Relationship of Hepcidin with Parasitemia and Anemia among Patients with Uncomplicated Plasmodium falciparum Malaria in Ghana

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Abstract. The pathogenesis of malarial anemia is incompletely understood. Hepcidin, a recently discovered peptide hormone, is a major regulator of iron metabolism and is thought to play a central role in the anemia of chronic inflammation. The specific aim of the study was to characterize the association between urinary hepcidin, hemoglobin, and parasitemia in 199 patients presenting for evaluation of Plasmodium falciparum malaria in Ghana. Urinary hepcidin was semi-quantitatively assessed using surface-enhanced laser desorption/ionization time-of-flight mass spectrometry. Urinary hepcidin (intensity/mmol creatinine) was associated with log parasitemia in 86 children (beta = 0.086, standard error [SE] = 0.035, \( P < 0.017 \)), 31 pregnant women (beta = 0.218, SE = 0.085, \( P < 0.016 \)), and 82 adults (beta = 0.184, SE = 0.043, \( P < 0.0001 \)). Urinary hepcidin was not significantly associated with hemoglobin or anemia. Urinary hepcidin is more strongly associated with parasitemia than hemoglobin or anemia among patients with acute P. falciparum malaria in Ghana.

INTRODUCTION

Malaria is a leading cause of morbidity and mortality and accounts for an estimated 500 million cases and 1 to 3 million deaths annually in developing countries. Anemia is one of the most common and severe outcomes of Plasmodium falciparum malaria, and its pathogenesis is incompletely understood. Hepcidin, a recently discovered peptide hormone synthesized primarily by hepatocytes, is considered a major regulator of iron homeostasis. Hepcidin regulates iron metabolism by inhibiting duodenal iron absorption at the intestinal epithelium and by reducing mobilization of iron from the liver and spleen. The synthesis of hepcidin is modulated by iron status, hypoxia, and inflammation. In the anemia of chronic inflammation, hepcidin is thought to block the release of iron from enterocytes, hepatocytes, and macrophages, leading to hypoferremia and limited iron availability for erythropoiesis. Although studies suggest that hepcidin is a key regulator in the anemia of chronic inflammation, the role of hepcidin in the pathogenesis of malarial anemia has not been characterized. We hypothesized that urinary hepcidin levels would be associated with high parasitemia among patients with uncomplicated P. falciparum malaria. To address this hypothesis, we measured urinary hepcidin among patients being evaluated for malaria in Ghana.

MATERIALS AND METHODS

Study subjects. The study subjects consisted of a consecutive sample of 290 symptomatic individuals (187 adults, 58 pregnant women, 103 children) seen at Kpone Health Center in Kpone On Sea, Ghana, from July to August 2005 for evaluation of malaria and malarial antigens in urine. The Kpone Health Center serves Kpone On Sea, a village in eastern coastal Ghana, an area that is endemic for Plasmodium falciparum malaria. Temperature was measured using a digital axillary thermometer. A fingertip blood sample was taken to prepare thick and thin blood films to determine the presence or absence of malaria parasites, level of parasitemia, and hemoglobin concentrations using a HemoCue machine (HemoCue Inc., Mission Viejo, CA). Subjects were eligible for the study if they were diagnosed with P. falciparum malaria, were not admitted for transfusion, had a hemoglobin level > 50 g/L, and had no evidence of cerebral malaria. Only one subject had received anti-malarial medication. Subjects were enrolled in the study after written informed consent was obtained, and for children, after written informed consent was obtained from the parent or guardian. Spot urine samples (5 mL) were collected, and urine samples were immediately aliquoted and frozen in cryotubes at –70°C. The study protocol was approved by the National Institutes of Health and the Noguchi Memorial Institute for Medical Research IRB CPN 025/-2-03. Analyses of urine were performed under approval by the Committee for Human Research of the Johns Hopkins Bloomberg School of Public Health.

Thick and thin Giemsa-stained blood films were analyzed for the number of parasites per 200 white blood cells. Slides were considered negative if no parasites were seen in 100 fields on the thick film. Urinary hepcidin was measured using surface-enhanced laser desorption/ionization time-of-flight mass spectrometry (SELDI-TOF MS). Urine samples were thawed, vortexed, and centrifuged at 12,000 rpm for 25 minutes. The supernatant (7 µL) of each sample was applied in triplicate (three spots) on the NP20 Ciphergen ProteinChip® and incubated in a moist chamber for 30 min. Unbound proteins are removed by washing three times with HPLC grade water for 10 min. Chip arrays are air-dried, and 1.0 µL of alpha-cyano-4-hydroxy-cinnamic acid (CHCA, Ciphergen) in 50% acetonitrile and 0.25% trifluoroacetic acid was added to the array surface and allowed to air dry. The arrays were analyzed on a Ciphergen ProteinChip® Reader (Model PBS II, Ciphergen). Data was collected using the following settings: two warming shots at laser intensity 175 (not collected); 10 shots at laser intensity 170 at every five positions between 20 and 80; high mass 4500 Da; mass deflector 100 Da, detector sensitivity 7; acquired mass range from a mass-over-charge (m/z) ratio of 500 to 4500. Peak annotation was conducted using Ciphergen ProteinChip Software (version 3.2.0), after baseline subtraction and adjustment. A peak is recorded for
hepcidin at the characteristic m/v of 2790 when the signal-to-noise ratio is > 3:1. Although the molecular weight of hepcidin is 2789, it is ionized as a charged (protonated) species on SELDI-TOF MS; thus, the average peak on low-resolution mass spectrometry is one unit higher than the average molecular weight because of the added proton. Urinary creatinine was measured using a commercial ELISA (Quidel Corporation, San Diego, CA) in Baltimore. Between run coefficients of variation (CV) for urinary creatinine were 11.9% and 5.9% for high and low controls, respectively. Urinary hepcidin concentrations were expressed as intensity per mmol/L creatinine.

Means and standard deviations were calculated for descriptive statistics. Anemia was defined as hemoglobin < 11 g/dL for children and pregnant women, < 12 g/dL for non-pregnant adult women, and < 13 g/dL for men. Both malaria parasitemia and urinary hepcidin (mmol/L creatinine) were continuous variables that were highly skewed and were transformed by log_e to normalize the distribution. Linear regression models were used to examine the relationship between urinary hepcidin and other variables such as parasitemia and hemoglobin.

RESULTS

Of the 290 subjects in the study series, urine samples were available from 199 patients (86 children, 82 adults, and 31 pregnant women) who were evaluated for uncomplicated Plasmodium falciparum malaria. Three subjects were excluded from the analysis because of a lack of age data. Characteristics of the study groups are shown in Table 1. The prevalence of anemia among children, adults, and pregnant women was 79.1%, 82.9%, and 90.3%, respectively. Linear regression models were used to examine the relationship between urinary hepcidin and parasitemia, hemoglobin, and anemia in each of the three groups and in all three groups combined, as shown in Table 2. In univariable linear regression models, log parasitemia was positively associated with urinary hepcidin (intensity/mmol creatinine) in 86 children (beta or regression coefficient = 0.086, standard error [SE] = 0.055, P < 0.017), 31 pregnant women (beta or regression coefficient = 0.218, SE = 0.085, P < 0.016), and 82 adults (beta or regression coefficient = 0.184, SE = 0.043, P < 0.0001). Hepcidin was not significantly associated with hemoglobin or anemia in any of the groups. In a multi-variable linear regression model, log_e urinary hepcidin was associated with log_e parasitemia (beta or regression coefficient = 0.174, SE = 0.026, P < 0.0001) but not hemoglobin (beta or regression coefficient = 0.022, SE = 0.058, P = 0.71). Anemia was not included in the same multi-variable model because the two variables are highly dependent. There were no significant interactions between log_e parasitemia and hemoglobin. We also ran an alternative model that included only subjects who had more severe anemia (hemoglobin < 9 g/dL). In a multi-variable model, log_e urinary hepcidin was associated with log_e parasitemia (beta or regression coefficient = 0.248, SE = 0.056, P < 0.0001) but not hemoglobin (beta or regression coefficient = 0.211, SE = 0.192, P = 0.28).

DISCUSSION

This study shows that urinary hepcidin concentrations are more strongly associated with malaria parasitemia than hemoglobin levels or anemia among patients with acute, uncomplicated malaria in Ghana. To our knowledge, this is the first study to characterize urinary hepcidin levels in patients with malaria. Hepcidin is thought to play a central role in the anemia of chronic inflammation, and factors that have been
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Implicated in the modulation of hepcidin expression include hypoxia\(^5\) and inflammation.\(^6\) In malaria, the destruction of parasitized and normal erythrocytes, as well as ineffective erythropoiesis contribute almost equally to anemia, but recent data suggest that these mechanisms alone or in combination do not adequately explain the anemia of malaria infection.\(^7\)

The present study provides some initial insight into the pathogenesis of anemia during malaria. High levels of parasitemia were associated with high levels of hepcidin. Hepcidin inhibits duodenal iron absorption at the intestinal epithelium\(^8\) and inhibits mobilization of iron from liver and spleen.\(^9\) Hepcidin binds to the iron exporter, ferroportin, inducing its internalization and degradation.\(^10\) Ferroportin is the only mammalian iron exporter identified to date and is necessary for maternalfetal iron transfer and iron efflux from duodenal enterocytes, macrophages, and hepatocytes.\(^11\) The finding from the present study suggests that during acute, uncomplicated malaria, the availability of iron for erythropoiesis may be limited through the upregulation of hepcidin.

Both inflammation and infection can increase hepcidin expression, as shown in different animal models, including mice challenged with turpentine\(^4\) and lipopolysaccharide,\(^5\) rats given Freund’s complete adjuvant\(^11\) and fish challenged with \textit{Streptococcus iniae} infection.\(^12\) The present study is limited in that serum IL-6 concentrations were not measured; however, elevated serum IL-6 levels are known to be associated with high \textit{P. falciparum} malaria parasitemia.\(^13\) An acute episode of malaria induces inflammation and elevates IL-6 levels. IL-6 has been implicated in the upregulation of hepcidin, which blocks the release of iron from enterocytes, hepatocytes, and macrophages, leading to hypoferremia and limited iron availability for erythropoiesis.\(^2\)

Hepcidin expression can also be upregulated by an iron-replete state.\(^14\) A limitation of this study is that indicators of iron status were not measured in the present study, but it is probably unlikely that an iron-replete state accounted for the high hepcidin levels in this population that included pregnant women and children, two high risk groups for iron deficiency. Although the study does not include a group of subjects without malaria, mean urinary hepcidin levels as measured by SELDI-TOF MS were 0.52 intensity/mmol Cr in healthy control subjects and 0.1 intensity/mmol Cr in subjects with iron deficiency anemia.\(^15\) The urinary hepcidin levels of 3–6 intensity/mmol Cr in the present study are comparable to mean levels of 6 intensity/mmol Cr among adults challenged with lipopolysaccharide in a study of anemia and experimental endotoxemia.\(^16\)

Hepcidin expression is upregulated by hypoxia,\(^4\) but in the present study, low hemoglobin levels or anemia was not associated with urinary hepcidin. In multi-variante analyses, parasitemia was the strongest factor associated with hepcidin, suggesting that parasitemia, and possibly inflammation associated with parasitemia, are stronger modulators of hepcidin expression than hypoxia.

Hepcidin is best measured in urine because the active 25 AA form of hepcidin is readily filtered by the glomerulus and passes into the urine. In the present study, urinary hepcidin was measured by SELDI-TOF MS, a method that is semiquantitative but has been shown to correlate well with an immunologic method developed by Ganz and colleagues for use in their own laboratory.\(^6\) A commercially available immunoassay is available that measures the 64 AA prohormone form of hepcidin, but the prohormone does not have biologic activity and correlates poorly with indicators of iron metabolism\(^7\)–\(^9\) and inflammation.\(^10\) The present study relied upon the use of a single Hemocue measurement, a method that has been used around the world in developing countries and has been extensively validated for hemoglobin measurement.\(^21\)–\(^23\)

Future studies are needed to examine longitudinal changes in hepcidin, hemoglobin, iron status indicators, and proinflammatory cytokines such as IL-6 among patients with malaria. The present study provides some initial insights and represents a first step in the elucidation of the role of hepcidin in the anemia that occurs in \textit{P. falciparum} malaria. Such investigations are important because anemia remains a common but not well understood complication of malaria.

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