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GSK3β: A plausible molecular target in the cytokine-modulating effect of exogenous insulin in a murine model of malarial infection

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Abstract. Malaria is a life-threatening disease caused by the *Plasmodium* sp. parasite. Infection results in heightened pro-inflammatory response which contributes to the pathophysiology of the disease. To mitigate the overwhelming cytokine response, host-directed therapy is a plausible approach. Glycogen synthase kinase-3β (GSK3β), a serine/threonine kinase plays a pivotal role in the regulation of inflammatory response during pathogenic infections. The present study was conducted to investigate the chemo-suppressive and cytokine-modulating effects of insulin administration in malaria-infected mice and the involvement of GSK3β. Intraperitoneal administrations of 0.3 and 0.5 U/kg body weight insulin each for four consecutive days into *Plasmodium berghei* NK65 (PbN)-infected mice resulted in chemo-suppression exceeding 60% and improved median survival time of infected mice (20.5 days and 19 days respectively compared to 15.5 days for non-treated control). Western analysis revealed that pGSK3β (Ser9) intensity in brain samples from insulin-treated (0.3 and 0.5 U/kg body weight) infected mice each were 0.6 and 2.2 times respectively than that in control. In liver samples, pGSK3β (Ser9) intensity from insulin-treated infected mice were significantly higher (4.8 and 16.1 fold for 0.3 and 0.5 U/kg bw respectively) than that in control. Insulin administration decreased both brain and liver pNF-κB p65 (Ser536) intensities (to 0.8 and 0.6 times for 0.3 U/kg bw insulin; and to 0.2 and 0.1 times for 0.5 U/kg bw insulin respectively compared to control). Insulin treatment (0.5 U/kg bw) also significantly decreased the serum levels of pro-inflammatory cytokines (TNF-α (3.3 times) and IFN-γ (4.9 times)) whilst significantly increasing the levels of anti-inflammatory cytokines (IL-4 (4.9 fold) and IL-10 (2.1 fold)) in PbN-infected mice. Results from this study demonstrated that the cytokine-modulating effects of insulin at least in part involve inhibition of GSK3β and consequent inhibition of the activation of NF-κB p65 suggesting insulin as a potential adjunctive therapeutic for malaria.

INTRODUCTION

In malaria as in most infections, host response contributes significantly to pathogenesis and clinical outcomes (Mackintosh *et al.*, 2004; Gowda & Wu, 2018). Dysregulated inflammatory response to the *plasmodial* parasite may lead to organ damage and death (Dieye *et al.*, 2016). Disease eradication can be addressed by employing host-directed strategies in addition to anti-parasitic therapeutics (Singh *et al.*, 2019). An attractive area of drug development for malaria treatment is the identification of novel pathways and targets associated with inflammation to mediate host immune response against infection, as the basis for effective adjunctive therapy. The objective of adjunctive therapy should be the improvement of clinical outcome and reduction of mortality, as well as prevention of long-term cognitive impairments. Even

though the pathophysiology of malaria is not fully understood, imbalanced cytokine production is a major factor contributing to disease severity (Auntino *et al.*, 2012).

In severe malaria (Wassmer *et al.*, 2017) and in experimental cerebral malaria (Frevert *et al.*, 2014), modulation of cytokine response is a common feature in which production of anti-inflammatory cytokines serves to down-regulate inflammation thus preventing detrimental immune reactions. Cytokine modulation is an attractive complementary strategy to manage malarial infection (Ghostner *et al.*, 2012). A key enzyme regulating pathogen-induced inflammatory response is glycogen synthase kinase-3β (GSK3β) which regulates the inflammatory response through the modulation of pro- and anti-inflammatory cytokine production (Wang *et al.*, 2014). GSK3 was first identified by Embi *et al.* (1980) as an important serine/threonine kinase in the regulation of glycogen metabolism mediated by insulin signalling. Significant findings from our laboratory and elsewhere revealed that LiCl, a GSK3 inhibitor, suppressed parasitaemia development (Zakaria *et al.*, 2010), modulated cytokine production (Ali *et al.*, 2017; Hassan *et al.*, 2019) and improved cognitive function (Dai *et al.*, 2012) in rodent models of malarial infection. Since GSK3 is an established target of lithium (Liu & Yao, 2016), and is pivotal in regulating inflammatory responses during infection, agents that can cause inhibition of GSK3β and modulate cytokine production may be potentially useful as adjunctive therapeutics for malaria. For example, erythropoietin a hematopoietic growth factor that can cause inhibition of GSK3β (Inkster *et al.*, 2018), has been reported to reduce mortality in cerebral malaria in mice and in a human clinical trial (Bienvenu & Picot, 2013). Insulin, a hormone that is also reported to possess anti-inflammatory effects and known to cause inhibition of GSK3β, a downstream component in PI3K/Akt signaling (Yang *et al.*, 2018) is another feasible candidate to be evaluated for immune modulation during malarial infection.

Insulin is reported to suppress production of pro-inflammatory cytokines whilst inducing anti-inflammatory mediator(s) production in obesity-induced inflammation (McArdle *et al.*, 2013). In another study in hyperglycaemia, the anti-inflammatory effects of insulin was proposed to be dependent upon its suppression of innate immune mechanisms involving transcription factors such as NF-κB (Sun *et al.*, 2014). NF-κB has an important role in inflammation and the regulation of cytokine production (Liu *et al.*, 2017). As such modulation of immune responses using insulin could represent an effective therapeutic strategy (Dandona *et al.*, 2007) in inflammation-related diseases including malaria. The aim of the present study is to evaluate the anti-malarial activity, cytokine-modulating effect of insulin treatment and the involvement of GSK3β in a murine model of malarial infection.

MATERIALS AND METHODS

Experimental animals

Institute of Cancer Research (ICR) strain of mice (male, 6-8 weeks old) were obtained from the Animal House Complex, Universiti Kebangsaan Malaysia (UKM) and housed at the Malaria Infection Laboratory, UKM. Permission and approval for animal studies were acquired from the UKM Animal Ethics Committee (UKMAEC) (reference number: FST/2018/HASIDAH/28-MAR./910-MAR.-2018-AUG.-2019). The animals were maintained at a constant room temperature (22°C), 50–70% relative humidity, a 12 hour light/dark cycle with unlimited access to pellets (rat chow, Barastoc brand from Ridley, Australia) and water.

Animal infection studies (four-day suppressive test)

Prior to infection studies, survivability of experimental animals (non-infected) administered with different doses of insulin was recorded for 30 days to ascertain that the dose of insulin to be further used for the four-day suppressive test did not affect animal survivability. For this, five groups of normal mice (n=6) were injected (ip) with different doses of insulin (0.1, 0.3, 0.5, 0.7 or 1.0 U/kg bw) for four consecutive days. These

doses were selected based on prior toxicity tests using normal non-infected mice (data not shown). The control group of animals were administered with 0.2 mL of 0.85% saline solution. Mice were observed for gross behavioural changes while body weights were recorded and survivability monitored for 30 days.

Chloroquine-sensitive *P. berghei* NK65 (PbN) strain, MRA-268 was obtained from the Malaria Research and Reference Reagent Resource Centre (MR4, Manassas, USA); http://www.beiresources.org, a part of BEI Resources, NIAID, NIH: deposited by Miracle Harrington. The parasite strain was maintained in ICR mice. Mice were randomly divided into eight groups (n=6) and injected intraperitoneally (ip) with 0.2 mL of infected blood (2×10^7 PbN-parasitised erythrocytes/mL). Starting at three hours post-infection (day 0), the experimental animals were injected (ip) over four consecutive days with either the anti-malarial reference drug (10 mg/kg body weight (bw) chloroquine) or the GSK3 inhibitor reference (100 mg/kg bw LiCl), 0.85% saline solution [control group A] or different doses of insulin (0.1, 0.3, 0.5, 0.7 or 1.0 U/kg bw) [test group B] in 0.2 mL volumes. The percentage of blood parasitaemia on day 4 post-infection (according to the four-day suppressive test by Peters (1975)) was determined from thin blood smears of tail blood from each animal. The average percentage of chemo-suppression (PC) was calculated by comparing the percentage of blood parasitaemia levels in control group A and test groups B:

$$PC = [(A - B)/A] \times 100$$

Brain and liver protein extraction and western analysis

Based on the four-day suppressive test results, brain and liver were obtained from infected mice administered with an effective dose of insulin (resulting in >60% chemo-suppression) at four days post-infection (Day 4) to determine phosphorylation states of GSK3β, Akt and NF-κB. Protein extraction of brain and liver were carried out according to Dai *et al.* (2012) but using a modified lysis buffer as described by Lee (2007). Protein concentration was measured according to Bradford (1976). Equal amounts of 40 µg protein samples were loaded into each lane of sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) gels. Separated proteins were transferred onto nitrocellulose membrane (Towbin *et al.*, 1979) and blocked with 3% BSA in Tris buffered saline-Tween 20 (TBST) (0.1% w/v Tween-20 in TBS). Membranes were further probed and incubated overnight at 4°C with primary monoclonal antibodies i.e. anti-GSK3β, anti-phosphoSer9-GSK3β, anti-Akt, anti-phosphoSer473-Akt, anti-NF-κB p65 or anti-phosphoSer536-NF-κB p65 (Cell Signalling, USA) prior to 2 h incubation with a corresponding secondary antibody, HRP-conjugated anti-mouse IgG (Cell Signalling, USA) at room temperature. Membranes were reprobed with anti-β-actin (Cell Signalling, USA) to ensure equal protein loading. Immunoreactive bands were detected using Western Lightning Plus-ECL Enhanced Chemiluminescence Substrate Kit (Perkin Elmer, USA). Band area intensity was quantified using a densitometer (Vilbert Lourmat 302, France).

Cytokine analysis

Mice were divided into five groups (n=6) and treatments were given as in the animal infection studies (four-day suppressive test) previously described above. Blood were collected through cardiac puncture of euthanised mice and further processed according to Phelan *et al.* (2002) to obtain serum. Whole blood samples were allowed to clot for 30 min at room temperature and centrifuged at 12,000 g for 30 minutes at 4°C. The levels of pro-inflammatory cytokines (TNF-α and IFN-γ) and anti-inflammatory cytokines (IL-4 and IL-10) in serum were determined using enzyme-linked immunosorbent assay (ELISA) kits (Genomax Techonologies, Singapore).

Statistical analysis

Statistical signicance of data between groups were evaluated using the Student's t-test and log rank test (for Kaplan-Meier survival analysis). Western blot analysis data obtained

were expressed as mean ± SD. Cytokine analysis data were expressed as mean ± SEM based on samples obtained from six mice per group. P value of < 0.05 between groups was considered statistically significant.

RESULTS

Insulin administration inhibited development of parasite in *P. berghei* NK65-infected mice

Insulin administration into *P. berghei* NK65 (PbN)-infected mice for four consecutive days resulted in suppression of parasite development in erythrocytes (Table 1). As shown the chemo-suppression increased from 39.55 ± 3.57% (at a dose of 0.1 U/kg bw insulin) to 67.25 ± 3.14% (at a dose of 0.3 U/kg bw insulin) but decreased when higher doses of 0.5, 0.7 and 1.0 U/kg bw insulin were employed (41.93 ± 2.94% at 1.0 U/kg bw insulin). Treatment with lithium chloride, a known GSK3 inhibitor at 100 mg/kg bw resulted in 72.71 ± 2.63% suppression whilst treatment with the anti-malarial reference drug, chloroquine (10 mg/kg bw) gave 96.70 ± 0.52% suppression. Table 1 also shows the effect of insulin treatment on median survival time of infected animals. Median survival time increased from 16.5 days (at a dose of 0.1 U/kg bw insulin) to 20.5 days (at a dose of 0.3 U/kg bw insulin) but decreased when higher doses of 0.5, 0.7 and 1.0 U/kg bw insulin were employed (16.5 days at 1.0 U/kg bw insulin). The median survival time of mice treated with lithium chloride (100 mg/kg bw) and chloroquine (10 mg/kg bw) was 21 days and >30 days respectively. Based on the results obtained for chemo-suppression (exceeding 60%) and optimum median survival time, we then used only organ samples from animals treated with 0.3 and 0.5 U/kg bw insulin for subsequent western analysis to evaluate phosphorylation states of Akt, GSK3β and NF-κB.

Insulin administration increased levels of phosphorylated Akt (Ser473) and GSK3β (SER9) in brain and liver of *P. berghei* NK65-infected mice

Insulin administration at 0.3 U/kg bw resulted in significant 1.5 fold increase and 0.6 times ($p>0.05$) of control in the relative densities of pAkt (Ser473) (Figure 1a) and pGSK3β (Ser9) (Figure 1b) respectively in brain of infected mice. At the dose of 0.5 U/kg bw, insulin administration resulted in significant 1.5 fold and 2.2 fold increase in the relative densities of pAkt (Ser473) (Figure 1(a)) and pGSK3β (Ser9) (Figure 1(b)) respectively in brain of infected mice as compared to the non-treated infected animals. In addition, administration of LiCl (100 mg/kg bw) resulted in significant 1.6 fold and 1.1 fold increase of phosphorylated brain pAkt (Ser473) (Figure 1(a)) and pGSK3β (Ser9) (Figure 1(b)) respectively.

Table 1. Chemo-suppressive effect of insulin on *P. berghei* NK65-infected mice

Compound/Drugs	Dose (/kg bw)	Average parasitaemia suppression on day 4 (%)	Median survival time (days)
Insulin	0.1 U	39.55 ± 2.05*	16.5
	0.3 U	67.25 ± 3.83**	20.5*
	0.5 U	62.12 ± 3.22**	19*
	0.7 U	51.30 ± 4.17*	18*
	1.0 U	41.93 ± 2.54*	16.5
Chloroquine (anti-malarial reference drug)	10 mg	96.70 ± 0.52*	>30*
LiCl (GSK3 inhibitor)	100 mg	72.70 ± 1.93*	21*
0.85% Saline (control)	0.2 mL	–	15.5

Parasitaemia suppression was calculated on day 4 post-infection and survivability of mice recorded throughout the experimental period (30 days). Data represent mean ± SEM for parasitaemia suppression and median survival time (n = 6). Median survival times were determined from Kaplan-Meier survival curves (not shown). * Significantly different from negative control at P<0.05.

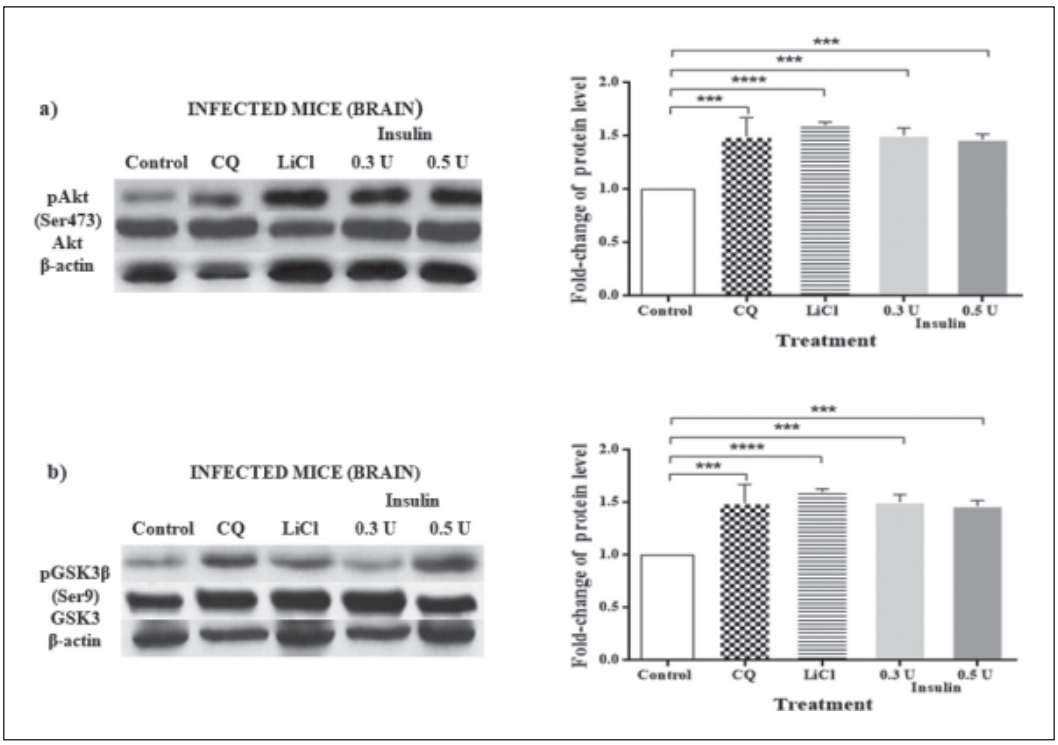

Figure 1. Western blot and relative densitometric quantitation of (a) pAkt (Ser473) and (b) pGSK3β (Ser9) in brain of *P. berghei* NK65-infected mice. Fold-change of protein level in mice administered with chloroquine (CQ; 10 mg/kg bw), lithium chloride (LiCl; 100 mg/kg bw), or insulin (0.3 and 0.5 U/kg bw). Total Akt and pAkt (Ser473) or GSK3β and pGSK3β (Ser9) from brain were measured and levels of phosphorylated GSK3β were normalised to total levels of Akt or GSK3β. Densitometric measurements are illustrated as mean ± SD of treated group compared to non-treated control. β-actin was used as internal loading control. Representative western blot images shown. Significant differences between tested and control groups were evaluated at $P<0.05$ (*).

In PbN-infected mice, insulin administration at 0.3 U/kg bw resulted in significant 2.8 fold and 4.8 fold increase in the relative densities of liver pAkt (Ser473) (Figure 2(a)) and pGSK3 (Ser9) (Figure 2(b)) respectively as compared to the non-treated infected controls. At the dose of 0.5 U/kg bw, insulin administration resulted in significant 2.2 fold and 16.1 fold increase in the relative densities of pAkt (Ser473) (Figure 2(a)) and pGSK3 (Ser9) (Figure 2(b)) respectively in liver of infected mice as compared to the non-treated infected animals. In addition, administration of LiCl (100 mg/kg bw) resulted in significant ($p<0.05$) 1.6 fold and 9.0 fold increase of phosphorylated liver pAkt (Ser473) (Figure 2(a)) and pGSK3β (Ser9) (Figure 2(b)) respectively. It is noteworthy that treatment with chloroquine also resulted in significant increase in pAkt (Ser473) and pGSK3β (Ser9) in brain and liver of infected animals (Figures 1 and 2). The significance of this observation on the effect of chloroquine will be discussed later. Based on the results from western analysis that showed higher and significant fold change in pGSK3β (Ser9), we next analysed cytokine levels in serum samples obtained from animals treated with 0.5 U/kg bw insulin only.

Insulin administration decreased levels of phosphorylated pNF-κB p65 (Ser536) in brain and liver of *P. berghei* NK65-infected mice

As compared to the non-treated infected controls, insulin administration at 0.3 U/kg

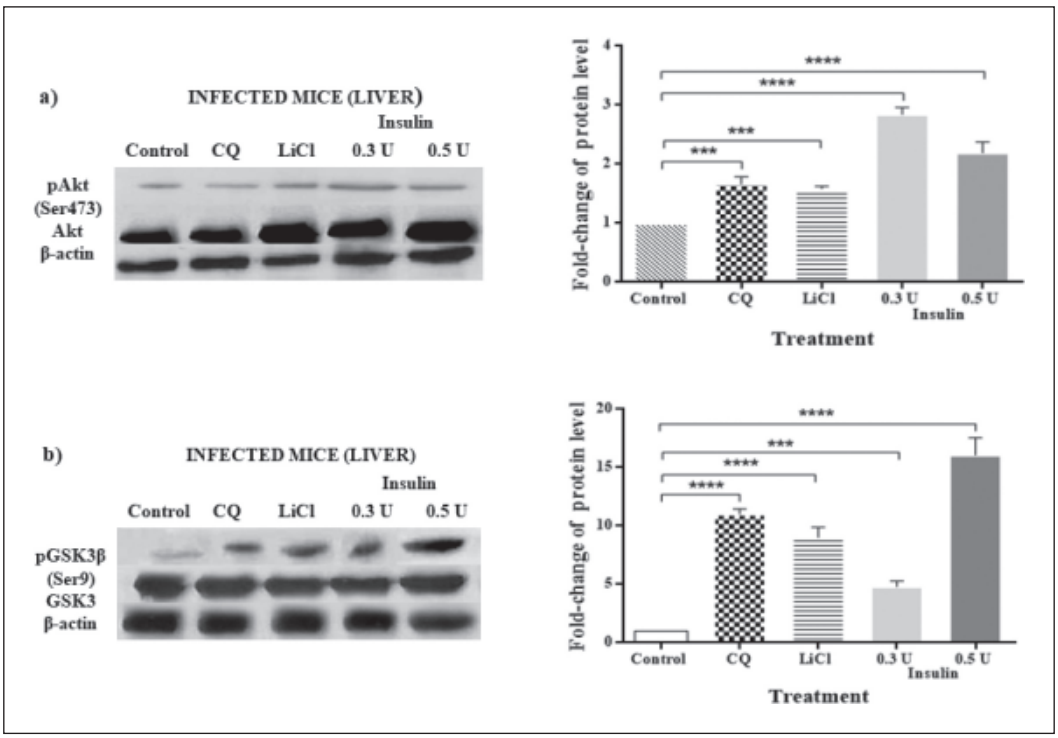

Figure 2. Western blot and relative densitometric quantitation of (a) pAkt (Ser473) and (b) pGSK3β (Ser9) in liver of *P. berghei* NK65-infected mice. Fold-change of protein level in mice administered with chloroquine (CQ; 10 mg/kg bw), lithium chloride (LiCl; 100 mg/kg bw), or insulin (0.3 and 0.5 U/kg bw). Total Akt and pAkt (Ser473) or GSK3β and pGSK3β (Ser9) from liver were measured and levels of phosphorylated GSK3β were normalised to total levels of Akt or GSK3β. Densitometric measurements are illustrated as mean ± SD of treated group compared to non-treated control. β-actin was used as internal loading control. Representative western blot images shown. Significant differences between tested and control groups were evaluated at $P<0.05$ (*).

bw resulted in decreased ($p<0.05$) relative densities of pNF-κB p65 (Ser536) in brain (Figure 3(a)) and liver (Figure 3(b)) of malaria-infected animals to 0.8 and 0.6 times the level in control respectively. At a higher dose of 0.5 U/kg bw, insulin administration resulted in decreased ($p<0.05$) relative densities of pNF-κB p65 (Ser536) in brain (Figure 3(a)) and liver (Figure 3(b)) of malaria-infected animals to 0.2 and 0.1 times the level in control respectively. Administration of LiCl (100 mg/kg bw) resulted in decreased relative densities of pNF-κB p65 (Ser536) in brain (Figure 3(a)) and liver (Figure 3(b)) of malaria-infected animals to 0.9 times ($p>0.05$) and 0.6 times ($p<0.05$) the level in control respectively. Next we analysed cytokine levels in sera obtained from insulin-treated infected mice to evaluate the host inflammatory cytokine response of the treatment.

Insulin administration modulated pro- and anti-inflammatory cytokines in *P. berghei* NK65-infected mice

As compared to the non-treated infected controls, insulin administration at 0.5 U/kg bw resulted in significant decrease in TNF-α (Figure 4(a)) and IFN-γ (Figure 4(b)) levels in sera to 3.3 and 4.9 times the level of that observed in control respectively. On the other hand, administration with 0.5 U/kg bw insulin increased the levels of anti-inflammatory cytokines IL-4 (Figure 4(c)) and IL-10 (Figure 4(d)) in sera of infected mice by 4.9 and

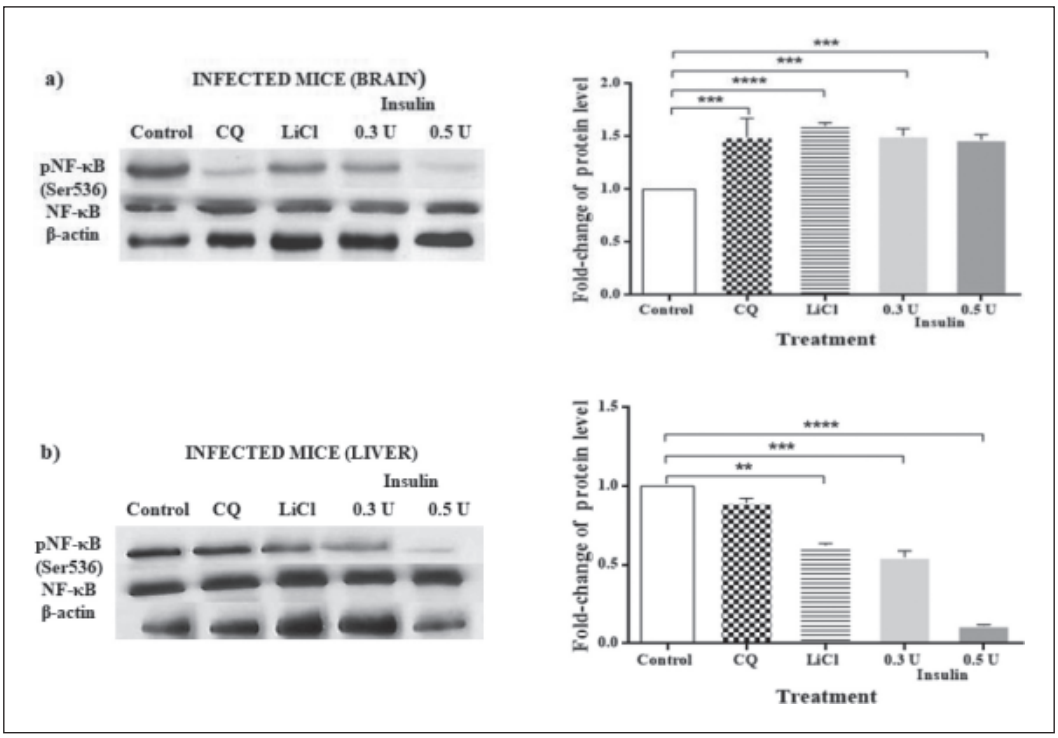

Figure 3. Western blot and relative densitometric quantitation of pNF-κB p65 (Ser536) in a) brain and b) liver of *P. berghei* NK65-infected mice. Fold-change of protein level in mice administered with chloroquine (CQ; 10 mg/kg bw), lithium chloride (LiCl; 100 mg/kg bw), or insulin (0.3 and 0.5 U/kg bw). Total NF-κB and pNF-κB p65 (Ser536) from brain and liver were measured and levels of phosphorylated NF-κB p65 were normalised to total levels of NF-κB. Densitometric measurements are illustrated as mean ± SD of treated group compared to non-treated control. β-actin was used as internal loading control. Representative western blot images shown. Significant differences between tested and control groups were evaluated at $P<0.05$ (*).

2.1 fold compared to non-treated mice respectively. In addition, administration of LiCl (100 mg/kg bw) resulted in significant decrease in TNF-α and IFN-γ levels in sera to 4.2 and 5.4 times the level of that observed in control respectively (Figures 4(a) and 4(b)). For the anti-inflammatory cytokines IL-4 and IL-10, administration with LiCl (100 mg/kg bw) increased the levels by 7.3 and 1.9 fold compared to non-treated mice respectively (Figures 4(c) and 4(d)). It is noteworthy that treatment with chloroquine also modulated the levels of pro- and anti-inflammatory cytokines in malaria-infected animals (Figures 4(a), 4(b), 4(c) and 4(d)). The significance of this effect by chloroquine will be addressed in the discussion.

DISCUSSION

During malarial infection, dysregulation of the inflammatory response signalling can cause imbalance in cytokine production leading to overwhelming levels of pro-inflammatory cytokines such as TNF-α and IFN-γ (Dunst *et al.*, 2017). Besides its glucose-lowering activity, insulin is now known to have anti-inflammatory effects (Dandona *et al.*, 2007; Sun *et al.*, 2014) which among others culminate in cytokine modulation (Aljiffry *et al.*, 2016). Additionally GSK3, an important downstream component during insulin signaling is phosphorylated and consequently inhibited (Kaidanovich-Beilin & Woodgett, 2011). Based on studies in our

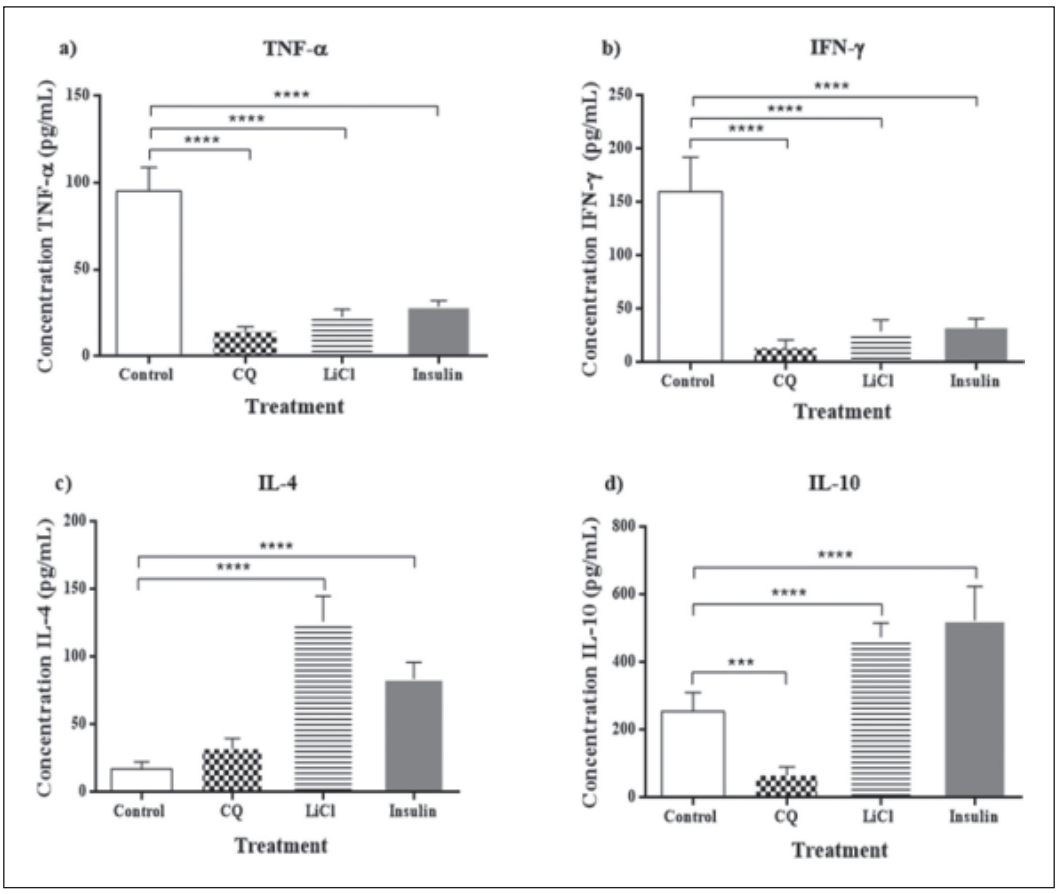

Figure 4. The levels of pro-inflammatory cytokines a) TNF-α and b) IFN-γ and anti-inflammatory cytokines c) IL-4 and d) IL-10 levels in serum of mice infected-PbN administered with insulin or LiCl treatment. Data are expressed as mean ± SEM; n = 6 ICR mice per group. Significant difference as compared with control infected group was evaluated at $P < 0.05$ (*), $P < 0.001$ (***), $P < 0.0001$ (****).

laboratory and elsewhere that LiCl (Zakaria *et al.*, 2010; Ali *et al.*, 2017) and other GSK3 inhibitors (Sudi *et al.*, 2018; Hassan *et al.*, 2019) were able to exhibit anti-malarial activity and modulate the inflammatory cytokine response in murine models of malarial infection, we extend our investigation on GSK3 inhibitors to study the effect of insulin administration on suppression of parasitaemia and cytokine production in mice infected with *P. berghei*. To our knowledge, there is currently no evidence about the anti-plasmodial and anti-malarial activities of insulin. The findings from the present study represent the first report that exogenous insulin exhibits anti-malarial activity in a murine model of malarial infection and corroborate previous reports that compounds with GSK3-inhibitory effect (Ali *et al.*, 2017; Hassan *et al.*, 2019) can elicit active (>60%) chemo-suppressive activity and improve animal survivability. On its own, insulin does not exhibit anti-plasmodial activity when tested *in vitro* (data not shown). Therefore, the observed reduction of parasitaemia in mice given insulin treatment is likely due to the parasite clearance by immune cells as a result of the immunomodulatory effect of insulin.

More importantly, the present study demonstrate that insulin administration resulted in modulation of inflammatory cytokines (decreased pro-inflammatory cytokine levels whilst increasing anti-inflammatory cytokines). Administration of chloroquine also resulted in decreased

pro-inflammatory cytokine levels and increased anti-inflammatory cytokines. In the present study, chloroquine was shown to exhibit both anti-malarial as well as cytokine-modulating activities. Malarial infection generally causes excessive inflammatory response with a surge in pro-inflammatory cytokines (cytokine storm) contributing to pathophysiology of the disease. Thus chloroquine possibly reduced inflammation through its cytokine-modulating effects in the host. These observations suggest that the anti-malarial activity of insulin may be attributed to its action in boosting the innate immune system (Pakpour *et al.*, 2012). We have also previously observed that chloroquine can inhibit liver GSK3β and modulate inflammatory cytokine levels in serum of *Burkholderia pseudomallei*-infected mice (Ganesan *et al.*, 2018).

We also postulate that improved survivability of infected animals with insulin treatment is likely due to the modulation of inflammatory response by insulin mediated via inhibition of GSK3β since western analysis revealed that insulin administration at 0.5 U/kg bw resulted in significant phosphorylation of GSK3β (Ser9) in brain and liver.

Furthermore, based on the increased phosphorylation (and consequent activation) of Akt, the inhibition of GSK3 is possibly a consequence of GSK3 phosphorylation by its upstream kinase, Akt (Beurel *et al.*, 2015) in the insulin signaling pathway. GSK3, a downstream target of PI3K/Akt signaling is a point of convergence for the host cytokine inflammatory response. Here we have shown that pNF-κB p65 an inflammatory transcription factor, the regulation of which is pivotal for the modulation of inflammatory cytokines, is decreased due to insulin treatment. It is known that the inhibition of activation of NF-κB can occur upon GSK3 inhibition (Medunjanin *et al.*, 2016).

Yan *et al.* (2016) proposed that the anti-inflammatory effects of insulin involve crosstalk between TLR4 and PI3K/Akt pathway. We propose that the cytokine-modulating effects of insulin observed here in this study may be explained by a similar mechanism via activation of the PI3K/Akt pathway. Based on the inhibitory action of insulin on GSK3, we conclude that GSK3 is a molecular target of anti-inflammatory (cytokine-modulating) action of this hormone.

As mentioned earlier, overwhelming cytokine production is a key factor contributing to pathogenesis and severity of malaria (Dieye *et al.*, 2016). It is known that dysregulation of GSK3β will result in overproduction of pro-inflammatory cytokines consequently leading to overwhelming inflammatory response in the host (Cortes-Vieyra *et al.*, 2012). Host-directed therapies to modulate the cytokine imbalance in malaria need to be explored. As such, adjunctive therapy based on modulation of host inflammatory response to infection is a rational strategy to reduce malaria-associated mortality and enhance the clinical efficacy of current anti-malarial therapy. Findings from this study demonstrated that the cytokine-modulating effects of insulin at least in part involve inhibition of GSK3β and consequent inhibition of the activation of NF-κB p65 suggesting insulin as a potential adjunctive therapeutic involving GSK3β as a molecular target for malaria. Adjunctive therapy should not only focus on reduction of mortality and improvement of clinical outcomes but also aim to prevent long-term cognitive impairment. Dai *et al.* (2012), showed that LiCl, a GSK3 inhibitor can restore neurocognitive functions in mice infected with cerebral malaria. A number of adjunctive therapies including erythropoietin have demonstrated encouraging results in experimental models of cerebral malaria and severe malaria (John *et al.*, 2010). In addition, erythropoietin, has been reported to cause GSK3 inhibition and improve cognitive deficits in patients with mood disorders (Inkster *et al.*, 2018). As such, insulin a physiological agonist that can cause inhibition of GSK3 is a plausible therapeutic for anti-malarial and to address cognitive impairments in cerebral malaria.

CONCLUSION

Targeting signaling pathways critical to host inflammatory response is a useful strategy for the development of effective adjunctive therapy for malaria. We have demonstrated that the cytokine-modulating effects of insulin at least in part involve inhibition of GSK3β mediated through the activation of the PI3K/Akt pathway. In conclusion, GSK3β is a plausible molecular target of insulin as a potential adjunctive therapeutic for malaria.

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Conflict of Interests

The authors declare that they have no conflict of interests.

REFERENCES

Ali, A.H., Sudi, S., Basir, R., Embi, N. & Sidek, H.M. (2017). The antimalarial effect of curcumin is mediated by the inhibition of glycogen synthase kinase-3β. *Journal of Medicinal Food* **20**(2): 152-161.

Aljiffry, M., Hassanain, M., Schricker, T., Shaheen, M., Nouh, T., Lattermann, R., Salman, A., Wykes, L. & Metrakos, P. (2016). Effect of insulin therapy using hyper-insulinemic normoglycemic clamp on inflammatory response in brain dead organ donors. *Experimental and Clinical Endocrinology & Diabetes* **124**(5): 318-323.

Autino, B., Corbett, Y., Castelli, F. & Taramelli, D. (2012). Pathogenesis of malaria in tissues and blood. *Mediterranean Journal of Hematology and Infectious Diseases* **4**(1): e2012061.

Bradford, M.M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye Binding. *Analytical Biochemistry* **72**(1-2): 248-254.

Beurel, E., Grieco, S.F. & Jope, R.S. (2015). Glycogen synthase kinase-3 (GSK3): regulation, actions, and diseases. *Pharmacology & Therapeutics* **148**: 114-131.

Bienvenu, A.L. & Picot, S. (2013). Cerebral malaria: protection by erythropoietin. *Tissue-Protective Cytokines* **982**(19): 315-324.

Cortés-Vieyra, R., Bravo-Patiño, A., Valdez-Alarcón, J.J., Juárez, M.C., Finlay, B.B. & Baizabal-Aguirre, V.M. (2012). Role of glycogen synthase kinase-3 beta in the inflammatory response caused by bacterial pathogens. *Journal of Inflammation* **9**(1): 1-9.

Dai, M., Freeman, B., Shikani, H.J., Bruno, F.P., Collado, J.E., Macias, R., Reznik, S.E., Davies, P., Spray, D.C. & Tanowitz, H.B. (2012). Altered Regulation of Akt Signaling with Murine Cerebral Malaria, Effects on Long-Term Neuro-Cognitive Function, Restoration with Lithium Treatment. *PloS One* **7**(10): e44117.

Dandona, P., Chaudhuri, A., Mohanty, P. & Ghanim, H. (2007). Anti-inflammatory effects of insulin. *Current Opinion in Clinical Nutrition & Metabolic Care* **10**(4): 511-517.

Dieye, Y., Mbengue, B., Dagamajalu, S., Fall, M.M., Loke, M.F., Nguer, C.M., Thiam, A., Vadivelu, J. & Dieye, A. (2016). Cytokine response during non-cerebral and cerebral malaria: evidence of a failure to control inflammation as a cause of death in african adults. *Peer Journal Preprints* **4**: 1-20.

Dunst, J., Kamena, F. & Matuschewski, K. (2017). Cytokines and chemokines in cerebral malaria pathogenesis. *Frontiers in Cellular and Infection Microbiology* **7**: 324-339.

Embi, N., Rylatt, D.B. & Cohen, P. (1980). Glycogen synthase kinase-3 from rabbit skeletal muscle: separation from cyclic-AMP-dependent protein kinase and phosphorylase kinase. *European Journal of Biochemistry* **107**(2): 519-527.

Frevert, U., Nacer, A., Cabrera, M., Movila, A. & Leberl, M. (2014). Imaging *Plasmodium* immunobiology in the liver, brain, and lung. *Parasitology International* **63**(1): 171-186.

Ganesan, N., Embi, N. & Hasidah, M. (2018). The anti-malarial chloroquine modulated cytokine levels and increased animal survivability via akt-mediated inhibition of GSKβ in *Burkholderia Pseudomallei* infected mice. *Tropical Biomedicine* **35**(3): 709-723.

Gostner, J.M., Schröcksnadel, S., Becker, K., Jenny, M., Schennach, H., Überall, F. & Fuchs, D. (2012). Antimalarial drug chloroquine counteracts activation of indoleamine (2, 3)-dioxygenase activity in human PBMC. *FEBS Open Biology* **2**: 241-245.

Gowda, D.C. & Wu, X. (2018). Parasite Recognition and Signaling Mechanisms in Innate Immune Responses to Malaria. *Frontiers in Immunology* **9**: 3006-3023.

Hassan, W., Basir, R., Ali, A., Embi, N. & Sidek, H. (2019). Anti-malarial and cytokine-modulating effects of andrographolide in a murine model of malarial infection. *Tropical Biomedicine* **36**(3): 776-791.

Inkster, B., Zai, G., Lewis, G. & Miskowiak, K.W. (2018). GSK3β: A plausible mechanism of cognitive and hippocampal changes induced by erythropoietin treatment in mood disorders? *Translational Psychiatry* **8**(1): 1-13.

John, C.C., Kutamba, E., Mugarura, K. & Opoka, R.O. (2010). Adjunctive therapy for cerebral malaria and other severe forms of *Plasmodium falciparum* Malaria. *Expert Review of Anti-infective Therapy* **8**(9): 997-1008.

Kaidanovich-Beilin, O. & Woodgett, J.R. (2011). GSK-3: Functional insights from cell biology and animal models. *Frontiers in Molecular Neuroscience* **4**: 40-65.

Lee, C. (2007). Protein extraction from mammalian tissues. *Methods Molecular Biology* **362**: 385-389.

Liu, T., Zhang, L., Joo, D. & Sun, S.-C. (2017). NF-κB signaling in inflammation. *Signal Transduction and Targeted Therapy* **2**(1): 17023-17031.

Liu, X. & Yao, Z. (2016). Chronic over-nutrition and dysregulation of GSK3 in Diseases. *Nutrition & Metabolism* **13**(1): 49-66.

Mackintosh, C.L., Beeson, J.G. & Marsh, K. (2004). Clinical features and pathogenesis of severe malaria. *Trends in Parasitology* **20**(12): 597-603.

Mcardle, M.A., Finucane, O.M., Connaughton, R.M., Mcmorrow, A.M. & Roche, H.M. (2013). Mechanisms of obesity-induced inflammation and insulin resistance: insights into the emerging role of nutritional strategies. *Frontiers in Endocrinology* **4**: 52-75.

Medunjanin, S., Schleithoff, L., Fiegehenn, C., Weinert, S., Zuschratter, W. & Braun-dullaeus, R.C. (2016). GSK-3β controls NF-κB activity via IKK γ / NEMO. *Science Reports* **6**: 1-11.

Pakpour, N., Corby-Harris, V., Green, G.P., Smithers, H.M., Cheung, K.W., Riehle, M. A. & Luckhart, S. (2012). Ingested human insulin inhibits the mosquito NF-κB-dependent immune response to *Plasmodium falciparum*. *Infection and Immunity* **80**(6): 2141-2149.

Peters, W., Portus, J. & Robinson, B. (1975). The chemotherapy of rodent malaria, xxii: the value of drug-resistant strains of *P. berghei* in screening for blood schizontocidal Activity. *Annals of Tropical Medicine & Parasitology* **69**(2): 155-171.

Phelan, H., Stahls, P., Hunt, J., Bagby, G.J. & Molina, P.E. (2002). Impact of Alcohol Intoxication on Hemodynamic, Metabolic, and Cytokine Responses to Hemorrhagic Shock. *Journal of Trauma and Acute Care Surgery* **52**(4): 675-682.

Rasoanaivo, P., Ramanitrahasimbola, D., Rafatro, H., Rakotondramanana, D., Robijaona, B., Rakotozafy, A., Ratsimamanga Urverg, S., Labaïed, M., Grellier, P. & Allorge, L. (2004). Screening extracts of madagascan plants in search of antiplasmodial compounds. *Phytotherapy Research: An International Journal Devoted to Pharmacological and Toxicological Evaluation of Natural Product Derivatives* **18**(9): 742-747.

Singh, B., Varikuti, S., Halsey, G., Volpedo, G., Hamza, O.M. & Satoskar, A.R. (2019). Host-directed therapies for parasitic diseases. *Future Medicinal Chemistry* **11**(16): 1999-2018.

Sudi, S., Hassan, W.R.M., Ali, A.H., Basir, R., Embi, N. & Sidek, H.M. (2018). A derivative of cinnamic acid, methyl-4-hydroxycinnamate modulates inflammatory cytokine levels in malaria-infected mice through inhibition of GSK3β. *Malaysian Applied Biology* **47**(3): 153-157.

Sun, Q., Li, J. & Gao, F. (2014). New insights into insulin: the anti-inflammatory effect and its clinical relevance. *World Journal of Diabetes* **5**(2): 89-96.

Towbin, H., Staehelin, T. & Gordon, J. (1979). Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: Procedure and some applications. *Proceedings of The National Academy of Sciences* **76**(9): 4350-4354.

Wang, H., Kumar, A., Lamont, R.J. & Scott, D.A. (2014). GSK3β and the control of infectious bacterial diseases. *Trends in Microbiology* **22**(4): 208-217.

Wassmer, S.C. & Grau, G.E.R. (2017). Severe Malaria: What's new on the pathogenesis front? *International Journal for Parasitology* **47**(2-3): 145-152.

Yan, H., Ma, Y., Li, Y., Zheng, X., Lv, P., Zhang, Y. & Li, J. (2016). Insulin inhibits inflammation and promotes atherosclerotic plaque stability via PI3K-Akt pathway activation. *Immunology Letters* **170**: 7-14.

Yang, L., Wang, H., Liu, L. & Xie, A. (2018). The role of insulin/IGF-1/PI3K/Akt/GSK3β signaling in Parkinson's disease dementia. *Frontiers in Neuroscience* **12**: 73-80.

Zakaria, N.A., Embi, N. & Sidek, H.M. (2010). Suppression of *Plasmodium berghei* parasitaemia by LiCl in an animal infection model. *Tropical Biomedicine* **27**(3): 624-631.