INVESTIGATION OF THE ASSOCIATION BETWEEN PROSTAGLANDIN D_2 (PGD_2) LEVELS AND MALARIA PATHOGENICITY AND SEVERITY

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ABSTRACT: The aim of the present study was to preliminarily investigate the possible role of PGD_2 in malaria pathogenicity/severity. Plasma and urine samples were collected from a total of 11 healthy Thai subjects and 47 Burmese patients with malaria (*Plasmodium falciparum* and *P. vivax* with different levels of parasitemia and mixed infection). There appeared to be the relationship between PGD_2 levels in both plasma and urine samples and malaria disease pathogenicity/severity. However, due to limited sample size and technical problems relating to the assay method for determination of PGD_2 (EIA), significant correlation was only clearly seen with urinary PGD_2 levels in both *P. falciparum* and *P. vivax*. *In vitro* study however, could not confirm the production of PGD_2 by *P. falciparum* (*Pf*PGD_2). Further study with larger sample size with improved assay performance is required to definitely conclude on the association between PGD_2 levels malaria disease pathogenicity/severity.

Keywords: prostaglandin D_2, malaria disease, pathogenicity

INTRODUCTION: Malaria has always been a major killer of populations throughout the tropics for thousands of years. Despite important advances in our understanding of the disease, it continues to be one of the greatest causes of serious illness and death in the world. Over 75% of 2-3 million deaths occurring in African children, and about 500 million new cases reported annually, is a challenge to drug therapy and discovery[1]. Of the four species of human malarial parasite, *Plasmodium falciparum* is remarkable for its high case fatality rate particularly in young children and alarming development of resistance to antimalarial drugs. Resistance to antimalarials is spreading throughout the world and is impeding efforts to control malaria[2]. Resistance of *P. falciparum* to the most affordable drugs, such as chloroquine and sulfadoxine/pyrimethamine and mefloquine, has become widespread throughout Africa and Asia, in particular Southeast Asia[2].

Several lines of evidence suggest the involvement of heme oxygenase-1 (HO-1) enzyme in the pathogenesis of cerebral malaria[3-5]. HO-1 is a rate-limiting enzymes in heme catabolism forming biliverdin, carbonmonoxide and iron from heme[6,7]. Biliverdin is rapidly reduced to bilirubin. It has been shown that HO-1 may suppress cerebral malaria and enzyme activity is induced by the prostanoid prostaglandin J_2 (PGJ_2), which is synthesized by the non-enzymatic dehydration of prostaglandin D_2 (PGD_2). It is therefore hypothesized that HO-1, PGJ_2, and PGD_2 may be involved in malaria pathogenicity, susceptibility and disease severity. In human, PGD_2 plays importance role in various pathological conditions, including brain, eye, and hematopoietic cells, *etc.* Malaria infection is also characterized by symptoms mediated by prostaglandins which are thought to derive from host cells[8]. Pathogenesis in malaria patients could enhance PGD_2 level, especially in cerebral malaria patients. However, PGD_2 is not only produced from human to protect their cells or organs from oxidative stress and induce expressions of cytoprotective mediators, but may also be secreted from parasites to protect malarial cell from oxidative stress. Parasite-produced prostaglandins (*Pf*PGD_2, *Pf*PGE_2 and *Pf*$PGF_{2\alpha}$) were found to accumulate in the culture medium of *P. falciparum*[8]. Intra-erythrocytic *P. falciparum* parasites might release *Pf*PGD_2[9] which may influence heme catabolism in the host cells near the sequestration sites, especially in brain, retina and microvasculature. Moreover, sequestration of parasitized erythrocytes might generate hemodynamic stress, which in

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turn, increases the production of *Pf*PGD_2 through induction of L-PGDS expression in vascular endothelial cells as reported in the case of fluid shear stress[10]. The discovery of prostaglandin production in *P. falciparum* (PfPGD_2) may indicate the possibility of a substantial contribution of the parasite to malaria symptoms/pathogenicity/severity[8]. The mechanism of PGD_2 synthesis by malarial cyclooxygenase (COX) was shown to be clearly distinguishable from that by mammalian COX[9]. *Pf*PGD_2 would therefore be a selective potential target for antimalarials without affecting (or with minimum) mammalian PGD_2 biosynthesis. The aim of the present study was to preliminarily investigate the possible role of PGD_2 in malaria pathogenicity/severity. This was done by investigating plasma and urinary PGD_2 levels in patients with malaria and healthy subjects, as well as the production of *pF*PGD_2 by *P. falciaprum in vitro* culture.

MATERIALS AND METHODS:

Investigation of prostaglandins D_2 (PGD_2) levels in plasma and urine of patients with malaria and healthy subjects

The study was conducted at Mae-Sot General Hospital, Mae-Sot, Tak Province. Approval of the study protocol was obtained from the Ethics Committee of the Ministry of Public Health of Thailand. Plasma and urine samples were collected from a total of 11 healthy Thai subjects and 47 Burmese patients with malaria. Plasma samples were collected from healthy subjects (control: n=6), patients with acute uncomplicated *P. falciparum* with low (<0.1% parasitemia: n=6), medium (0.1-1.0% parasitemia: n=6), and high (>1.0% parasitemia: n=2) parasitemia. In addition, 3 and 6 samples respectively, were collected from patients with *P. vivax* (0.1-1.0% parasitemia) and mixed infection with *P. vivax* and *P. falciparum* (0.1–1.0% parasitemia). Urine samples (n = 5 each) were collected from healthy subjects (control), patients with acute uncomplicated *P. falciparum* with low, medium, and high parasitemia, as well as *P. vivax* patients with low (<0.1% parasitemia) and medium (0.1-1.0% parasitemia) parasitemia.

Plasma (3 ml whole blood each with EDTA anti-coagulant) and urine (2 ml each without preservative) samples from patients with malaria were collected prior to antimalarial treatment during the acute phase of malaria infection. Urine samples were immediately stored at -80°C until analysis. For whole blood samples, indomethacin (10 μM final concentration) was added immediately after collection to prevent *ex vivo* formation of eicosanoids, which have the potential to interfere with the PGD_2 assay. To ensure the stability of PGD_2, plasma sample was immediately separated after collection. Briefly, plasma samples were diluted with cold acetone at the ratio of 1:1 (v:v) and incubated on ice for 5 min. Precipitated protein was removed through centrifugation at 3,000 x g for 10 min and stored at -80°C until analysis. Before analysis, samples were evaporated to dryness under a stream of nitrogen gas.

Investigation of prostaglandins D_2 production in *Plasmodium falciparum* (*Pf*PGD_2) culture *in vitro*

K1 (chloroquine-resistant) and 3D7 (chloroquine-sensitive) *P. falciparum* clones were maintained in culture *in vitro* according to the method of Trager and Jensen with modification[11]. Suspension of human type O^+ erythrocytes (5%) was prepared in culture medium consisting of powdered RMPI 1640 (GIBCO Laboratories, USA) diluted in sterile water with 25 mM HEPES (*N*-2-hydroxyethylpiperazine-*N*-2 ethanesulfonic acid, Sigma, USA), 32 mM $NaHCO_3$ (GIBCO), and 10% human type B+ fresh frozen plasma (in acid-citrate-dextrose anticoagulant). Stock cultures were maintained in 5 ml of the 5% erythrocyte suspension in 25-cm^2 tissue culture flasks (Corning Co. Ltd., USA). The flasks were flushed with a gas mixture consisting of 5% O_2, 5% CO_2, and 90% N_2 and incubated at 37°C. Subculture was performed every 2-3 days in order to maintain parasitemia at lower than 2%.

For each experiment, stock *P. falciparum* culture was further diluted in culture medium containing non-infected type O^+ human erythrocytes to yield a final 1.5% hematocrit and 0.25-0.5% parasitemia. The parasites were synchronized using 5% *D*-sorbital to enforce them to ring stage. The culture of both of K1 and 3D7 clones were adjusted to 1 and 6% parasitemia. Culture medium was collected at 0 (control) and at 24 and 48 hours incubation

(a half cycle and one cycle of erythrocytic phase of *P. falciparum*, respectively) and stored at -80°C until analysis.

Determination of PGD_2 levels

PGD_2 levels in plasma and urine samples collected from healthy subjects and patients with malaria including *P. falciparum* culture medium were determined using Prostaglandin D_2-MOX Express™ EIA kit (Cayman Chemical, USA). Briefly, dried samples were resuspended in 100 µl of EIA buffer. Fifty µl plasma (1:10 dilution), urine (1:5 dilution) and culture medium were added in 96-well plates coated with goat anti-rabbit IgG antibodies. The tracer (50 µl) and the PGD_2 specific antibody (50 µl) were added to each well and the plates were incubated overnight at 4 °C. Following incubation, wells were washed five times with 10 mM phosphate buffer (pH 7.4) containing Tween 20 (0.05%) pH 7.4 and 200 µl Ellman's reagent [69 mM acetylthiocholine and 54 mM 5,50-dithio-bis (2-nitrobenzoic acid) in 10 mM phosphate buffer pH 7.4] was added to each well. Microtitre plates were then incubated in the dark at room temperature (25 °C) for 60-90 min. This allowed the bound enzyme tracer to react with Ellman's reagent to yield a yellow solution that could be measured photometrically with a microplate reader at 410 nm. A standard curve was developed using PRISM™ software version 5.0 (GraphPad, San Diego, CA, USA) and concentrations of PGD_2 in plasma, urine and culture medium samples relative to those standards were determined.

Data analysis

Comparison of PGD_2 levels in plasma and urine samples collected from all patient groups and healthy subjects were performed using Kruskall Wallis and Mann-Whitney *U* tests for data not conforming to normal distribution. Spearman correlation test was applied to the data not conforming to normal distribution to assess the relation between the two quantitative variables. Comparison of *Pf*PGD_2 levels in culture medium of the resistant K1 and sensitive 3D7 *P.falciparum* clones was performed using Mann-Whitney *U* test for data not conforming to normal distribution. Statistical significance level was set at $\alpha = 0.05$ for all tests.

RESULTS:

Investigation of association between PGD_2 levels and malaria pathogenicity/severity

The association between malaria pathogenicity /severity and plasma and urinary levels of PGD_2 were investigated in samples collected from patients with *P. falciparum* with different severity and *P. vivax* infections during the acute phase infection, in comparison with healthy subjects. Median (range) values of plasma PGD_2 concentrations in healthy subjects, patients with *P. falciparum* with low, moderate, high parasitemia, and patients with *P. vivax* and mixed infection with *P. falciparum* and *P. vivax* were 408 (141-525), 428 (245-627), 420 (271-470), 793 (629-957), 251 (235-258), and 213 (194-318) pg/ml, respectively (Table 1). Median plasma PGD_2 concentration in patients with *P. falciparum* malaria with high parasitemia was significantly higher than healthy subjects ($p = 0.0023$), and patients with low ($p = 0.035$), moderate ($p = 0.035$), *P.vivax* ($p = 0.035$) and mixed infection ($p = 0.023$). In addition, median plasma PGD_2 concentrations in patients with *P. falciparum* with moderate and low parasitemia and healthy subjects were significantly higher than those with mixed infection ($p = 0.045$, 0.040, and 0.032, respectively). There was no significant correlation ($p = 0.185$, $r^2 = 0.242$) between plasma PGD_2 levels and severity of *P. falciparum* infection (Figure 1).

Median (range) values of urinary PGD_2 concentrations in healthy subjects, patients with *P. falciparum*

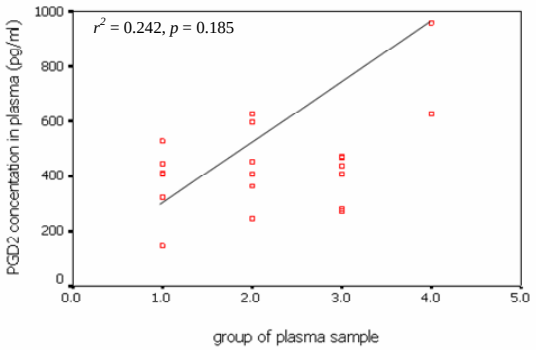


Figure 1 Scatter plot for correlation between plasma PGD_2 concentration and severity of *P. falciparum* infection classified by level of parasitemia (Group 1: control-healthy subjects; Group 2: patients with low parasitemia; Group 3: patients with moderate parasitemia; Group 4: patients with high parasitemia)

with low, moderate, high parasitemia, and patients with *P. vivax* with low and high parasitemia were 48 (17-145), 135 (59-162), 156 (93-209), 164 (141-259), 160 (73-306), and 207 (174-218) pg/ml, respectively (Table 2). Due to technical problem relating to PGD_2 analysis, one urine sample from the group of *P. falciparum* with low parasitemia was excluded from data analysis. Median value of urinary PGD_2 concentration in healthy subjects was significantly lower than patients with *P. falciparum* with low (p=0.043), moderate (p=0.023), high (p=0.005) parasitemia, and patients with *P. vivax* with low (p=0.035) and moderate (p=0.004) parasitemia. In addition, the median PGD_2 level in patients with *P. falciparum* with low and moderate parasitemia was also significantly lower than those with *P. vivax* infection with moderate parasitemia (p=0.023 and 0.025, respectively). There was a significant correlation between PGD_2 levels in urine and severity of *P. falciparum* (p<0.0001, r^2 = 0.586) and *P. vivax* (p<0.0001, r^2=0.642) infection (Figure 2).

Investigation of *Pf*PGD_2 production in *Plasmodium falciparum* culture *in vitro*

To prove that *P. falciparum* can synthesize *Pf*PGD_2, the concentration of *Pf* PGD_2 was determined in *P. falciparum* culture *in vitro*. *Pf*PGD_2 concentration of the complete RPMI media collected from K1 and 3D7 *P. falciparum* clones at 0 (control), 24 and 48 hr were measured by EIA technique. No measureable concentration of *Pf*PGD_2 was detected in the complete medium culture collected from both clones during both incubation periods.

DISCUSSION: Prostaglandin D_2 (PGD_2) is bio-synthesized in the brain by a soluble, glutathione independent lipocalin-type PGD_2 synthase (L-PGDS). It accumulates in the cerebrospinal fluid, where it induces physiologic sleep in humans[12]. PGD_2 is also synthesized in mast cells and leukocytes by a cellular, myeloid-type, glutathione -dependent PGD_2 synthase. With respect to malaria infection, the link between PGD_2 and disease pathogenicity/severity through induction of hemeoxygenase-1 (HO-1) enzyme has been proposed in the present study. It is speculated that PGD_2 derived from host cells and/or from the intra-erythrocytic *P. falciparum* parasites may be involved in malaria pathogenicity/severity through stimulating HO-1 expression. In our recent *in vitro* study in human retinal pigment epithelial (RPE) cells (ARPE-19, D407), lines of evidence (dose-dependent stimulatory effect of PGD_2 on HO-1 expression and modulatory effects of DP_2 agonists and antagonists) have been provided to suggest that PGD_2 mediates stimulation of HO-1 mRNA

Table 1 Median (range) concentrations of PGD_2 in plasma associated with malarial pathogenicity

Group	Number of sample	Median (range) plasma PGD_2 concentration[#] (pg/ml)
Healthy subjects (control)	6	408 (141 - 525)[a]
P. falciparum with		
low parasitemia	6	428 (245 - 627)[b]
moderate parasitemia	6	420 (271 - 470)[c]
high parasitemia	2	793 (629 - 957)[d]
P. vivax	3	251 (235 - 258)
Mixed infection with *P. falciparum* and *P. vivax*	6	213 (194 - 318)

[#] *Measured by enzyme immunoassay (EIA) technique*
[a] *Significantly lower than P. falciparum with high (p 0.0023) parasitemia, and significantly higher than patients with mixed infection (p 0.025) by Mann-Whitney U test*
[b] *Significantly lower than P. falciparum with high parasitemia (p 0.035), and significantly higher than patients with P. vivax (p 0.0045) and mixed infection (p 0.04) by Mann-Whitney U test*
[c] *Significantly lower than P. falciparum with high parasitemia (p 0.035), and significantly higher than patients with mixed infection (p 0.0045) by Mann-Whitney U test*
[d] *Significantly higher than patients with mixed infection (p 0.035) by Mann-Whitney U test*

Table 2 Median (range) concentrations of PGD_2 in urine associated with malarial pathogenicity

Group	Number of sample	Median (range) plasma PGD_2 concentration[#] (pg/ml)
Healthy subjects (control)	5	48 (17 - 145)[a]
P. falciparum with		
low parasitemia	4	135 (59 - 162)[b]
moderate parasitemia	5	156 (93 - 209)[c]
high parasitemia	5	164 (141 - 259)
P. vivax with		
low parasitemia	5	160 (73 - 306)
moderate parasitemia	5	207 (174 - 218)

[#] *Measured by enzyme immunoassay (EIA) technique*
[a] *Significantly lower than P. falciparum with low (p 0.043), moderate (p 0.023), high (p 0.005) parasitemia, and patients with P. vivax (p 0.035) and mixed infection (p 0.004) by Mann-Whitney U test*
[b] *Significantly lower than P. vivax with moderate parasitemia (p 0.0023) by Mann-Whitney U test*
[c] *Significantly lower than P. vivax with moderate parasitemia (p 0.025) by Mann-Whitney U test*

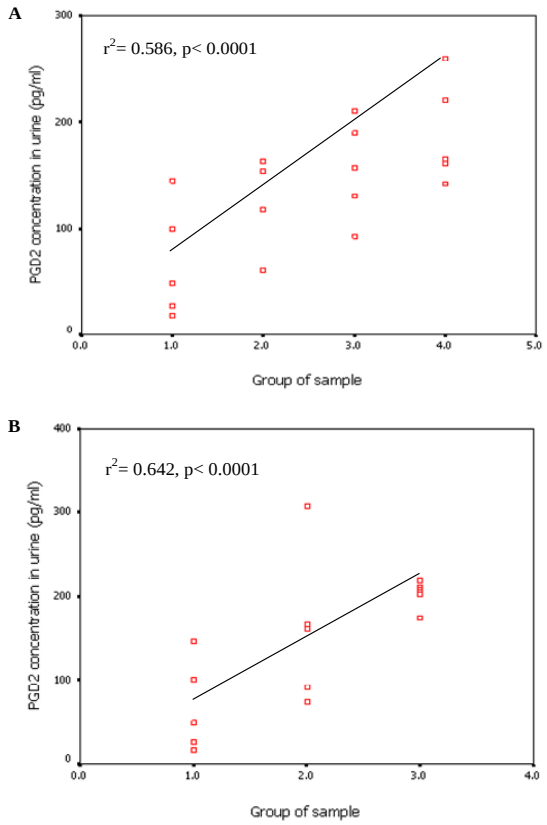


Figure 2 Scatter plot for correlation between urinary PGD_2 concentration and severity of infection classified by type of (a) *P. falciparum* malaria infection and level of parasitemia (Group 1: control-healthy subjects; Group 2: *P. falciparum* patients with low parasitemia; Group 3: *P. falciparum* patients with moderate parasitemia; Group 4: *P. falciparum* patients with high parasitemia); and (b) *P. vivax* infection and level of parasitemia (Group 1: control-healthy subjects; Group 2: *P. vivax* patients with low parasitemia; Group 3: *P. vivax* patients with moderate parasitemia, respectively).

and protein expression *via* acting through binding to DP_2 receptors linking the PGD_2-DP_2 with heme homeostasis[13]. A clinical study conducted in Kenyan children with cerebral malaria demonstrated no significant increase in blood carboxyhemoglobin levels was detected[14], which is consistent in part with the current proposal that the enhanced heme degradation may be localized only in host cells near the sequestered site such as micro-vasculature[4,5].

In the present study, the possible association between plasma and urinary PGD_2 levels and malaria pathogenecity/severity was investigated in patients with malaria with diferrent pathogenicity/ severity. PGD_2 concentrations in plasma of healthy subjects were found to be about 8-10 fold of that in urine (Table 1-2). There appeared to be a significant correlation between malaria disease severity of both *P. falciparum* and *P. vivax* infection and the level of urinary PGD_2. Patients with malaria with all pathogenicity and severity had markedly higher PGD_2 levels (about 3-4 fold) than healthy subjects. For plasma PGD_2 on the other hand, markedly and significantly higher PGD_2 levels were observed in patients with *P. falciparum* with high parasitemia, of which the median level was about 2-fold of the healthy subject and the groups with low and moderate parasitemia. Interestingly, PGD_2 concentrations in patients with *P. vivax* and mixed infection with *P. falciparum* and *P. vivax* were only about 50 and 30% of healthy subjects and *P. falciparum* patients with high parasitemia. It is noted however that the parasite isolates, plasma and urine samples were not obtained from the same patients, and this could increase the variability of the results. As plasma PGD_2 levels in healthy subjects represents baseline PGD_2 in human plasma, and since plasma PGD_2 levels were markedly high in patients with *P. falciparum* with high parasitemia, it is possible that the levels were the summation of PGD_2 produced by both *P. falciparum* (*Pf*PGD_2) and human hosts themselves. This speculation will be true if the EIA assay for PGD_2 is not only specific to human, but also the parasite PGD_2. *Pf*PGD_2 produced can induce several pathological effects in host including intravascular hemolysis, tissue and microvascular damages from heme toxicity, oxidative stress, hyperthermia, infection and inflammation. On the other hand, PGD_2 produced by human hosts can result in the increase of oxidative stress in infected erythrocytes, and inducing PGD_2 production by the parasite into erythrocytes and microvascular environment[8]. Based on the present finding, urinary PGD_2 concentrations may provide a more reliable and useful tool for predicting severity of malaria disease. Plasma PGD_2 is not an appropriate matrix due to rapid degradation of PGD_2 in the presence of plasma protein especially albumin, which complicated the analysis of PGD_2[15]. Plasma samples as well as tissue homogenates for determination of PGD_2 should be extracted immediately after collection to remove proteins and to stabilize PGD_2.

Indomethacin was immediately added to whole blood sample after collection to prevent *ex vivo* formation of eicosanoids which have the potential to interfere with the EIA assay. For urine sample, the addition of indomethacin is not required.

PGD_2 production in *P. falciparum* culture *in vitro* was next performed in order to provide direct evidence for the production of *Pf*PGD_2 by the parasite by measuring the levels of *Pf*PGD_2 in *P. falciparum* culture *in vitro*. Unfortunately, no measureable concentration of *Pf*PGD_2 was detected in the complete medium culture collected from both *P. falciparum* clones (chloroquine resistant- K1 and chloroquine sensitive- 3D7) following both 24 and 48 hours incubation periods. A number of reasons would explain this finding. The limitation of the EIA assay based on the parent compound PGD_2 is its unreliability and difficulty of result interpretation. Antigenic protein conjugates of PGD_2, synthesized for the production of antisera show considerable amounts of decomposition. Thus, the resulting antibody response is heterogeneous with poor specificity. Furthermore, PGD_2-EIA antiserum derived from human PGD_2 used in this EIA method might be non-specific to *Pf*PGD_2. There was evidence that human PGD_2 structure is different from *Pf*PGD_2[9]. The absence of exogenous arachidonic acid substrate added to the incubation reaction might also be another reason for the observation of undetectable *Pf*PGD_2. Kubata and colleagues[9] previously reported the accumulation of prostaglandins (*Pf*PGD_2, *Pf*PGE_2 and *Pf*$PGF_{2\alpha}$) in culture medium of *P. falciparum* and indicated that the capacity to produce prostaglandins of parasite cell was increased by cultivation with exogenous arachidonic acid. The effect of the addition of exogenous arachidonic acid was observed when parasite cell were grown in complete medium supplemented with 33 mM arschidonic acid for 48 hr before harvesting. Almost all of the parasite-produced prostaglandins accumulated in the culture medium but not in the cells. Arachidonic acid content in phospholipids of *P. falciparum*-infected RBC plasma membrane was found to be much lower than that of normal red blood cells. Since *Plasmodium* is incapable of *de novo* biosynthesis of fatty acids, *P. falciparum* directly or indirectly uses an exogenous source of arachidonic acid.

Based on results obtained from limited sample size in patients with malaria, there appeared to be association between malaria pathogenicity/ severity and urinary/plasma PGD_2 concentrations at certain level. *In vitro* study however, could not confirm the production of *Pf*PGD_2 in *Plasmodium falciparum* culture. Further study with larger sample size in patients with malaria and confirmation of the *in vitro* study with extended incubation period is required to provide definitive conclusion. In addition, further study should be performed to improve the sensitivity and specificity of the assay method for PGD_2 that could differentiate between the *Pf*PGD_2 and human PGD_2.

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