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Hemozoin Regulates iNOS Expression by Modulating the Transcription Factor NF-κB in Macrophages

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Abstract

Hemozoin (Hz) is released from ruptured erythrocytes during malaria infection caused by *Plasmodium* sp., in addition the malaria infected individuals are prone to bacterial sepsis. The molecular interactions between Hz, bacterial components and macrophages remains poorly investigated. In this report, we investigated the combinatorial immune-modulatory effects of phagocytosed Hz, Interferon gamma (IFNγ) or lipopolysaccharide (LPS) in macrophages. Macrophages were treated with various concentrations of commercial synthetic Hz, and surprisingly it did not result in inducible nitric oxide synthase (iNOS) expression. However, when macrophages were pretreated with Hz and then challenged with IFNγ or LPS, there was a differential impact on iNOS expression. There was an increase in iNOS expression when macrophages were pre-treated with Hz and subsequently treated with IFNγ when compared to IFNγ alone. Whereas iNOS expression was reduced when Hz phagocytosed macrophages were stimulated with LPS compared to LPS alone. Furthermore, there was an increased activation of NF-κB in Hz phagocytosed macrophages that were challenged with IFNγ. The interaction between Hz and macrophages has an impact on iNOS expression.

Keywords: Hemozoin; Inducible nitric oxide synthase; Lipopolysaccharide; Macrophage; Malaria; Sepsis

Abbreviations: Hz: Hemozoin; NF-κB: Nuclear Factor Kappa-Light-Chain-Enhancer of Activated B Cells; iNOS: Inducible Nitric Oxide Synthase; IFNγ: Interferon Gamma; LPS: Lipopolysaccharide

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Introduction

Malaria is one of the most devastating diseases worldwide with high incidence of morbidity and mortality [1]. The co-infection of malaria and Gram-negative bacterial sepsis is also a major concern that results in severe clinical complications [2-4]. Hemozoin (Hz) is released following the rupture of the erythrocytes, which is formed by the parasite – *Plasmodium* sp., as a detox mechanism during the intra-erythrocytic cycle [5]. Macrophages (in particular liver-resident) play an important role in elimination of malarial parasites and subsequent phagocytosis of hemozoin [6,7]. The release of hemozoin has physiological relevance as it can modulate the immune system, and it may continue to be present in many organs, such as liver, spleen for extended period in the life time [8]. Hemozoin also induces lung inflammation and correlates with acute respiratory distress syndrome, and may impact the pulmonary microvasculature [9,10]. Hemozoin is reported to modulate the immune functions of immune cells such as macrophages and dendritic cells. Some reports suggest Hz as an inert, bystander; other suggests contradictory findings as immune-activating or suppressive effects [11-14]. Macrophages are also one of important components of the immune system being involved in both the innate and adaptive immune responses. Macrophages are the major phagocytotic cells of the immune system and are essential for generating beneficial inflammation that is necessary for host defence and bactericidal activity [15,16]. In response to bacterial infection, the molecular signalling events in macrophages leads to expression of iNOS (inducible nitric oxide

synthase) and are important sources of iNOS-derived nitric-oxide (NO), which together with the respiratory burst of reactive oxygen species (ROS), leads to effective bactericidal activity [15,17].

The molecular interactions between Hz, bacterial components, inflammatory mediators and macrophages remains poorly investigated. In this study, we investigated the combinatorial immune-modulatory effects of synthetic Hz and bacterial endotoxins and pro-inflammatory agents. Here we report the effect of phagocytosis of hemozoin on subsequent production of iNOS by macrophages in response to pro-inflammatory agents-IFNγ and LPS.

Material and Methods

Culture of RAW 264.7 macrophage cell line

Murine macrophage/monocyte cell line RAW 264.7, was cultured in 1×DMEM media containing 10% FBS (Hyclone) supplemented with penicillin (100 U/mL), streptomycin (100 µg/ml), and maintained at 37°C in a humidified air containing 5% CO_2 [18].

Phagocytosis of hemozoin

RAW 264.7 macrophages were treated with Hz (100 µg/ml) and incubated for 3 h and 18 h. The macrophages were also pretreated with Hz and followed by stimulation with pro- and anti-inflammatory agents - IFNγ (100 ng/ml), LPS (1 µg/ml) and IL4 (20 ng/ml) for 8 h, and observed under microscope at 40× magnification (Nikon) for phagocytosis and morphological attributes.

Expression of iNOS by macrophages

Hemozoin crystals (Catalogue # tlrl-hz, InvivoGen) was suspended in sterile molecular grade water, and sonicated in bath sonicator (Bransonic® Ultrasonic Baths, Thomas Scientific) prior to use. RAW 264.7 macrophages were stimulated with synthetic hemozoin with various concentration 10, 20, 50, 100, and 250 µg/ml for 16 h. LPS TLR-grade (1 µg/ml) (Alexis) was used as positive control. The macrophages were washed with 1×PBS and lysed with 1×RIPA buffer containing protease inhibitor cocktail (539131, Calbiochem) and 1 mM phenylmethylsulfonyl fluoride (PMSF), and processed for immunoblotting, as described before [18]. Antibodies used were anti-iNOS/NOS Type II (610332, BD Transduction Laboratories) and anti-β-actin (Sigma).

Expression of iNOS by macrophages in response to pro- and anti-inflammatory agents

Macrophages were pretreated with hemozoin and stimulated with - IFNγ [100 ng/ml] (BD Pharmingen), LPS [1 µg/ml] (Alexis Biochemicals) and IL4 [20 ng/ml] (RnD Systems), and processed for Western immunoblotting for iNOS as described previously [19].

Effect of hemozoin on transcriptional activity

RAW 264.7 macrophages were pre-treated with hemozoin (100 µg/ml for 14h) and stimulated with IFNγ (100 ng/ml) and LPS (1 µg/ml) for 15, 30, 60 min. The cells were washed with 1×PBS, and lysed with 1×RIPA buffer, containing protease and phosphatase inhibitor cocktail (539131 and 524625 respectively, Calbiochem) and 1 mM PMSF. The lysates were immunoblotted for phospho-NF-κB p65 (Ser536) (3031S, Cell Signaling) and NF-κB p-65 (3034, Cell Signaling).

Result and Discussion

Effect of hemozoin on expression of iNOS

Previous studies have used Hemozoin (Hz), isolated from parasitized RBCs or synthesized in the research labs to investigate the role of Hz in immune-modulation. The methods described to isolate natural Hz or synthesize Hz are time consuming, laborious, and there could be chance of contamination of the Hz with other cellular components or chemical components [20]. The concentration of Hz used in these experiments ranged from 10 µg/ml to 400 µg/ml for *in-vitro* cellular assays, and has been previously described [20]. We came across commercial synthetic Hemozoin crystals (Catalogue # tlrl-hz, InvivoGen) and sought to investigate its effects on expression of iNOS in macrophages, as this source of Hz is readily available. Based on the previous literature [20], we chose to use 10 µg/ml to 250 µg/ml of Hz. Macrophages were stimulated with various concentrations of hemozoin (Hz) for 16 h, and immunoblotted for iNOS. Hemozoin had no effect on expression of iNOS, compared to LPS alone, which was used as positive control **(Supplementary Figure 1).** Other researchers have used Hz isolated from parasitized RBCs and synthetic Hz and observed differing results [8,14,20,21]. The stimulation with different sources of Hz have reported to induce differential gene expression in macrophages [8,14,20,22]. Since we did not observe iNOS expression in response to hemozoin, we checked if hemozoin was phagocytosed by macrophages. Microscopic imaging suggests that Hz was phagocytosed by macrophages in less than 3 h **(Figure 1)** and did not result in any notable changes in cell morphology.

We further investigated the effect of bacterial components (LPS) and inflammatory mediators (IFNγ) as one would expect in a sepsis conditions. The macrophages were, pretreated with Hz and challenged with pro-inflammatory agents (IFNγ or LPS) to mimic the release of inflammatory mediators such as iNOS. An increased expression of iNOS protein was observed when macrophages were pre-treated with Hz, and stimulated with IFNγ when compared to IFNγ alone **(Figure 2).** Surprisingly, we observed unexpected results. The expression of iNOS was reduced, when macrophages were pre-treated with Hz, and then stimulated with LPS, when compared to LPS alone (**Figure 2).** In controls, the macrophages were pretreated with Hz stimulated with IL4 or IL4 alone (an anti-inflammatory agent), and as expected in both the approach, there was no production of iNOS.

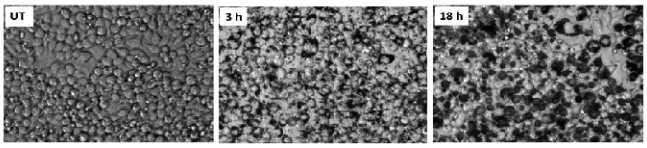

Figure 1 Phagocytosis of hemozoin by macrophage. The RAW 264.7 macrophages were incubated with synthetic hemozoin for 3 h and 18 h, visualized under microscope at 40× magnification. UT: Untreated.

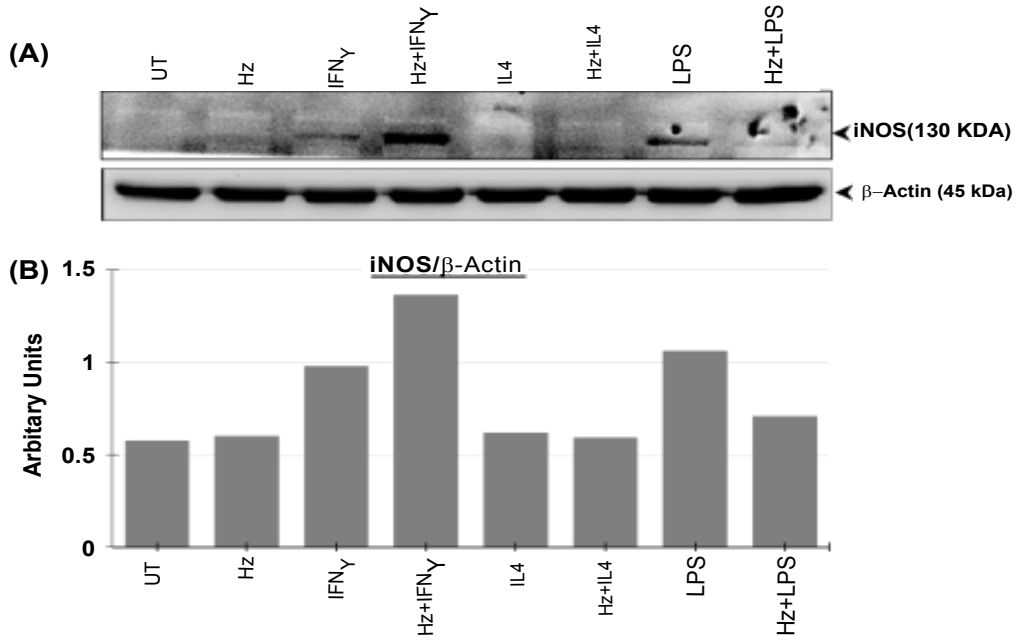

Figure 2 Expression of iNOS. The macrophages were pre-treated with hemozoin (Hz) (100 µg/ml for 14 h) and stimulated with IFNγ (100 ng/ml), IL4 (20 ng/ml) and LPS (1 µg/ml) for 12 h, and immunoblotted for iNOS. Pre-treatment with Hz and subsequent challenge with IFNγ resulted in increased expression of iNOS as compared to LPS (A). Densitometric analysis of the immuno-blot (B). UT: Untreated.

This result shows that the phagocytosis of the Hz by macrophages alters the immune-modulatory effects, which affects the iNOS production. A similar observation, indicating enhanced iNOS expression when macrophages (RAW 264.7 cells) were fed with Hz and treated with IFNγ has been reported [14]. We further checked, if the pretreatment with Hz and subsequent challenge with IFNγ or LPS or IL4 had any morphological effects on macrophages. There was no apparent change in the morphological appearance of macrophages post Hz phagocytosis **(Figure 3).**

Modulation of transcriptional activation of NF-κB

We then investigated the activation of the key transcription factor - NF-κB, a regulator for iNOS expression. When macrophages were pretreated with Hz and then challenged with IFNγ, there was a gradual increase in phosphorylation rate with the highest amount of phosphorylation of NF-κB at 60 minutes in comparison with IFNγ alone. However, we observed an opposite effect when macrophages were pretreated with Hz and then challenged with LPS. Initially there was phosphorylation of NF-κB at 15 minutes, but then the rate of phosphorylation decreases in comparison to LPS at 60 minutes **(Figure 4).** This transcriptional activity of NF-kB may relate to the result that is observed in **Figure 2**. These results indicate that Hz in some ways exerts differential expression of iNOS in combinatorial response to hemozoin and pro-inflammatory mediators by regulating the activation of transcription factor NF-κB. What molecular events cause this phenomenon and how it affects the iNOS expression is for further investigation.

Conclusion

The results demonstrate hemozoin and inflammatory mediators exerts differential iNOS expression in macrophages. Additionally, based on our results, and previous publications, it is evident that different sources for acquiring hemozoin (natural, synthetic or commercial) may be confounding factors and may result in different experimental outcomes, and thus experiments should be judicially planned.

Conflict of Interest/Disclosures

The authors associated with this manuscript declare that there is no conflict of interest.

Author Contributions

Conceived and designed experiments: JWC, RR. Performed experiments: RR. Analyzed data: JWC RR. Contributed reagents/materials/analysis tools: AHC. Wrote paper: JWC, RR. Edited the manuscript: JWC, RR, MK, AR.

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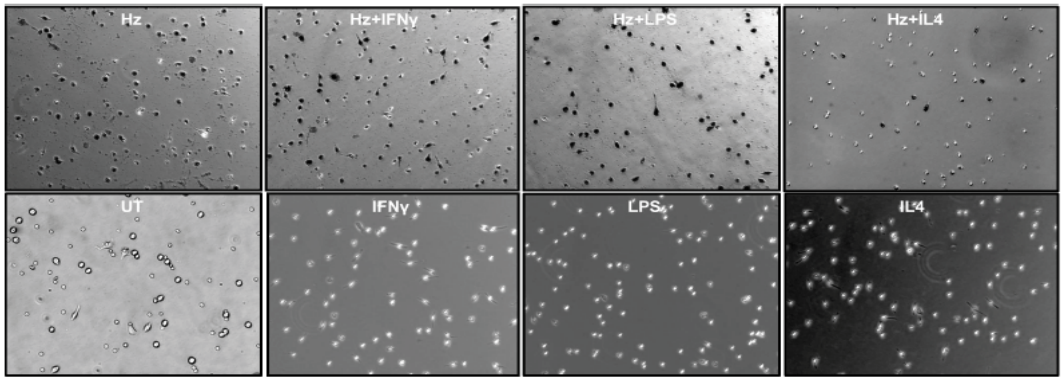

Figure 3 Effect of hemozoin and pro- and anti-inflammatory agents. Macrophages were treated with hemozoin (100 µg/ml for 14 h) and were stimulated with IFNγ (100 ng/ml), LPS (1 µg/ml) and IL4 (20 ng/ml) for 8 h. The cells were visualized under microscope at 20× magnification.

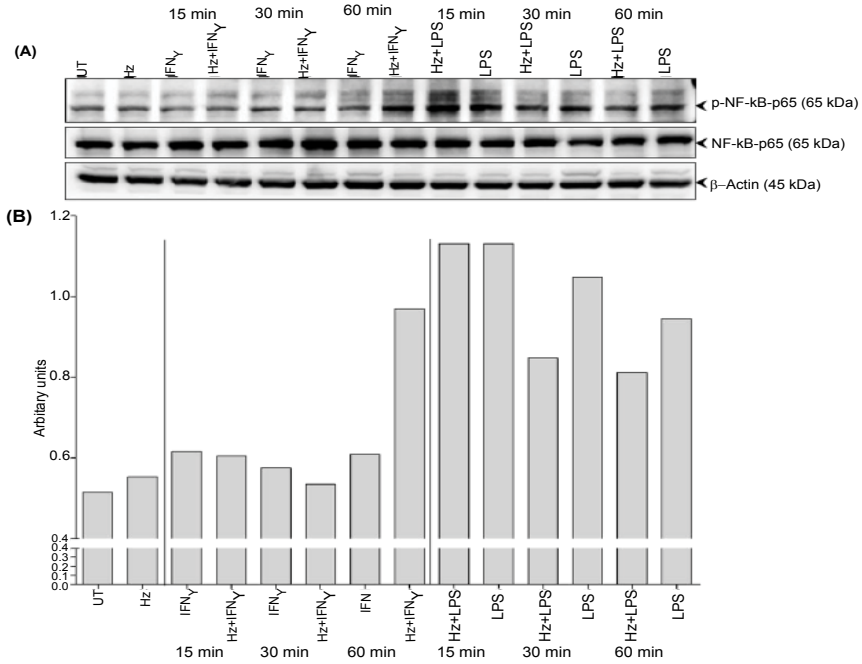

Figure 4 Regulation of transcription factor NF-kB. The macrophages were treated with hemozoin (Hz) (100 µg/ml for 14h) and stimulated with IFNγ (100 ng/ml) and LPS (1 µg/ml). The lysates were immunoblotted with p-NF-kB p-65 and NF-kB p-65 antibodies. Pre-treatment with Hz and subsequent challenge with IFNγ resulted in activation of NF-kB, whereas compared with LPS (A). (B) Densitometric analysis of the immuno-blot. The expression of p-NF-kB p-65 and NF-kB p-65 was normalised with β-Actin. The ratios of normalised p-NF-kB p-65/NF-kB p-65 are presented (B). UT: Untreated.

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