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Hemozoin Induces Macrophage Chemokine Expression through Oxidative Stress-Dependent and -Independent Mechanisms

Maritza Jaramillo,† Marianne Godbout,* and Martin Olivier2†‡

Chemokine production has been associated with the immunopathology related to malaria. Previous findings indicated that hemozoin (HZ), a parasite metabolite released during schizogony, might be an important source of these proinflammatory mediators. In this study we investigated the molecular mechanisms underlying HZ-inducible macrophage (Mφ) chemokine mRNA expression. We found that both Plasmodium falciparum (HZ) and synthetic HZ increase mRNA levels of various chemokine transcripts (MIP-1α/CCL3, MIP-1β/CCL4, MIP-2/CXCL2, and MCP-1/CCL2) in murine B10R Mφ. The cellular response to HZ involved ERK1/2 phosphorylation, NF-κB activation, reactive oxygen species (ROS) generation, and ROS-dependent protein-tyrosine phosphatase down-regulation. Selective inhibition of either IkBα or the ERK1/2 pathway abolished both NF-κB activation and chemokine up-regulation. Similarly, blockade of HZ-inducible Mφ ROS with superoxide dismutase suppressed chemokine induction, strongly reduced NF-κB activation, and restored HZ-mediated Mφ protein-tyrosine phosphatase inactivation. In contrast, superoxide dismutase had no effect on ERK1/2 phosphorylation by HZ. Collectively, these data indicate that HZ triggers ROS-dependent and -independent signals, leading to increased chemokine mRNA expression in Mφ. Overall, our findings may help to better understand the molecular mechanisms through which parasite components, such as HZ, modulate the immune response during malaria infection. The Journal of Immunology, 2005, 174: 475–484.
activation of oxidative stress-dependent and -independent pathways. A better understanding of the transductions signals through which HZ modulates the host immune response might be helpful in defining specific therapeutic targets to tame proinflammatory mediator overproduction during malaria infection.

Materials and Methods

Materials

Hemin chloride, polymyxin B (Poly B) sulfate, LPS (Escherichia coli, serotype 0111:B4), 3% H2O2 (v/v), and superoxide dismutase (SOD) were purchased from Sigma-Aldrich. Isotopes [α-32P]dUTP (3000 Ci/mmol) and [γ-32P]ATP (3000 Ci/mmol) were obtained from PerkinElmer. BAY 11-7082 was purchased from BIOMOL. Apigenin and PD 98059 were obtained from Calbiochem.

Cell and culture conditions

The murine Mφ cell line B10R, derived from the bone marrow of B10A.Bcrf (B10R) mice (17), was provided by Dr. D. Radzioch (McGill University, Montreal, Canada). Cells were maintained in DMEM (Invitrogen Life Technologies) supplemented with 10% heat-inactivated FBS (HyClone) plus 100 μg/ml streptomycin and 2 mM t-glutamine at 37°C and 5% CO2.

Cell viability assays

(3-(4,5-Dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenil)-2-(4-sulfophenyl)-2H-tetrazolium (inner salt) assays for cell viability were performed and indicated no cytotoxic or cytostatic effect from the various specific inhibitors at the concentrations used (data not shown). Briefly, B10R Mφ were seeded in 96-well plates (3 × 104 cells/well) and were stimulated for 3 h with increasing concentrations of the various inhibitors (100 μM). Then, cells were incubated with 10 μl of a 201/i2 solution of 2 mg/ml (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenil)-2-(4-sulfophenyl)-2H-tetrazolium (inner salt; Promega) and 3 mM phenazine methosulfate (Sigma-Aldrich) for 1 h, and OD was read at 492 nm.

β-Hematin (sHZ) preparation

β-Hematin was synthesized as previously described (9). Briefly, 45 mg of hemin chloride (Sigma-Aldrich) was 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, was added, and the suspension was stirred with a magnet for 2–3 h at 60°C. After addition of 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 or four times in 2% SDS and then in water to wash out the 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. Pj HZ was extracted as described previously (9) and was provided by Dr. D. C. Gowda (Pennsylvania State University College of Medicine, Hershey, PA). To assess the level of endotoxins in the HZ preparations, the Limulus amebocyte lysate test (E-toxate kit; Sigma-Aldrich) was performed, and no endotoxin contamination was detected.

Heme quantitation

The total heme content was determined, as described by Sullivan et al. (18), by adding 20 mM NaOH/2% SDS to the HZ preparations, incubating the suspension at room temperature for 2 h, and then reading the OD at 400 nm (DGB UV/visible spectrophotometer; Beckman Coulter). Twenty-five micrograms of sHZ equals 26 mmol of heme content, and 25 μg of Pj HZ equals 29 mmol of heme content. It should be noted that the concentrations of HZ used to stimulate Mφ (25–75 μg/ml) were chosen based on reported estimates of the HZ concentrations encountered during malaria infection (12, 19, 20) as well as on previous studies in which these amounts of HZ were shown to be effective both in vitro and in vivo (12, 21). Briefly, by reference to standard hematological measurements and assuming that 1) a 70-kg adult has a 1% synchronized Pj parasitemia (a relatively mild parasitoid load), 2) 50% of the hemoglobin in an infected cell is degraded, and 3) all heme in the degraded hemoglobin is converted into HZ, it has been estimated that as much as 200 μmol of HZ (~3 μmol/kg) is released into the circulation of a Pj-infected human patient at schizogeny (12). In addition, it was calculated that 106 Pj trophozoite-infected RBC contain between 339 and 652 ng of HZ (equal to 0.5–1.0 nmol of heme content) (19, 20). According to these data, the concentrations of HZ used in the current study (~26–29 nmol/ml heme content) could easily be released by a patient with a mild parasitemia load.

RNase protection assay (RPA)

mRNA expression studies were performed using an RPA kit (RiboQuant; BD Pharmingen), as we described previously (22). Total RNA was isolated from stimulated cells with TRizol reagent (Invitrogen Life Technologies) according to the manufacturer’s protocol. One of the multiprobe templates was labeled with [α-32P]dUTP using T7 RNA polymerase. Then, 3 × 107 cpm of labeled probe was allowed to hybridize with 10 μg of total RNA for 16 h at 56°C. mRNA probe hybrids were treated with RNase A and phenol-chloroform-extracted. Protected hybrids were resolved on a 5% denaturing polyacrylamide sequencing gel and exposed to radiographic film overnight at ~80°C. Laser densitometry was performed using an Omega 2000 digital imaging and analysis system (Alexis). The multiprobe templates (BD Pharmingen) used in this study were MCK-5, for the murine chemokines lymphotactin, RANTES, eotaxin, MIP-1α, MIP-1β, MIP-2, inducing protein-10, MCP-1, and TCA-3, and a custom template for murine cyto- kines IL-4, IL-12p40, IL-10, IL-1α, IL-1β, IL-2, IL-6, and IFN-γ. The template sets included housekeeping genes ml-32 and/or GAPDH.

EMSA

Cell stimulation was terminated by the addition of ice-cold PBS, nuclear extracts were prepared according to the microscale protocol, and EMSA was performed using 6 μg of nuclear proteins, as we described previously (22). Briefly, nuclear extracts were incubated for 20 min at room temperature with 1.0 μl of binding buffer (100 mM HEPES [pH 7.9], 40% glycerol, 0.1 M KCl, 10 mM EDTA, 1.0 mM DTT, 5 μM DmEDETA, and 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 [γ-32P]dATP radiolabeled dsDNA oligonucleotide. This mixture was incubated for 20 min at room temperature, and the reaction was stopped using 5 μl of 0.2 M EDTA. 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. The dsDNA oligonucleotide containing a consensus binding site for NF-κB–Rel homodimeric and heterodimeric complexes (5'-AGTTGAGGAGGACTTCCCCAGGC-3') was obtained from Santa Cruz Biotechnology. The oligonucleotides containing NF-κB–binding sites of the murine chemokine promoters were synthesized in our laboratory as follows: NF-κB/MIP-2, 5'-GAGCTCAGGGAATTTCCCTGGTCC-3'; NF-κB/MCP-1, 5'-AAGGGTCTGGGAACCTCAATACGTC-3'; and NF-κB/MIP-1α, 5'-GGAGTATCCAGCTCCGTAG-3'. The nonspecific probe Oct-2A (5'-GGAGTATCCAGCTCCGTAG CATGGAAATCTCCTGTCG-3'), which was used to confirm the specificity of the DNA/nuclear protein reaction, was also synthesized in our laboratory. Color 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) or p50 (Santa Cruz Biotechnology) in the presence of all components of the binding reaction described above for 1 h at 4°C.

Western blotting

Cells were collected after stimulation, lysed in cold buffer containing 20 mM Tris-HCl (pH 8.0), 0.14 M NaCl, 10% glycerol (v/v), 1% IgE (v/v), 25 μM nitrophenyl guanidinobenzoate, 10 μM sodium fluoride, 1 mM sodium orthovanadate, 25 μg/ml leupeptin, and aprotonin. The lysates (20 μg/lane) were subjected to SDS-PAGE, and the separated proteins were transferred onto a polyvinylidine difluoride membrane (Millipore). After a 1-h blocking period in TBST containing 5% milk, the membranes were incubated overnight in TBST/5% BSA at 4°C with one of the following rabbit polyclonal Abs (New England Biolabs): phospho-ERK1/2 (Thr202/Tyr204), ERK1/2, phospho-MEK1/2 (Ser217/20), MEK1/2, phospho-IκBα (Ser32), and IκBα. Proteins were then detected with an anti-rabbit, HRP-conjugated, goat Ab (Affini-Pure; Jackson ImmunoResearch Laboratories) and subsequent visualization by ECL (ECL Western blotting detection system; Amersham Biosciences).

Phosphatase activity

After stimulation, Mφ were washed in PBS and lysed in a buffer containing 50 mM Tris-HCl (pH 7.0), 0.1 M EDTA, 0.1% MEGTA, 0.1% 2-ME (v/v), 1% IgE (v/v), 25 μg/ml aprotinin, and 25 μg/ml leupeptin. Cellular protein-tyrosine phosphatase (PTP) activity was determined by evaluating the capacity of cell lysates to hydrolyze para-nitrophenyl phosphate (pNPP; Roche), as we previously described (25). Briefly, 20 μg of total proteins were incubated with 180 μl of a reaction containing 50
mM HEPES (pH 7.5), 0.1% 2-ME (v/v), and 10 mM pNPP. After a 10- to 60-min incubation at 37°C, PTP activity was monitored, reading the OD at 405 nm (DGB UV/visible spectrophotometer; Beckman Coulter).

### Oxidative stress generation

To monitor reactive oxygen species (ROS) generation, M<sub>Φ</sub>/H9278 were seeded in 96-well plates (3 × 10<sup>4</sup> cells/well) and, after two washes with PBS, they were incubated for 30 min with H<sub>2</sub>DCFDA, 2',7'-dichlorofluorescein diacetate (Calbiochem), a commonly used fluorogenic probe, diluted to a final concentration of 100 μM. Cell washing was followed by a 1-h exposure to either H<sub>2</sub>O<sub>2</sub> or shZ with or without SOD, and fluorescence was read at 485/530 nm using an LS50 luminescence spectrometer (PerkinElmer).

### Statistical analysis

Statistically significant differences between groups were determined by ANOVA module of StatView Plus SE software (Abacus Concepts) and Fisher’s least significant difference test. A value of \( p < 0.05 \) was considered statistically significant. All data are presented as the mean ± SEM.

### Results

**Pf HZ and shZ increase M<sub>Φ</sub> chemokine mRNA expression**

M<sub>Φ</sub> have been identified as one of the main sources of proinflammatory mediators in response to Pf infection (2). Thus, we initially established whether HZ induced chemokine mRNA expression in the murine M<sub>Φ</sub> cell line B10R. As depicted in Fig. 1A, when cells were stimulated for 2 h with 10–75 μg/ml of either Pf HZ or its synthetic form, shZ, a concentration-dependent increase in various chemokine transcripts was detected. Maximal values over the negative control were obtained for MIP-1β (3- and 4-fold), MIP-1α (2- and 3-fold), MIP-2 (140- and 170-fold), and MCP-1 (3- and 4-fold) when 75 μg/ml Pf HZ or shZ was added, respectively. In addition, time-course experiments performed with an intermediate concentration of shZ (25 μg/ml) revealed that chemokine induction occurred very rapidly (0.5–1 h poststimulation), peaked after 2 h, and transiently decreased over an 8-h period (Fig. 1B). Subsequent experiments presented in this section are those after cell stimulation with shZ; however, all of them were performed in parallel with Pf HZ, and the results were the same as those obtained with shZ.

**Synthetic HZ-dependent chemokine mRNA up-regulation is not due to LPS contamination**

To demonstrate that chemokine expression in response to shZ was not due to the presence of LPS, cells were incubated for 2 h in culture medium (DMEM/10% FBS) containing either LPS (100 ng/ml) or shZ (25 μg/ml) and treated, or not, with 5–10 μg/ml Poly B for 30 min before cell stimulation. Because Poly B binds to LPS and blocks its activity (26), this compound has the ability to inhibit LPS-inducible M<sub>Φ</sub> activation. As shown in Fig. 2A, Poly B did not exert any inhibitory effect on shZ-dependent chemokine modulation; however, this compound dramatically reduced the chemokine increase in response to LPS, reaching 100% of inhibition in the presence of 10 μg/ml Poly B. These data are in line with those of our previous study showing that the synergistic effect of shZ on IFN-γ-mediated NO production was not reduced in the presence of Poly B and was not affected in TLR4-deleted M<sub>Φ</sub>-derived from LPS-unresponsive mice. In contrast, the ability of LPS to induce NO synthesis was suppressed upon Poly B pretreatment and was abolished in TLR4-deleted M<sub>Φ</sub> (9). In addition, we found that although LPS, at very low concentrations (0.1 and 1 ng/ml), led to the up-regulation of various proinflammatory cytokine transcripts (IL-12, IL-1β, IL-10, and IL-6), shZ was unable to do so even when added at 25 μg/ml (Fig. 2B). This set of experiments indicates the absence of LPS in the shZ preparations.

**FIGURE 1.** PfHZ and shZ increase chemokine mRNA levels in murine M<sub>Φ</sub>. Cells were treated with 10–75 μg/ml PfHZ or shZ for 2 h (A) or with 25 μg/ml shZ over an 8-h period (B), and chemokine mRNA expression was monitored using an mCK5 multiprobe RPA system (left panels). Densitometric quantification of chemokine mRNA levels over the negative control values after normalization to GAPDH (right panels). ◼, PfHZ; □, shZ. The results shown are representative of one of three independent experiments.
and suggests that the two agonists exert different biological effects on Mφ.

Activation of the ERK1/2 pathway is required for shHZ-inducible chemokine modulation

The role of ERK1/2 MAPK on chemokine induction is well documented (22, 27). Therefore, we tested the ability of shHZ to activate this signaling cascade. A transient phosphorylation of the immediate ERK1/2 upstream activator MEK1/2 (Fig. 3A) and of ERK1/2 (Fig. 3B) occurred after cell exposure to shHZ (25 μg/ml). Both MEK1/2 and ERK1/2 phosphorylation were detected at 30 min poststimulation and remained sustained for up to 2 h. Whereas MEK1/2 phosphorylation declined thereafter, that of ERK1/2 was still detectable after 4 h. To investigate the involvement of the ERK1/2 pathway in shHZ-mediated chemokine up-regulation, cells were incubated for 1 h with increasing concentrations of specific inhibitors directed against MEK1/2 (PD 98059) and ERK1/2 (apigenin) before shHZ stimulation (2 h). As shown in Fig. 4A, 5 μM PD 98059 inhibited the expression of all four chemokines. In correlation with these observations, cell exposure to 20 μM apigenin resulted in a marked reduction of their chemokine transcripts (Fig. 4B, ∼45% for MIP-1β, ∼50% for MIP-1α, ∼60% for MIP-2, and ∼40% MCP-1), whereas maximal inhibitory concentrations (40 μM) completely abrogated chemokine expression. These results indicate that ERK1/2-dependent signals are necessary for Mφ chemokine modulation in response to the malarial pigment.

Involvement of the transcription factor NF-κB on shHZ-dependent chemokine induction

NF-κB proteins control the expression of multiple genes involved in inflammatory processes such as those encoding chemokines (28). Of interest, NF-κB binding sites have been found in the promoter regions of several murine chemokine genes, including those of MIP-2 (23) and MCP-1 (24). Based on these observations, we sought to establish whether this NF was activated in Mφ by shHZ. As shown in Fig. 5A, NF-κB nuclear translocation was maximal at 1 h poststimulation and then decreased over a 4-h period. To define the nature of the shHZ-induced NF-κB complex, supershift assays were performed using Abs directed at p50 and p65, two ubiquitous members of the NF-κB family. As illustrated in Fig. 5B, the complex binding was diminished and partially supershifted in the presence of an anti-p50 Ab and was almost completely abrogated by an anti-p65 Ab. These data indicated that shHZ activates DNA binding of both p50 and p65 NF-κB subunits in Mφ. To address the question of whether shHZ not only led to NF-κB nuclear translocation but also to its binding to one or more chemokine genes, nuclear extracts from shHZ-stimulated cells were incubated with oligonucleotides containing specific sequences of the NF-κB-binding sites present in the murine MIP-2 and MCP-1 promoters. We observed that after 1 h, shHZ led to maximal NF-κB binding to both sites. Whereas NF-κB binding to the MIP-2 sequence was sustained for up to 4 h, NF-κB binding to the MCP-1 promoter was transient, but still detectable at 4 h poststimulation (Fig. 5C). Given that specific blockade of the ERK1/2 pathway ablated chemokine expression, we next determined its involvement in shHZ-inducible IκBα phosphorylation and subsequent NF-κB nuclear translocation. As illustrated in Fig. 6A, shHZ led to a rapid phosphorylation of IκBα (15 min poststimulation). This intracellular event appeared to be under the control of MEK1/2-ERK1/2-dependent signals, because specific inhibitors directed against these MAPKs abrogated the effect of shHZ on IκBα. Similarly, cell exposure to either apigenin or PD 98059 caused a concentration-dependent diminution of NF-κB nuclear translocation (Fig. 6B). These data prompted us to further evaluate the role of NF-κB in chemokine modulation. As a control experiment, Mφ were incubated for 1 h with BAY 11-7082 (1 or 5 μM), an inhibitor of IκBα phosphorylation (29), before shHZ stimulation, and NF-κB nuclear translocation was monitored by EMSA (Fig. 6C). As expected, cells incubated with BAY 11-7082 showed a concentration-dependent
reduction in the binding of the NF-κB complex. We were thus interested in examining the effect of this inhibitor on chemokine induction. To this end, after cell stimulation, as described in the previous experiment, total RNA was extracted and subjected to RPA analysis. As illustrated in Fig. 6D, Mφ exposure to 5 μM BAY 11-7082 abrogated the expression of all four transcripts. These data suggest that chemokine modulation in response to sHZ involves ERK1/2-dependent NF-κB activation.

Chemokine mRNA up-regulation is mediated through sHZ-inducible ROS generation

Mφ from Pfi-infected mice were shown to release oxygen metabolites (30) and in vitro oxidative killing of Pf was described in peritoneal Mφ (31). Similarly, in response to Pf HZ (32) or sHZ (33), peritoneal Mφ produced ROS. Therefore, we assessed whether sHZ generated ROS in B10R Mφ (Fig. 7A). When cells were stimulated with 10–50 μg/ml sHZ, a concentration-dependent induction of ROS was detected, which was statistically significant compared with that in untreated Mφ (OD values) and was equivalent to 30% of the oxidative stress generated upon administration of exogenous H2O2 (500 μM). In addition, we found that most of the ROS produced by sHZ-stimulated Mφ corresponded to superoxide anion (O2−). As shown in Fig. 7B, in the presence of increasing concentrations of SOD, an enzyme that detoxifies O2− (34), sHZ-inducible ROS were significantly reduced, reaching 91% inhibition when 300 U/ml SOD was added. Next, we elucidated whether sHZ-mediated ERK1/2 phosphorylation, NF-κB activation, and chemokine modulation were due to ROS generation. Whereas SOD pretreatment (300 U/ml) did not affect ERK1/2 phosphorylation (data not shown), NF-κB nuclear translocation in response to sHZ was strongly reduced in the presence of this enzyme (Fig. 7C). Importantly, cell incubation with 300 U/ml SOD abrogated the expression of all chemokine transcripts (Fig. 7D). This set of experiments demonstrated that the malarial pigment leads to a mild oxidative stress in Mφ, which seems to be in part responsible for NF-κB activation and is required for sHZ-inducible chemokine mRNA up-regulation.

Mφ PTP inactivation in response to sHZ is ROS dependent

Intracellular signaling is regulated by the equilibrium between protein-tyrosine kinase (PTK) and PTP activity. Knowing that PTP inactivation occurs in response to ROS, including O2− (35, 36) and H2O2 (37), the ability of sHZ to reduce PTP activity was examined as a possible mechanism explaining Mφ signal transduction activation. To this end, time-course experiments were conducted in the presence of sHZ (25 μg/ml) from 5 min to 4 h, and Mφ PTP activity was monitored. As shown in Fig. 8A, sHZ rapidly down-regulated Mφ PTP activity, leading to a significant diminution (~40% reduction compared with basal activity) after a 30-min treatment and a more dramatic decrease (~55%) after 2 h. Next, we evaluated whether the effect of sHZ on Mφ PTP activity was due to its ability to induce ROS. When cells were treated with

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**FIGURE 4.** The ERK1/2 pathway is involved in chemokine mRNA induction in response to sHZ. Mφ were incubated for 1 h with increasing concentrations of either PD 98059 (A) or apigenin (B) before sHZ stimulation (25 μg/ml for 2 h), and their effects on chemokine mRNA expression were evaluated by RPA (left panels). Integrated density values of chemokine mRNA levels were normalized to GAPDH (right panels). The results shown are representative of one of three separate experiments. □, Nil, untreated; ■, sHZ with or without PD 98059 or apigenin.
75–300 U/ml SOD before sHZ stimulation, Mφ PTP activity was restored in a concentration-dependent manner, reaching up to 93% of basal PTP activity in the presence of 300 U/ml SOD (Fig. 8B). Collectively, these data indicate that Mφ PTP inactivation in response to sHZ is an early event that seems to be dependent on oxidative stress.

Discussion

In *Pf* malaria, Mφ have been proposed as one of the main sources of proinflammatory mediators (2); therefore, it is of paramount importance to identify the parasite components participating in chemokine induction and to define the underlying regulatory mechanisms. In this regard, Sherry et al. (12) reported that both *Pf* HZ and sHZ lead to the release of chemokines MIP-1α and MIP-1β in murine Mφ and human PBMC. More recently, we demonstrated that sHZ also induces chemokine transcription and secretion in vivo (10). Confirming and extending these previous findings, we show that either *Pf* HZ or sHZ increases mRNA levels of MIP-1α, MIP-1β, MIP-2, and MCP-1 in B10R murine Mφ through a mechanism that is oxidative stress and ERK1/2 dependent and involves PTP down-regulation and NF-κB activation. Chemokine up-regulation was detected at early times of stimulation and occurred in a concentration-dependent manner. These data suggest that upon its release into the bloodstream, HZ might interact with resident Mφ and circulating monocytes and lead to rapid chemokine production. In this context, HZ could stimulate the immunological response at sites of parasite sequestration by inducing potent chemotacticants and activators. However, at higher parasitemias and subsequently higher HZ concentrations, the malarial pigment might also contribute to an exacerbated proinflammatory response (e.g., leukocytosis and high ROS production), causing tissue damage and microvascular flow disturbance.

Our results, demonstrating the capacity of sHZ to trigger Mφ oxidative stress, are in line with previous studies reporting the same phenomenon after Mφ treatment with *Pf* HZ (32) or sHZ (33). However, these studies associated HZ-inducible ROS with depression of Mφ functions. Human monocyte-derived Mφ fed with *Pf* HZ displayed a long-lasting oxidative burst, but were unable to repeat the phagocyte cycle. Moreover, their ability to generate oxidative stress in response to PMA was irreversibly suppressed (32). Similarly, the ability of sHZ to reduce LPS-mediated cytokine and NO production in Mφ was attributed to sHZ-inducible oxidative stress (33). In contrast, we found that ROS appear to be one of the mechanisms through which the malarial pigment leads to Mφ chemokine induction. These inconsistencies probably stem from the fact that, as discussed by others (33) and by us (9), the effects of HZ most likely depend on cell susceptibility to oxidative stress, which can vary according to the cell type as well as

![FIGURE 5. Synthetic HZ leads to NF-κB nuclear translocation and binding to the murine MIP-2 and MCP-1 promoters.](image-url)
its tissue source. Whereas HZ was shown to exert a down-regulating effect on NO production by peritoneal Mφ/H9278, it did not modulate this activity in microglial cells (33), and it was able to increase it in bone marrow-derived Mφ/H9278 (9). In this context, it is conceivable that rather than a unique effect, HZ might lead to a localized negative or positive ROS-mediated regulation of Mφ/H9278 function depending on their antioxidant defenses.

In addition to its capacity to activate ROS, sHZ caused a marked reduction of Mφ/H9278 PTP activity. Moreover, in the presence of SOD, an enzyme involved in O$_2^-$ catabolism (34), basal PTP activity was almost completely restored, indicating that this down-regulatory event depends mostly on sHZ-inducible O$_2^-$ generation. Consistent with this, PTPs possess reduction-oxidation (redox)-sensitive cysteine residues in their active sites (38), and their activity is inhibited in the presence of either O$_2^-$ (35, 36) or H$_2$O$_2$ (37). Of interest, our in vitro data revealed that peroxovanadium (bpV (phen)), a potent and selective PTP inhibitor (39), suppressed basal Mφ phosphatase activity (40) and increased chemokine mRNA levels in B10R Mφ (our unpublished observations). Moreover, we demonstrated that PTP inhibition strongly up-regulated the in vivo expression of various chemokine transcripts and enhanced their production in response to infection (41). In parallel, we detected a greater chemokine induction in Mφ/H9278 deficient in Src homology region 2 domain-containing phosphatase-1, one of the major Mφ/H9278 PTPs (42), compared with wild-type Mφ (our unpublished observations). In light of these findings, it seems plausible to propose ROS-mediated PTP inactivation as a possible mechanism for HZ-inducible Mφ/H9278 chemokine modulation. Even though the Mφ PTP(s) targeted by sHZ remains to be identified, a general explanation that could account for our results is that upon HZ stimulation, the intracellular equilibrium, in which basal PTP activity predominates over that of PTK, would be altered toward a higher PTK activity due to an increase in ROS and subsequent reduction of PTP activity via their redox-sensitive residues.

Current data provide evidence that a mild oxidative stress can act as a second messenger leading to redox-responsive transcription factor activation and subsequent chemokine gene expression (22, 43). In agreement with these studies, we found that chemokine modulation in response to sHZ required nuclear translocation and

![FIGURE 6. Role of NF-κB in Mφ sHZ-dependent chemokine mRNA up-regulation. Total proteins from untreated or sHZ-stimulated Mφ (15 min), pretreated, or not, with apigenin or PD98059 (μM), were subjected to Western blotting, and IκBα phosphorylation was monitored with a phospho-IκBα Ab. IκBα protein levels were monitored using an IκBα Ab (A). Labeled NF-κB probe was incubated with nuclear extracts from either untreated or sHZ-stimulated cells (25 μg/ml for 1 h), pretreated, or not, with increasing concentrations (micromolar) of apigenin, PD 98059 (B), or BAY 11-7082 (C), and EMSA was performed. Binding specificity was tested by adding to nuclear extracts from 1-h treated cells a 100-fold molar excess of cold NF-κB oligonucleotide or a nonspecific Oct-2A probe. D. After a 1-h exposure to BAY 11-7082, Mφ were additionally stimulated with 25 μg/ml sHZ for 2 h. Then, total RNA was extracted, and changes in chemokine mRNA levels were monitored by RPA. □, Nil, untreated; ■ sHZ with or without BAY 11-7082. These results are representative of one of three separate experiments.](http://www.jimmunol.org/)

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binding of the redox-sensitive NF-κB to murine chemokine promoters. Importantly, blockage of oxidative stress with SOD significantly reduced NF-κB translocation, indicating a key role for O$_2^-$ generation in this signaling event. Our previous work (44) demonstrated the induction of NF-κB activity in response to specific PTP inhibitors, and others reported that SOD pretreatment abrogated Mφ IkBα tyrosine phosphorylation (45) and NF-κB activation (46) by silica with or without pervanadate. Therefore, it can be envisaged that NF-κB induction by sHZ involves O$_2^-$-dependent PTP inactivation and subsequent PTK up-regulation. This is in concert with the demonstration that hypoxia/reoxygenation activates NF-κB via tyrosine phosphorylation of IkBα (47), which is mediated by the PTK c-Src (48) and is dependent on the PTK Syk in response to H$_2$O$_2$ (49). Even though ROS do not phosphorylate IkBα on its serine residues, they induce IkB kinase (IKK) activation as well as p65 serine phosphorylation and nuclear translocation (49). Moreover, it was recently reported that SOD treatment reversed increased O$_2^-$ levels and prevented the activation of IKKa, IKKβ, and the p65 NF-κB subunit (50). Thus, according to this evidence and the knowledge that IKKs phosphorylate p65 (51), it is plausible that IKK activation is required for sHZ-inducible p65 nuclear translocation.

Blockage of ROS with SOD markedly down-regulated NF-κB nuclear translocation in response to the malarial pigment, but did not abolish it, suggesting that ROS-independent mechanisms are also involved. Analysis of possible alternative second messengers revealed that sHZ leads to rapid phosphorylation of ERK1/2 MAPK. Because this event was not affected by SOD administration (data not shown), these data indicate that ERK1/2 activation is not dependent on O$_2^-$ generation. Consistent with previous studies suggesting the involvement of MEK/ERK1/2 in NF-κB activation (52–54) and chemokine modulation (22, 27), sHZ-inducible IkBα serine phosphorylation, NF-κB nuclear translocation, as well as chemokine up-regulation were impaired in the presence of specific inhibitors of the ERK1/2 pathway. These results are perfectly in line with our published data showing that the up-regulating effect
of sHZ on IFN-γ-mediated NO production occurred via ERK1/2-dependent NF-κB binding to the murine inducible NO synthase promoter (9). Thus, it is likely that through activation of the same signals leading to PTP down-regulation, NF-κB-dependent mechanisms, which appear to elicit independent activation. A more detailed characterization of the various signaling pathways involved will help in understanding how sHZ chemokine modulation in response to HZ. Given the importance of establishing the correlation between the clinical manifestations of malaria and the effects of HZ on Mφ functions, our work may contribute to better define the role of HZ in malaria pathogenesis and might be useful in the development of specific therapeutic targets to tame proinflammatory mediator overproduction.

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References


