, 2, 1, 0 h before/after IFN-γ. Subsequent experiments presented in this section are those following cell stimulation with sHZ, β-hematin; however, all of them were performed in parallel in the presence of *Pf* HZ and the results were the same as those obtained with sHZ.

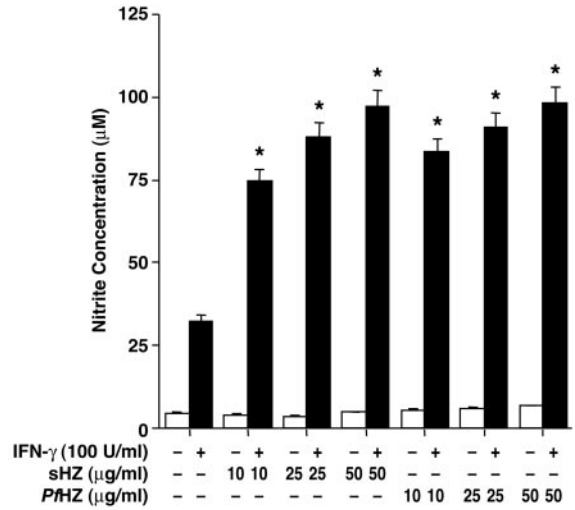

FIGURE 1. *Pf* HZ and sHZ increase IFN-γ-inducible NO generation in murine Mϕ. Cells were stimulated with increasing 10–50 μg/ml doses of either *Pf* HZ or sHZ and/or 100 U/ml IFN-γ for 24 h. Then, supernatants were collected and submitted to the colorimetric Griess reaction to evaluate nitrite production. □, Untreated, sHZ, or *Pf* HZ; ■, IFN-γ ± sHZ or *Pf* HZ. Results are representative of one of three independent experiments performed in triplicate (mean ± SEM, $n = 3$). *, $p < 0.05$, sHZ or *Pf* HZ + IFN-γ vs IFN-γ.

sHZ-dependent up-regulation of IFN-γ-mediated NO synthesis is not due to LPS contamination

To demonstrate that the sHZ up-regulatory effect on IFN-γ-mediated NO generation was not due to the presence of LPS, cells were incubated for 24 h in culture medium (DMEM 10% FBS) containing either 100 ng/ml LPS or 25 μg/ml sHZ plus 100 U/ml IFN-γ, treated or not with 1–10 μg/ml Poly B for 30 min before cell stimulation. Because Poly B binds to LPS and blocks its activity (32), this compound has the capacity to inhibit LPS-inducible Mϕ NO synthesis. As depicted in Fig. 2*A*, Poly B did not exert any inhibitory effect on sHZ + IFN-γ-dependent NO generation; however, this compound dramatically reduced NO induction by LPS in a dose-dependent manner, reaching 100% of inhibition when maximal Poly B concentrations were added. To rule out the possibility of LPS contamination, TLR4 DEL Mϕ derived from LPS-unresponsive mice and B10R Mϕ were stimulated either with IFN-γ ± sHZ or with LPS for 24 h and nitrite production was measured. As shown in Fig. 2*B*, the effect of LPS was detected in B10R Mϕ but was completely abrogated in TLR4 DEL Mϕ. In contrast, the synergistic effect of sHZ on the IFN-γ-mediated NO induction was present in both B10R and TLR4 DEL Mϕ. These data confirmed that the sHZ-dependent up-regulatory event is not due to the presence of LPS in the sHZ preparations.

sHZ up-regulation of IFN-γ-inducible NO synthesis depends on the increase of iNOS mRNA and protein levels

Because the iNOS enzyme is responsible for the high output of NO synthesis in Mϕ (2) and its regulation takes place mostly at the transcription level (33), we were interested to establish whether the sHZ synergistic effect on IFN-γ-dependent NO production was due to an increase on iNOS expression. As shown in Fig. 3*A*, kinetic analysis (0–24 h) revealed that iNOS mRNA and protein levels were not increased by sHZ alone; however, their IFN-γ-mediated up-regulation was significantly enhanced by sHZ in a

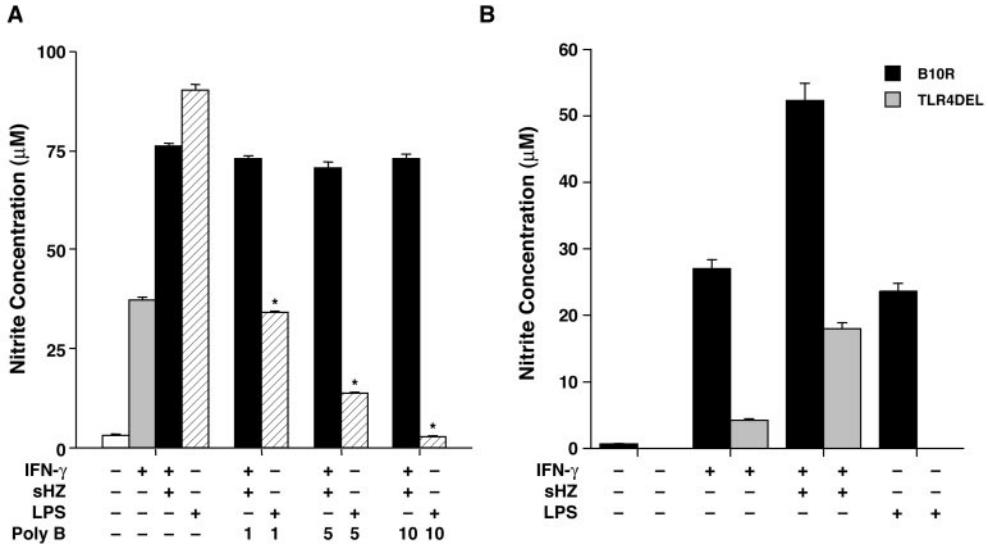

FIGURE 2. sHZ-dependent up-regulation of IFN-γ-mediated NO synthesis is not due to LPS contamination. *A*, DMEM 10% FBS medium, containing 100 ng/ml LPS or 25 μg/ml sHZ plus 100 U/ml IFN-γ was incubated or not with 1–10 μg/ml Poly B for 30 min at 37°C and 5% CO_2. Then, cells were stimulated with one the various conditioned mediums for 24 h and nitrite production was monitored as described in Fig. 1. □, Untreated; ▨, IFN-γ; ■, sHZ + IFN-γ ± Poly B; ▨, LPS ± Poly B. *, $p < 0.05$, LPS + Poly B vs LPS. *B*, B10R and TLR4 DEL Mϕ were stimulated either with 100 U/ml IFN-γ ± 25 μg/ml sHZ or with 100 ng/ml LPS for 24 h and nitrite production was measured. ■ B10R; ▨, TLR4 DEL. Results are representative of one of three separate experiments performed in triplicate (mean ± SEM, $n = 3$).

time-dependent manner (already detectable after 8 h of stimulation and maximal at 24 h). In correlation with these observations, the NO synthesis in response to sHZ + IFN-γ also occurred in a time-dependent manner, showing a slight but significant 4-fold increase following an 8-h treatment, and a maximal 35-fold induction over negative control after 24 h (Fig. 3*B*).

The synergistic effect of sHZ on IFN-γ-dependent NO induction is not reduced in presence of an iron chelator

A characteristic feature of the malaria pigment is the presence of a central ferric iron (Fe^{3+}) on the heme group, which constitutes the structural basis of HZ (34). To investigate whether the sHZ-mediated up-regulation of IFN-γ-inducible NO synthesis was simply an effect of HZ iron, B10R Mϕ were treated for 1 h with increasing 100–400 μM doses of desferrioxamine (DFX), a commonly used iron chelator (35). Then, IFN-γ ± sHZ were added and nitrite production was monitored after 24 h. As shown in Fig. 4, in presence of DFX, even at a very high dose (400 μM) (36), the synergistic effect of sHZ was not significantly reduced, confirming that the noticed sHZ-mediated up-regulation of IFN-γ-inducible NO is not due to sHZ iron.

Role of the Jak2/STAT1α pathway on sHZ + IFN-γ-dependent Mϕ NO regulation

Having established that the observed sHZ up-regulatory effect on NO synthesis was not an iron-dependent event, we were interested to investigate whether sHZ was acting by specifically activating Mϕ signaling pathways. STAT proteins are latent cytoplasmic transcription factors that are phosphorylated by Jak in response to cytokines, such as IFN-γ. Phosphorylated STAT proteins translocate to the nucleus, where they transiently turn on specific sets of cytokine-inducible genes (37). In Mϕ, the importance of the Jak2/STAT1α pathway on NO modulation by IFN-γ has been previously demonstrated (28). Therefore, we wished to examine the potential contribution of this signaling cascade to the sHZ + IFN-γ-mediated NO generation. As depicted in Fig. 5, when cells were treated with increasing 1–50 μM doses of AG 490, a Jak2 tyrosine kinase-specific inhibitor, before sHZ + IFN-γ stimulation, we observed a dose-dependent reduction on NO generation. A 71% inhibition was detected at 25 μM AG 490 and a 92% reduction at maximal inhibitor concentrations. In addition, because we have previously shown that AG 490 abrogates IFN-γ-dependent NO production (38), an inhibition control was performed by incubating cells with IFN-γ in the absence or in the presence of 50 μM AG 490; as expected, high doses of this compound completely blocked NO synthesis by IFN-γ. Thereafter, to more directly address the question of whether activation of the Jak2-STAT1α pathway was taking place following cell treatment with sHZ alone or in combination with IFN-γ, the phosphorylation status of both Jak2 and STAT1α was evaluated at various time points. As shown in Fig. 6, *A* and *B*, sHZ did not cause Jak2 and STAT1α phosphorylation and did not exert any up-regulatory effect on the IFN-γ-induced phosphorylation of both kinases. Next, knowing that upon IFN-γ-induced STAT1α nuclear translocation, this factor is able to bind to defined GAS present in the murine iNOS promoter and initiate transcription (37), we tested the capacity of sHZ to induce STAT1α binding to the murine *iNOS* gene and/or to enhance this regulatory event in response to IFN-γ (Fig. 6*C*). As expected, IFN-γ significantly and transiently induced STAT1α nuclear translocation and its subsequent binding to the iNOS promoter, being maximal following a 1-h treatment and progressively decreasing thereafter up to 4 h of stimulation. However, and in agreement with our data obtained by Western blot, sHZ did not lead to STAT1α binding to the GAS/iNOS-containing sequence and was not able to potentiate it in response to IFN-γ. The specificity of this binding complex was demonstrated by the fact that unlabeled GAS/iNOS oligonucleotide could compete effectively for binding while an unrelated Oct-2A probe could not. In addition, supershift assays allowed us to confirm that the IFN-γ-induced complex was in fact STAT1α. As illustrated in Fig. 6*D*, when nuclear extracts from sHZ + IFN-γ-treated cells (1 h) were incubated with a specific Ab against STAT1α, the complex binding was completely abrogated. Altogether, this set of experiments suggests that even though IFN-γ-dependent activation of the Jak2/STAT1α pathway

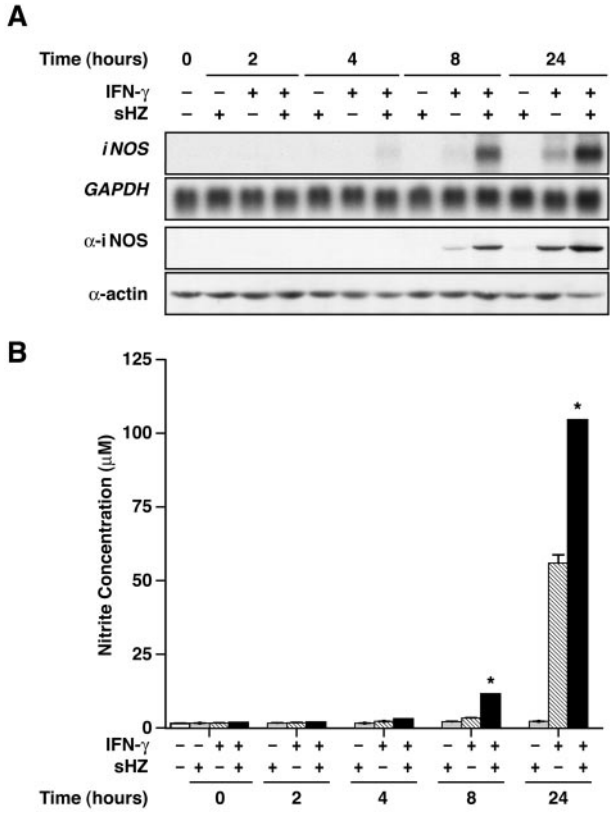

FIGURE 3. sHZ synergizes with IFN-γ to increase iNOS mRNA and protein levels and to generate NO (time course). *A*, B10R Mϕ were stimulated with 25 μg/ml sHZ and/or 100 U/ml IFN-γ for different time periods (0–24 h). Total RNA was extracted and iNOS mRNA levels were monitored by Northern blot analysis. GAPDH probe was used to confirm equal RNA loading (*upper panels*). Following cell stimulation as described above, protein lysates were subjected to Western blotting to evaluate iNOS protein expression. Equal protein levels were verified by using an anti-actin Ab (*lower panels*). *B*, Nitrite production from either untreated or stimulated (sHZ and/or IFN-γ) cells was measured at various time points (0–24 h) by the Griess reaction. □, Untreated; ▨, sHZ; ▧, IFN-γ; ■, sHZ + IFN-γ. Results are representative of one of three independent experiments performed in triplicate (mean ± SEM, $n = 3$). *, $p < 0.05$, sHZ + IFN-γ vs IFN-γ.

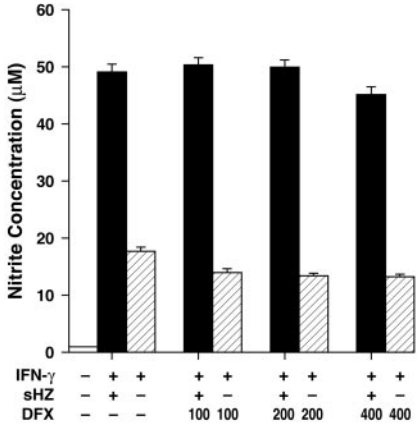

FIGURE 4. The synergistic effect of sHZ on IFN-γ-dependent NO induction is not reduced in the presence of an iron chelator. B10R cells were pretreated for 1 h with 100–400 μM DFX, and were further incubated with 100 U/ml IFN-γ ± 25 μg/ml sHZ for 24 h. Then, supernatants were collected and nitrite production was monitored. □, Untreated; ▨, IFN-γ ± DFX; ■, sHZ + IFN-γ ± DFX. Results are representative of one of three separate experiments performed in triplicate (mean + SEM, $n = 3$).

seems to be essential for Mϕ iNOS promoter activation and NO induction in response to sHZ + IFN-γ, this is not the mechanism through which sHZ exerts its synergistic effect on this cellular regulation.

Activation of the ERK pathway is required for Mϕ NO generation in response to sHZ + IFN-γ

Involvement of the ERK pathway on cytokine- or LPS-mediated iNOS regulation has been reported in a variety of cell types (39, 40) including Mϕ (41). In an attempt to identify the transductional mechanisms through which sHZ is able to potentiate IFN-γ-inducible NO production, time course experiments (0–4 h) were conducted to evaluate ERK1/2 phosphorylation in response to sHZ alone or in combination with IFN-γ. As depicted in Fig. 7, sHZ treatment resulted in a rapid (0.5 h) increase of ERK1/2 phosphorylation, reaching maximal values at 2 h poststimulation. As expected, IFN-γ also led to a time-dependent up-regulation of ERK1/2 activation, following similar kinetics of induction to that of sHZ. Of interest, a greater induction of phospho-ERK1/2 was generated by the combination of the two mediators, sHZ and IFN-γ, following a 2-h treatment; thus, suggesting that both stimuli additively up-regulate the activation of the ERK pathway in Mϕ. These data prompted us to investigate the functional importance of this signaling cascade on the sHZ + IFN-γ-inducible NO production. To this end, cells were treated with increasing doses of specific inhibitors directed either against mitogen-activated protein kinase (MAPK) kinase 1/2, PD 98059, or ERK1/2, apigenin, before sHZ + IFN-γ stimulation, and their effect on iNOS mRNA and protein expression was monitored by Northern and Western blot analyses, respectively (Fig. 8*A*). We found that intermediate doses of both PD 98059 and 20 μM apigenin caused a considerable decrease of iNOS mRNA and protein levels whereas a more dramatic diminution was seen when maximal doses of these compounds were added. In agreement with these observations, NO

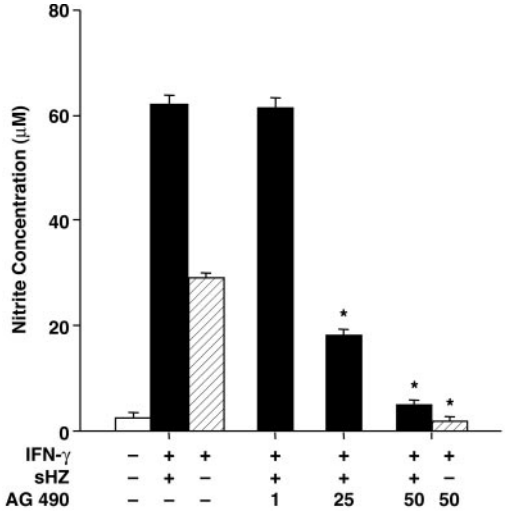

FIGURE 5. Effect of a Jak2-specific inhibitor (AG 490) on NO induction by IFN-γ and sHZ. Cells were treated (1 h) with 1–50 μM AG 490 before stimulation with 100 U/ml IFN-γ plus 25 μg/ml sHZ, or with 50 μM AG 490 before IFN-γ treatment. After 24 h, supernatants were collected and submitted to the Griess reaction to evaluate nitrite production. □, Untreated; ■, sHZ + IFN-γ ± AG 490; ▨, IFN-γ ± AG 490. Results are representative of one of three independent experiments performed in triplicate (mean ± SEM, $n = 3$). *, $p < 0.05$, sHZ + IFN-γ + AG 490 vs sHZ + IFN-γ; IFN-γ + AG 490 vs IFN-γ.

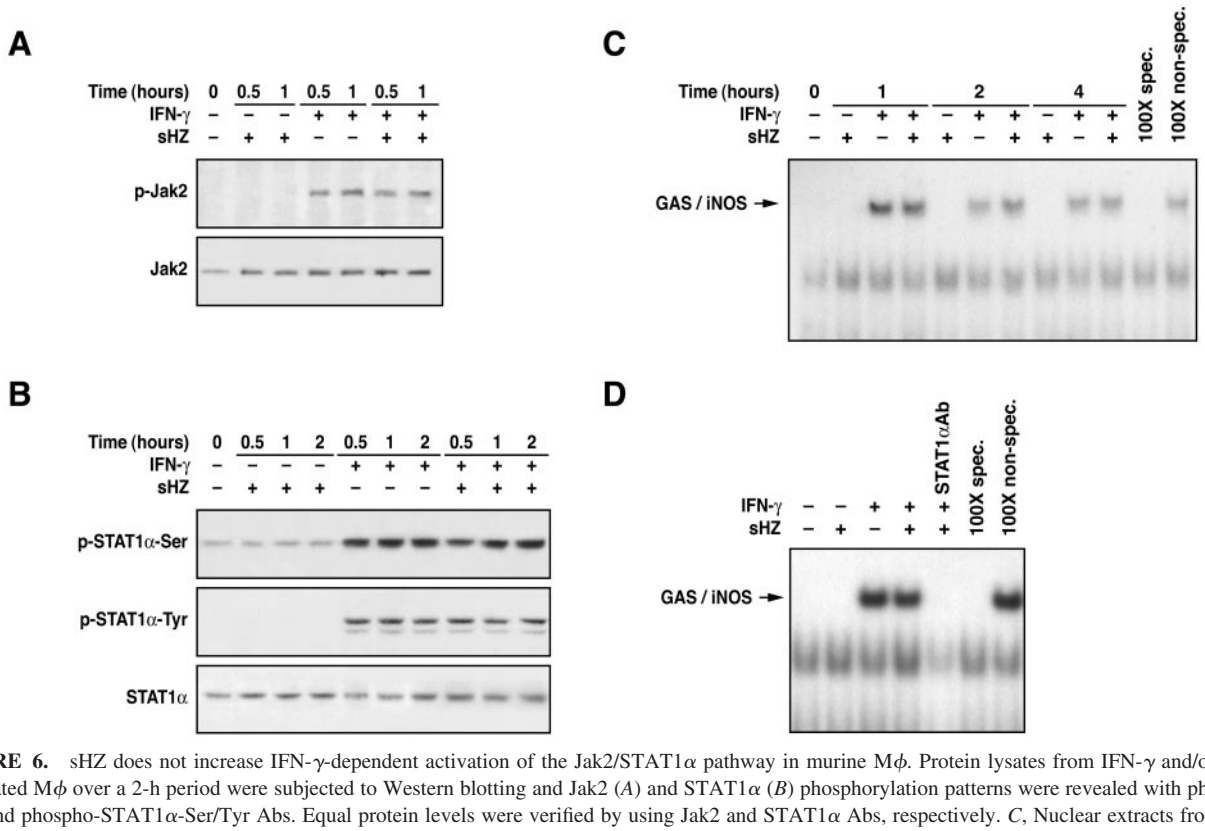

FIGURE 6. sHZ does not increase IFN-γ-dependent activation of the Jak2/STAT1α pathway in murine Mϕ. Protein lysates from IFN-γ and/or sHZ-stimulated Mϕ over a 2-h period were subjected to Western blotting and Jak2 (*A*) and STAT1α (*B*) phosphorylation patterns were revealed with phospho-Jak2 and phospho-STAT1α-Ser/Tyr Abs. Equal protein levels were verified by using Jak2 and STAT1α Abs, respectively. *C*, Nuclear extracts from cells either left untreated or stimulated with IFN-γ and/or sHZ for different time periods (0–4 h) were incubated with a [γ-^{32}P]-labeled GAS/iNOS probe and were subjected to EMSA. *D*, For supershift assays, nuclear extracts from cells treated with IFN-γ and sHZ (1 h) were incubated or not with a specific Ab against STAT1α for 1 h before EMSA. Binding specificity was tested by adding to nuclear extracts from 1-h IFN-γ-treated cells a 100-fold molar excess of either a cold GAS/iNOS oligonucleotide or a nonspecific Oct-2A probe. These results are representative of one of three separate experiments.

generation was also down-regulated in a dose-dependent manner by these specific inhibitors (Fig. 8*B*). Although PD 98059 treatment (40 μM) resulted in a partial but significant diminution (58% inhibition) on NO synthesis, maximal concentrations of apigenin led to a more marked reduction (79% inhibition). In parallel, we observed that the IFN-γ-dependent NO generation was almost suppressed in the presence of high doses (40 μM) of these compounds, 83% inhibition by PD 98059 and 91% by apigenin. This series of experiments indicate that the noticed sHZ + IFN-γ-mediated iNOS and subsequent Mϕ NO modulation are, at least in part, dependent on the activation of the ERK1/2 MAPK pathway by both sHZ and IFN-γ.

sHZ synergizes with IFN-γ to induce NF-κB binding to the murine iNOS promoter in Mϕ

The murine *iNOS* gene contains an array of putative transcription factor recognition boxes including two NF-κB sites (33). To test the capacity of sHZ to induce NF-κB binding to the murine iNOS promoter and/or to potentiate this regulatory event in response to IFN-γ, nuclear extracts from cells stimulated with sHZ and/or IFN-γ (0–4 h), were incubated with a labeled oligonucleotide containing a NF-κB binding site present in the iNOS promoter, and EMSA analysis was performed. As shown in Fig. 9*A*, in Mϕ treated with sHZ, the appearance of the specific binding complex was rapid, occurring within 1 h of stimulation and returning almost to basal levels after 4 h. In contrast, in IFN-γ-treated cells this binding complex was not observed at 1 h but rather required 2 h before it became slightly detectable and was maximal only following a 4-h treatment. In the presence of both sHZ and IFN-γ, a much higher NF-κB/iNOS binding activity was detected than when they were added separately, being maximal at 2 h and still significant after 4 h. The specificity of this binding complex was demonstrated by incubating nuclear extracts with a 100-fold molar excess of either unlabeled NF-κB/iNOS oligonucleotide (100× spec.) or unrelated Oct-2A probe (100× nonspec.). To identify the nuclear protein(s) that specifically bind to the NF-κB/iNOS site, we next performed supershift assays using Abs directed toward p50 and p65, two ubiquitous members of the NF-κB family. As illustrated in Fig. 9*B*, sHZ + IFN-γ activate DNA binding of both p50 and p65 NF-κB subunits to the iNOS promoter because the complex binding was diminished and partially supershifted by the anti-p50 Ab and almost completely abrogated by the anti-p65 Ab. In summary, these results indicate that sHZ and IFN-γ act in synergy to induce NF-κB binding to the murine *iNOS* gene.

NF-κB is involved on sHZ + IFN-γ-dependent NO generation in Mϕ

Having found that sHZ was able to induce NF-κB binding to the iNOS promoter and to increase it in response to IFN-γ, we further evaluated the potential role of this transcription factor in the sHZ + IFN-γ-mediated NO regulation. Both ERK and NF-κB activation have been shown to be required for cytokine-mediated NO synthesis (39). In addition, our previous results indicated that specific inhibitors of the ERK pathway strongly reduced NO synthesis by sHZ + IFN-γ. Therefore, we examined the effect of these compounds on the sHZ + IFN-γ-inducible NF-κB binding to the *iNOS* gene. As illustrated in Fig. 10*A*, when cells were treated either with apigenin or PD 98059, the sHZ + IFN-γ-enhanced NF-κB binding activity decreased in a dose-dependent manner. To more directly

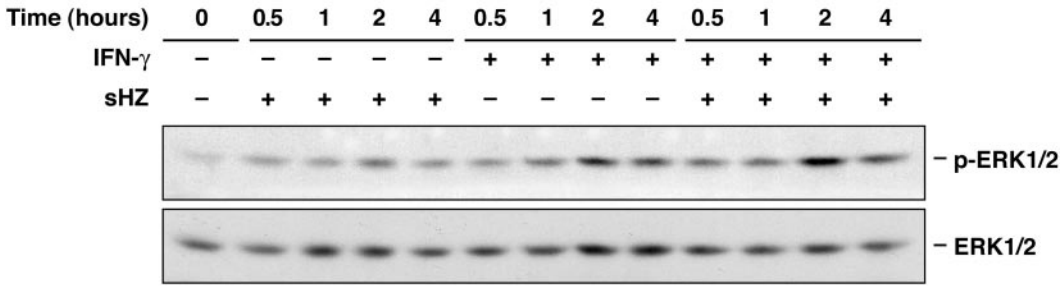

FIGURE 7. Time course of ERK1/2 phosphorylation in response to sHZ ± IFN-γ. Protein lysates from IFN-γ and/or sHZ-stimulated Mϕ over a 4-h period were subjected to Western blotting and ERK1/2 phosphorylation status was determined by using a phospho-ERK1/2 Ab. Equal protein levels were verified with an anti-ERK1/2 Ab. Results are representative of one of three separate experiments.

address the putative contribution of NF-κB on the sHZ + IFN-γ-dependent iNOS modulation, Mϕ were treated with increasing 0.5–1.5 μg/ml concentrations of CAPE, a chemical compound which has been shown to inhibit NF-κB nuclear translocation (42), before sHZ + IFN-γ stimulation. Our EMSA analyses revealed that CAPE leads to a significant and dose-dependent reduction in the binding of the NF-κB complex to the iNOS promoter (Fig. 10*B*). Based on these data, we examined the effect of this NF-κB inhibitor on the sHZ + IFN-γ-mediated increase of iNOS mRNA and protein expression. As depicted in Fig. 10*C*, maximal doses of 1.5 μg/ml CAPE caused a considerable decrease at both levels of iNOS induction. In line with these findings, NO generation in response to sHZ + IFN-γ was also down-regulated by this compound (Fig. 10*D*). A partial but significant reduction (38% inhibition) was observed when CAPE was added at 1.0 μg/ml and a more marked one (70% inhibition) at 1.5 μg/ml. Similarly, this last pretreatment caused a 67% inhibition in Mϕ stimulated with IFN-γ alone. This series of experiments indicates that ERK-dependent NF-κB activation is necessary for maximal sHZ + IFN-γ-mediated Mϕ iNOS and NO modulation.

Discussion

The production of elevated NO levels is a hallmark of malaria infection (5, 6). Among the various host cytokines, IFN-γ has been identified as an important Mϕ activator during plasmodial infection (43) and, thus, as central in the regulation of NO synthesis (8). In addition to host products, purified malarial GPI was shown to induce NO release in murine Mϕ and to synergize with IFN-γ in regulating NO production (13). In the present study, we demonstrate that another parasite metabolite, *Pf* HZ as well as its synthetic form, sHZ, are able to significantly enhance IFN-γ-dependent NO generation in murine Mϕ. Upon first consideration our results would seem inconsistent with those presented by other groups indicating that peritoneal Mϕ preconditioning with HZ results in reduction of cytokine-and/or LPS-induced NO production (44, 45). Of interest, contradictory results regarding Mϕ TNF-α (15, 17, 45, 46) regulation by HZ have also been reported. Based on previous studies as well as on our data, a dual role for HZ on Mϕ modulation can be proposed. Acute phase malaria is characterized by high production of reactive oxygen species (ROS) (47), and HZ has been shown to increase ROS in Mϕ (48). Depending on the susceptibility to ROS, activation of the same signaling pathway has been found to lead to ROS-dependent transcription factor up- or down-regulation in different cell types (26, 49). Therefore, it is possible that HZ leads to a localized negative or positive effect on Mϕ functions, depending on the cell susceptibility to ROS according to its tissue source. In fact, that HZ exerted a down-regulating effect on NO production by peritoneal Mϕ but not by microglial cells was attributed to differences in their antioxidant defenses (48). Additionally, because data from both human and rodent malaria indicate that Mϕ cytotoxic activities are maximal at the beginning of the infection and decline as the infection progresses (50), including the capacity to generate ROS (51), it is probable that HZ leads to Mϕ activation at the early stages of

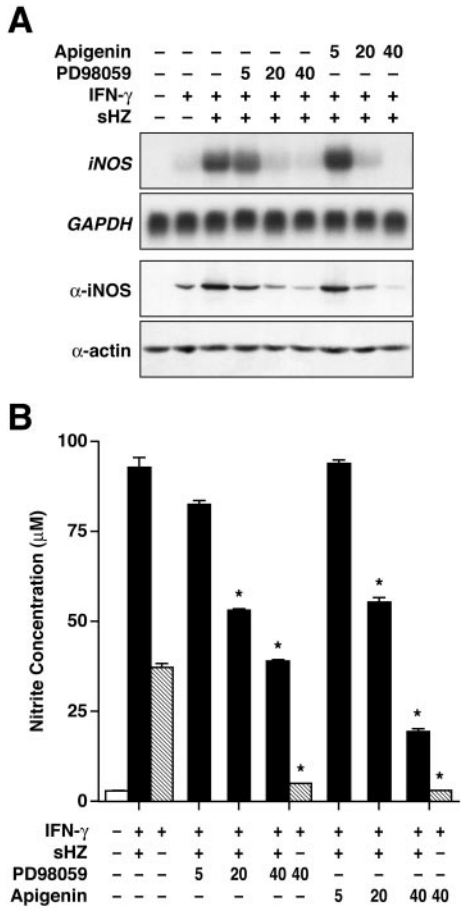

FIGURE 8. Involvement of the ERK1/2 pathway on iNOS and NO up-regulation by sHZ and IFN-γ. *A*, B10R Mϕ were treated (1 h) with either PD 98059 or 5–40 μM apigenin before stimulation with sHZ and IFN-γ (24 h), and their effects on iNOS mRNA (*upper panels*) and protein (*lower panels*) expression were evaluated by Northern blot and Western blot, respectively. *B*, Following cell stimulation as described above, nitrite production was measured by the Griess assay. □, Untreated; ■, sHZ + IFN-γ ± PD 98059 or apigenin; ▨, IFN-γ ± PD 98059 or apigenin. Results are representative of one of three independent experiments performed in triplicate (mean ± SEM, $n = 3$). ∗, $p < 0.05$, sHZ + IFN-γ + PD 98059/apigenin vs sHZ + IFN-γ; IFN-γ + PD 98059/apigenin vs IFN-γ.

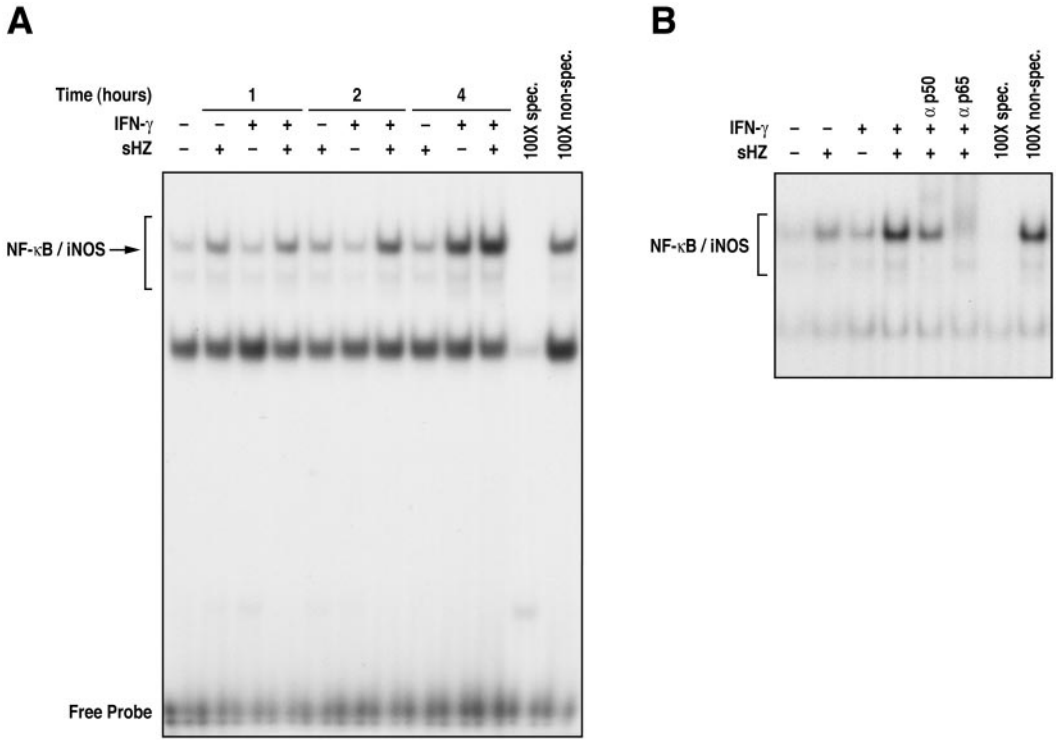

FIGURE 9. sHZ synergizes with IFN-γ to induce NF-κB binding to the murine iNOS promoter in Mϕ. *A*, Labeled NF-κB/iNOS probe was incubated with nuclear extracts from cells either nonstimulated or treated with sHZ and/or IFN-γ for different time periods (0–4 h) and EMSA analysis was performed. Binding specificity was tested by adding to nuclear extracts from 2-h sHZ + IFN-γ-treated cells a 100-fold molar excess of either a cold NF-κB/iNOS oligonucleotide or a nonspecific Oct-2A probe. *B*, For supershift assays, nuclear extracts from cells treated with IFN-γ and sHZ (2 h) were incubated or not with specific Abs against the p50 and p65 NF-κB isoforms for 1 h before EMSA. These results are representative of one of three separate experiments.

infection but after repetitive parasite cycles and subsequent Mϕ overloading with HZ, negative regulatory effects could be predominant. Further in vivo studies will be required to clarify the potential dual role of HZ on malaria infection.

Mϕ iNOS expression is a prerequisite for the production of high output NO. In line with this, we found that the synergism between HZ and IFN-γ in NO induction resulted from the up-regulatory effect exerted by HZ on the IFN-γ-mediated iNOS mRNA and protein expression. That HZ strongly potentiated IFN-γ-induced iNOS and NO expression but did not act alone suggests an effect of HZ on iNOS promoter induction only when cells are stimulated with IFN-γ. Concerning the biological implications of this synergistic interaction, the requirement for a dual stimulation (IFN-γ + HZ) for high-output Mϕ, NO might represent just another example of a more generalized mechanism, reported for IFN-γ in combination with parasite, bacterial, or viral infection (52), to generate an efficient double-lock system that limits the production of toxic doses of highly reactive molecules to the sites of infection. In this context, NO overproduction in HZ-loaded Mϕ would only occur as long as IFN-γ persists in the inflammatory milieu.

The cloning of the murine *iNOS* gene was accompanied by the demonstration that its induction by IFN-γ is mainly transcriptional (33) and it is regulated by the coordinate activity of various transcription factors (53). Because the molecular mechanisms underlying synergistic gene induction have been shown to involve cooperative kinase and transcription factor activation (52), we tested the hypothesis of whether the synergistic iNOS regulation in response to HZ + IFN-γ correlated with the activation of complementary Mϕ metabolic signaling cascades. We found that even though IFN-γ-dependent activation of the Jak2/STAT1α pathway is essential for Mϕ iNOS expression and NO synthesis in response to HZ + IFN-γ, HZ exerted its up-regulatory effect via alternative pathways, at least in part, mediated through an increase of IFN-γ-induced ERK1/2 phosphorylation and NF-κB binding to the murine iNOS promoter, leading to maximal iNOS induction.

Despite the incapacity of HZ to induce Jak2/STAT1α activation and to enhance it in response to IFN-γ, selective inhibition of Jak2 suppressed the IFN-γ + HZ-dependent NO synthesis. These results clearly indicate the requirement of this signaling cascade and are perfectly in line with previous studies showing that Jak2/STAT1α-mediated signals are essential for Mϕ NO induction by IFN-γ either alone or in combination with other agonists (28). Because STAT1α has been found to increase *iNOS* activity not only by direct binding of the murine *iNOS* gene but also by inducing gene expression of another transcription factor, IFN-γ responsive factor (IRF)-1 (54), it seems plausible that STAT1α might be participating in the IFN-γ + HZ-mediated synergistic action over the iNOS promoter by exerting both direct and indirect positive effects.

Analysis of possible alternative second messengers responsible for the HZ-dependent increase of IFN-γ-inducible iNOS expression indicated the involvement of the ERK1/2 MAPK pathway. We found that HZ not only induces ERK1/2 phosphorylation but also enhances this event in response to IFN-γ. Moreover, blockage of the ERK pathway resulted in a significant reduction of both iNOS expression and NO induction by HZ + IFN-γ, thereby suggesting a key role for these kinases in this regulatory process, as previously reported in response to several proinflammatory stimuli (39, 55). Even though the exact mechanisms underlying the ERK1/2 involvement in HZ + IFN-γ-mediated iNOS regulation remain to be fully characterized, according to our data and those

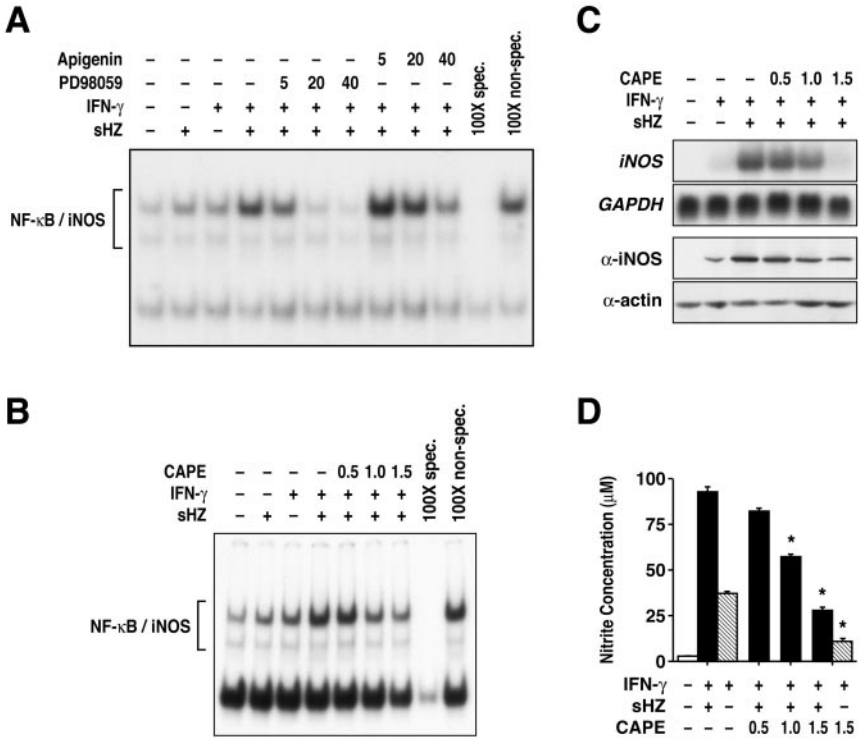

FIGURE 10. Role of NF-κB on sHZ + IFN-γ-dependent iNOS and NO regulation in Mϕ. Nuclear proteins from sHZ + IFN-γ-stimulated cells (2 h) pretreated or not with increasing doses of apigenin, PD 98059 (*A*), or CAPE (*B*) (1 h) were incubated with a NF-κB/iNOS probe and subjected to EMSA. *C*, B10R Mϕ were treated (1 h) with 0.5–1.5 μg/ml CAPE before stimulation with sHZ + IFN-γ (24 h), and its effect on iNOS mRNA (*upper panels*) and protein (*lower panels*) expression were evaluated by Northern and Western blot, respectively. *D*, Following cell stimulation as described in (*C*), nitrite production was measured by the Griess reaction. □, Untreated; ■, sHZ + IFN-γ ± CAPE; ▨, IFN-γ ± CAPE. These results are representative of one of three independent experiments performed in triplicate (mean ± SEM, $n = 3$). *, $p <$ 0.05, sHZ + IFN-γ + CAPE vs sHZ + IFN-γ; IFN-γ + CAPE vs IFN-γ.

reported by others, at least two alternative but not exclusive scenarios can be envisaged; one, by contributing to maximal IFN-γ-dependent STAT1α induction, and another one, by inducing NF-κB activity in response to both mediators.

Although STAT1α activation occurs primarily upon Jak2-inducible tyrosine phosphorylation, full STAT1α activation requires serine phosphorylation (56), which appears to be mediated by ERK1/2 in IFN-γ signaling (38, 57). Of interest, both ERK1/2 and STAT1α serine phosphorylation were found to be impaired by Jak2 inactivation (58, 59). Therefore, it is conceivable that the noticed abrogation of HZ + IFN-γ-inducible NO synthesis by selective blockage of Jak2 is due to the central role played by this kinase in *iNOS* transcriptional regulation, both by directly phosphorylating STAT1α on its tyrosine residue and by increasing ERK-dependent STAT1α serine phosphorylation.

A number of studies have associated synergistic iNOS modulation to a dual up-regulating effect on NF-κB activation (60–62). In agreement with these findings, and consistent with the synergistic iNOS induction by the combination of HZ and IFN-γ, HZ led to more rapid NF-κB binding to the iNOS promoter and strongly augmented this activity in response to IFN-γ. In addition, our data indicated that the HZ + IFN-γ-enhanced NF-κB capacity to bind the *iNOS* gene seems to be under the control of the ERK pathway. This is in line with previous reports showing that inactivation of the ERK pathway down-regulated both NF-κB activity and NO production (39, 63). According to the current data, the major contribution to synergistic iNOS promoter activation appears to be the cooperative action of NF-κB with STAT1α and/or IRF-1 (52, 64). Therefore, in addition to its independent action, it is very likely that NF-κB is contributing to the HZ + IFN-γ-dependent iNOS promoter activation by acting in synergy with STAT1α and/or IRF-1.

Given that blockage of the ERK pathway did not completely inhibit the HZ-dependent increase of IFN-γ-inducible NO generation, it is plausible to think that HZ might be exerting its up-regulatory effect by activating other signaling pathways in addition to ERK. In fact, increase in intracellular cAMP has been shown to modulate NO production in IFN-γ-treated Mϕ (65) and cAMP-dependent NF-κB activation was found to contribute to Mϕ iNOS expression (66). Interestingly, preliminary experiments revealed that HZ is able to induce Mϕ CREB activation (data not shown). Therefore, the potential involvement of cAMP-dependent pathways on HZ + IFN-γ-mediated iNOS expression should not be ruled out. Further study of the various kinases and transcription factors involved is essential to understanding transcriptional control of the murine *iNOS* gene and subsequent NO production in response to HZ + IFN-γ.

Altogether, our data allow us to propose a mechanism for the synergistic HZ + IFN-γ-mediated Mϕ iNOS and NO modulation, which requires IFN-γ-elicited transduction signals through activation of the Jak2/STAT1α pathway as well as an up-regulatory effect on ERK1/2 MAPK and NF-κB transcription factor by both mediators. Through this mechanism, IFN-γ produced during malaria infection may transform a given dose of HZ into an activating stimulus, further increasing IFN-γ-inducible Mϕ NO production in the local environment following schizont rupture. This would, in turn, lead to parasite clearance but it could also contribute to the malaria pathology related to NO overproduction (e.g., immunosuppression and cerebral malaria). Even though NO generation during murine malaria is well-documented, the ability of human Mϕ to produce NO remains controversial (2). Therefore, the capacity of HZ to increase human monocyte/Mϕ NO production in response to IFN-γ needs to be investigated to better understand the differences regarding the regulation and role(s) of NO in both human and experimental malaria. Because *Pf* infection remains a major public health concern around the world, it is thus of paramount importance to determine how the host immune system responds to parasite metabolites that may play a key role by acting as cell activators. Our work may contribute to a better understanding of the molecular mechanisms underlying the action of the malarial pigment HZ on Mϕ, which might be essential to the development of strategies for the management of this disease.

Acknowledgments

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