Interleukin-1β and Interferon-γ are Associated with Malaria-Induced Hypoinsulinemic Hypoglycemia in Plasmodium berghei Anka-Infected Mice

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ABSTRACT

Malaria-induced hypoglycemia is recognized as a serious complication of malaria and has one of the strongest associations with mortality in children. It has been speculated that oxidative stress and pro-inflammatory response during parasite infection were involved in its pathophysiology. Hence, this study aimed to investigate the development of malaria-induced hypoglycemia during Plasmodium berghei ANKA (PbANKA) infection with particular attention to the involvement of c-peptide, interleukin-1β (IL-1β), and interferon-γ (IFN-γ). ICR mice were infected with $1 \times 10^7$ parasitized erythrocytes of PbANKA, and parasitemia was monitored, and the development of hypoglycemia was assessed by measuring plasma glucose levels. The change of c-peptide level was evaluated. The pro-inflammatory response of IL-1β and IFN-γ were also quantified in plasma. It was found that PbANKA infection resulted in hypoglycemia as indicated by a significantly ($P < 0.05$) decrease in plasma glucose levels on day 4 post-infection and associated with parasitemia. The c-peptide was slightly increased at day 2 post-infection, and then significantly ($P < 0.05$) decreased since day 4. Furthermore, we observed a significantly ($P < 0.05$) increased IL-1β, firstly responded, at day 2 post-infection followed by increasing the IFN-γ level at day 4 in PbANKA-induced hypoglycemia. Our findings support the idea that hypoinsulinemic hypoglycemia in the PbANKA infected mice may be involved in the high IL-1β and IFN-γ against the parasite infection.

Keywords: Hypoglycemia; IFN-γ; IL-1β; malaria; Plasmodium berghei

ABSTRAK

Hipoglisemia yang disebabkan oleh malaria dikenali sebagai komplikasi malaria yang serius dan mempunyai salah satu kaitan paling kuat dengan kematian yang berlaku dalam kalangan kanak-kanak. Spekulasi bahawa tekanan oksidatif dan tindak balas pro-radang semasa jangkitan parasit terlibat dalam patofisiologinya. Oleh itu, kajian ini bertujuan untuk mengkaji perkembangan hipoglikemia yang disebabkan oleh malaria semasa jangkitan Plasmodium berghei ANKA (PbANKA) dengan perhatian khusus dilakukan terhadap penglibatan c-peptida, interleukin-1β (IL-1β) dan interferon-γ (IFN-γ). Tikus ICR telah dijangkiti dengan $1 \times 10^7$ eritrosit parasit PbANKA dan parasitemia dipantau dan perkembangan hipoglikemia dinilai dengan mengukur tahap glukosa pada plasma. Perubahan tahap c-peptida ini dinilai. Tindak balas pro-radang IL-1β dan IFN-γ juga dikira pada plasma. Jangkitan PbANKA didapati telah mengakibatkan hipoglikemia dan berlakunya penurunan ketara ($P < 0.05$) dalam paras glukosa plasma pada hari ke-4 selepas jangkitan dan dikaitkan dengan parasitemia. C-peptida meningkat sedikit pada hari ke-2 selepas jangkitan dan kemudian menurun dengan ketara ($P < 0.05$) pada hari ke-4. Seterusnya peningkatan ketara ($P < 0.05$) IL-1β berlaku, yang mula bertindak balas, pada hari ke-2 selepas jangkitan diikuti dengan meningkatkan tahap IFN-γ pada hari ke-4 dalam hipoglikemia yang disebabkan oleh PbANKA. Penemuan kajian menyokong idea bahawa hipoglikemia hipoinsulinemia pada tikus yang dijangkiti PbANKA mungkin terlibat dalam peningkatan IL-1β dan IFN-γ terhadap jangkitan parasit.

Kata kunci: Hipoglisemia; IFN-γ; IL-1β; malaria; Plasmodium berghei
**INTRODUCTION**

Malaria is a significant widespread disease in tropical and subtropical areas of the world, affecting humans. There were an estimated 229 million cases of malaria and 409,000 malaria deaths in 87 endemic countries, especially children aged under 5 years. Nearly 94% of the cases in 2019 were in the African Region (215 million cases), followed by the South-East Asia Region (3%) (WHO 2020). Reports indicated that the high parasitemia resulting from infection is associated with the derangement of some physiological systems, including severe hemolytic anemia, hemoglobinuria, metabolic acidosis, abnormal bleeding, microvascular impediment, liver damage, jaundice, kidney impairment, pulmonary edema, electrolyte imbalance, cerebral malaria, and hypoglycemia (White et al. 2014). Hypoglycemia is a serious complication of malaria and has one of the strongest associations with mortality in children (Planche & Krishna 2006). Malaria-induced hypoglycemia has been attributed to increased utilization of the host’s glucose stores by malaria parasites. It is because malaria parasites depend on glucose as a nutrient source, and it cannot store energy in the form of glycogen; they rely entirely on an exogenous supply of glucose (Madrid et al. 2015). The pathogenesis of malaria-induced hypoglycemia is not well characterized. Studies have shown that parasite’s depletion of gluconeogenic substrates plays a significant role in the reported hypoglycemia induced by malaria (Ogetii et al. 2010). Hypoglycemia during malaria infection has also been attributed to the disproportionate release of pro-inflammatory and inflammatory cytokines, so it has been associated with being a critical factor in the pathogenesis of malaria (Ogetii et al. 2010; Planche & Krishna 2006; Richards 1997). It has been reported that tumor necrosis factor (TNF) might cause malaria-associated hypoglycemia with hyperinsulinemia and insulin resistance in rodent malaria models (Elased & Playfair 1994). However, the cause of hypoglycemia induced by malaria has not been fully elucidated but is likely to be multifactorial, and other cytokines could be involved, such as interleukin-1β (IL-1β) and interferon-γ (IFN-γ). Several hypotheses have been suggested about the involvement of cytoadherence of parasitized erythrocytes and pancreatitis due to oxidative stress (Taverne et al. 1995). Additionally, the level of these cytokines and hypoglycemia in *Plasmodium berghei* infected mice have not yet been reported. Hence, this study was aimed to investigate the levels and correlation of IL-1β and IFN-γ in hypoglycemia induced by *Plasmodium berghei* infection in experimental mice.

**MATERIALS AND METHODS**

**REAGENTS**

All reagents used in this study were analytically graded and procured from certified local and international suppliers.

**EXPERIMENTAL MICE**

Healthy male, 4-6 weeks old ICR mice weighing 25-30 g were employed throughout the experiment. They were purchased from Nomura Siam International Co, Ltd., Thailand, and maintained under the standard conditions with 12 h light/12 h dark cycle at room temperature. They were provided with a pellet diet and clean water *ad libitum* and acclimatized for one week before the commencement of the experiment. According to international laboratory animal use and care guidelines, all animal handling and care were carried out throughout the experiment and approved by the Animal Ethics Committee, Walailak University.

*Plasmodium berghei*

*Plasmodium berghei* strain ANKA (PbANKA) was obtained from MR4 (Malaria Research and Reference Reagent Resource Center). Cryopreservative stock was thawed completely at 37 ℃ in a water bath and intraperitoneally injected into naïve mice. The parasites were maintained by serial passage of blood from infected mice to noninfected ones on a weekly basis.

**DETERMINATION OF PARASITEMIA**

Propagation of PbANKA in mice was monitored by microscopic examination of Giemsa-stained blood smears. Tail blood was collected and smeared on a microscopic slide. After being fixed with 100% methanol, the slide was then stained with 10% Giemsa solution at room temperature for 15 min. Parasitized erythrocytes were counted under a light microscope with a 100× oil immersion lens, and the percentage of parasitemia was calculated using the following formula (1).

\[
\text{% parasitemia} = \frac{\text{Number of parasitized erythrocytes} \times 100}{\text{Total number of erythrocytes}}
\]  

(1)

**DETERMINATION OF PLASMA GLUCOSE**

Glucose levels were measured in plasma samples collected by cardiac puncture using a commercial kit.
for the hexokinase endpoint method (Megazyme, Ireland) following the manufacturer’s protocol. Samples were read at 340 nm, and the results were expressed as mg of glucose per dL.

DETERMINATION OF C-PEPTIDE
C-peptide has been frequently used as a measure of insulin secretion. Since C-peptide is the part of proinsulin that is cleaved before co-secretion with insulin from pancreatic β-cells, it degrades slower than insulin (half-life of 20-30 min versus 3-5 min) and is excreted from the body at a constant rate (Leighton et al. 2017). According to the manufacturer’s instructions, levels of c-peptide were measured in plasma collected by cardiac puncture using a competitive ELISA assay (Sigma-Aldrich, St. Louis, Missouri, USA). Results were presented as ngmL⁻¹.

DETERMINATION OF IL-1β AND IFN-γ
The levels of IL-1β and IFN-γ were measured in the plasma collected by cardiac puncture using the sandwich ELISA assay, according to the manufacturer’s instructions (Sigma-Aldrich, St. Louis, Missouri, USA). Results were presented as pgmL⁻¹.

IN VIVO EXPERIMENTAL DESIGN
ICR mice were intraperitoneally injected with $1 \times 10^7$ parasitized erythrocytes of PbANKA and randomly divided into 5 groups (3 mice of each). On day 2, 4, 6, 8, and 10 post-infections, parasitemia was determined, and mouse blood was then collected from groups 1, 2, 3, 4, and 5, respectively, by cardiac puncture into heparinized vacuum tubes. Plasma was subsequently prepared and used to determine the plasma glucose, c-peptide, IL-1β, and IFN-γ levels. The uninfected group was also performed as healthy control.

STATISTICS
Data were organized and performed with GraphPad Prism 6.0 (GraphPad Software, Inc., San Diego, CA). Results were expressed as mean ± standard error of mean (SEM) and analyzed using one-way analysis of variance (ANOVA) and Tukey’s multiple comparison posttest. Statistical significance was considered if $P$-value < 0.05 at 95% confidence intervals.

RESULTS
MALARIA-INDUCED HYPOGLYCEMIA DEVELOPMENT DURING PbANKA INFECTION
Parasitemia was firstly detectable on day 2 post-infection with a parasitemia of $0.27 \pm 0.11\%$ and reached $33.67 \pm 13.65\%$ at day 10 (Figure 1(A)), and PbANKA infected mice died within 12 days (Figure 1(B)). Next, we observed that plasma glucose level was significantly decreased ($P < 0.05$) in infected ICR mice at day 4 post-infection ($164.17 \pm 6.43$ mglm⁻¹) until the infected mice died ($65.90 \pm 26.07$ mglm⁻¹ on day 10 post-infection) (Figure 1(C)), suggesting hypoglycemia development in the presence of PbANKA infection. Additionally, we observed a strong negative correlation ($R^2 = 0.9637$) between parasitemia and glucose levels (Figure 1(D)).

ASSOCIATION OF C-PEPTIDE LEVEL WITH PARASITEMIA
We investigated the relationship between parasitemia and c-peptide during PbANKA infection in ICR mice. As indicated in Figure 2(A), after 4 days of infection with PbANKA, c-peptide level was significantly lower ($P < 0.05$) in mice ($1.16 \pm 0.48$ ngmL⁻¹) compared with day 0 post-infection ($1.40 \pm 0.25$ ngmL⁻¹). As expected, c-peptide negatively correlated ($R^2 = 0.8208$) with parasitemia (Figure 2(B)).

PBANKA INFECTION INCREASED THE PRO-INFLAMMATORY RESPONSE DURING HYPOGLYCEMIA
The participation of oxidative stress products in the development of malaria-induced hypoglycemia was assessed by analysis of IL-1β and IFN-γ. We observed that the plasma levels of IL-1β in PbANKA infected mice were significantly higher ($P < 0.05$) at day 2 post-infection ($28.58 \pm 11.23$ pgmL⁻¹) (Figure 3(A)). In addition, PbANKA infection significantly increased ($P < 0.05$) levels of IFN-γ on day 4 post-infection ($14.66 \pm 5.20$ pgmL⁻¹) when compared with respective control on day 0 post-infection ($2.11 \pm 0.38$ pgmL⁻¹) (Figure 3(B)). Therefore, IL-1β was firstly responded followed by IFN-γ in the development of hypoglycemia during PbANKA infection.

DISCUSSION
In the current study, the association of IL-1β and IFN-γ with hypoglycemia caused by malaria was investigated in PbANKA infected ICR mice. Clinical reports have notified hypoglycemia during malaria infection, and it is an important life-threatening complication of malaria infection. In our study, PbANKA showed a significant ($P < 0.05$) reduction in plasma glucose and agreed with our previous observation (Ounjaijean et al. 2019). The cause of hypoglycemia is most likely multifactorial; the heavy utilization of glucose by the malaria parasite is a possible reason (Planche & Krishna 2006). It might be explained by parasites ability to increase the uptake of...
FIGURE 1. Hypoglycemia development during malaria infection. Naïve ICR mice were intraperitoneally infected with $1 \times 10^7$ parasitized erythrocytes of PbANKA. (A) % parasitemia, (B) % survival, and (C) glucose levels were measured on different days post-infection. (D) Correlation of % parasitemia and glucose levels was also estimated. Results were expressed as mean ± SEM of 5 mice per group. * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$ as compared to day 0 post-infection.

FIGURE 2. Assessment of c-peptide during malaria-induced hypoglycemia. Naïve ICR mice were intraperitoneally inoculated with $1 \times 10^7$ parasitized erythrocytes of PbANKA. (A) Levels of c-peptide was measured on different days post-infection. (B) Correlation between % parasitemia and c-peptide levels was also estimated. Results were presented as mean ± SEM of 5 mice per group. * $P < 0.05$ and *** $P < 0.001$ as compared to day 0 post-infection.
glucose through the process of glycolysis facilitated by the hexose transporters and impair glucose production caused by the inhibition of gluconeogenesis (Tjhin et al. 2013; van Niekerk et al. 2016). It has been reported that the overall glucose utilization rate of parasitized erythrocytes increased by about 100-fold compared to uninfected ones (Roth 1990). The invasion of PbANKA into erythrocytes requires a large amount of energy consumption, which increases during maturation. Therefore, high parasitemia is associated with high glucose requirements, leading to hypoglycemia, as indicated by the strong negative correlation between parasitemia and plasma glucose.

In addition, host illness could increase tissue metabolism (Madrid et al. 2015; Sengupta et al. 2020), depletion of glucose stores due to starvation, malabsorption of glucose due to decreased splanchnic blood flow, and impairment of gluconeogenesis induced by cytokines, which attributed to hypoglycemia in the host (Roe & Pasvol 2009). Liver injury-induced hypoglycemia is one of the causes of hypoinsulinemic hypoglycemia. Liver damage during malaria infection might induce hypoglycemia since the liver plays a significant role in the various controlled glucose homeostasis pathways, including glycogenesis, glycogenolysis, glycolysis, and gluconeogenesis (Han et al. 2016). The liver is the primary site for gluconeogenesis (90%), while the kidney can perform the same steps but only contributes 10%. The liver and renal cortex contain gluconeogenic enzymes such as pyruvate carboxylase, PEP carboxykinase, fructose 1,6-bisphosphatase, and glucose 6-phosphatase not found in all cell types. Thus, gluconeogenesis is restricted to specific tissues, primarily in the liver and renal cortex (Zhang et al. 2019). Liver and renal damage in PbANKA-infected mice was confirmed by a considerable increase in liver enzymes (AST, ALT, and ALP), BUN, and creatinine levels (Boonyapranai et al. 2021). PbANKA infection resulted in intrahepatic obstruction and hydropic degeneration, leading to necrosis of liver cells (Asmilia et al. 2020). Incretion of inflammatory cytokine during PbANKA infection related to inhibition of gluconeogenesis. Gluconeogenesis is an energy-demanding activity. Because IL-1β and IFN-γ reduce mitochondrial ATP synthesis (Barlow et al. 2018), it is hypothesized that lower cellular ATP levels lead to the suppression of hepatic gluconeogenesis (Zhang et al. 2019). Alteration of hepatic phosphoprotein levels in mice infected with malaria suggests the probability of liver damage (Maniam et al. 2012). Furthermore, the kidney is a critical organ involved in the regulation of plasma glucose. Malaria-associated acute kidney injury can affect glucose release and reabsorption, resulting in hypoglycemia (Ramos et al. 2019). Differentiation of monocytes from a resting to an inflammatory state requires a shift in energy metabolism due to high glucose consumption and rapid ATP generation by glycolysis (Mills et al. 2017), whereas inflammatory cytokines promote glucose uptake and metabolism by different host cell types (Metzger et al. 2004; Vogel et al. 1991). IL-1β also causes hypoglycemia in the animal model, where no hyperinsulinemia is measured, and in insulin-resistant diabetic mice (del Rey & Besedovsky 1989). IL-1β induces hypoglycemia, which is related to
inhibiting hepatic gluconeogenesis and enhanced glucose consumption in peripheral tissues (Metzger et al. 2004).

According to the current study, IL-1β was first responded followed by IFN-γ in developing of hypoglycemia during PbANKA infection. Innate and adaptive immunity is essential for controlling parasite growth and malaria infection severity (Arora et al. 2018; Liehl et al. 2015). Macrophages and dendritic cells (DCs) recognize pathogen-associated molecular patterns (PAMPs) on the pathogen’s surface via complementary pattern recognition receptors (PRRs), promoting malaria pathogen engulfment and destruction (Barnes 2018). In addition to phagocytosis, the activation of TLR/MyD88 signaling pathway is linked to the production of pro-inflammatory cytokines, such as IL-1β, TNF-α, and IL-12 (Franklin et al. 2009). IL-1β is produced by activated macrophages, a potent endogenous pyrogen that promotes an acute inflammatory response as part of the first line of defense against invading pathogens (Mayer-Barber & Yan 2017), and is involved in various cellular activities, including cell proliferation, differentiation, and apoptosis. In addition, activated macrophages and DCs promote NK cell activation and Th1 differentiation by IL-12, MHC I, and MHC II. The IFN-γ has been associated with the control of parasitemia, protection from malaria, and delayed reinfection (Arora et al. 2018). The NK cell and Th1 are key sources of the IFN-γ needed to drive macrophage hyperactivation, which plays a vital role in the antimalaria defense (Arora et al. 2018; Tukwasibwe et al. 2020). Intracellular malaria infects engulfed by macrophages but is not destroyed within ordinary phagosomes. Only in hyperactivated macrophages are sufficient levels of reactive oxygen intermediates and reactive nitrogen intermediates produced to kill such parasites efficiently (Mak et al. 2013).

Insulin is an important hormone to regulate the circulating level of plasma glucose. The current report found that insulin, as indicated by c-peptide levels, was significantly (P < 0.05) decreased, and a negative correlation between the c-peptide level and pro-inflammatory was also found. It might be explained by the secretion of pro-inflammatory cytokines such as IL-1β and IFN-γ and increased nitric oxide production. These mechanisms were thought to be a key mediator of impaired β-cell secretory function and β-cell apoptosis (Fei et al. 2008; Maedler et al. 2004; Thomas et al. 2002), which may cause hypoinsulinemia in illness mice with parasitemia. The pro-inflammatory cytokines such as IL-1β and IFN-γ have been reported to trigger destructive signaling pathways within β-cells, leading to elevation of reactive oxygen species, β-cell dysfunction, and eventually to apoptosis (Nano et al. 2021). The IL-1β exerts its effects predominantly through the NF-κB pathway, whereas IFN-γ acts mainly through the JAK/STAT1 pathway but may also activate STAT3 (Nano et al. 2021; Shi et al. 2019). NF-κB activation by IL-1β and IFN-γ leads to activate crosstalk between the canonical and non-canonical NF-κB pathways via p100 upregulation. Incretion of p52 via processing of p100 into the active subunit p52 related to impaired insulin secretion (Meyerovich et al. 2018). IL-1β and IFN-γ are also related to increasing of mitochondrial superoxide production, which results in pancreatic β-cell failure and β-cell loss (Barlow et al. 2018). Additionally, IL-1β and IFN-γ cause reduced mitochondrial ATP synthesis by defective oxidative phosphorylation and impairing ATP production, responsible for the insulin secretory defect in illness mice with parasitemia (Barlow et al. 2018).

**CONCLUSION**

Taken together, pro-inflammatory molecules including IL-1β and IFN-γ might have a critical role in developing hypoinsulinemic hypoglycemia during malaria propagation. The results also suggested that high parasitemia, liver injury, severe illness of host, and high levels of proinflammatory molecules, may be related to hypoglycemia in malaria infection. However, further research is needed for a better understanding of malaria-induced hypoglycemia.

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