Adenosine Triphosphate Depletion of Erythrocytes Simulates the Phenotype Associated with Pyruvate Kinase Deficiency and Confers Protection against *Plasmodium falciparum* In Vitro

Kodjo Ayi, W. Conrad Liles, Philippe Gros, and Kevin C. Kain

**Background.** Erythrocytes from individuals with pyruvate kinase deficiency (PKD) are resistant to invasion by *Plasmodium falciparum* parasites, and erythrocytes infected with ring-stage parasites are preferentially cleared by macrophages in vitro. However, the underlying molecular basis of protection is unknown. In the present study, we examined adenosine triphosphate (ATP) levels in PKD erythrocytes (ie, erythrocytes from individuals with PKD) and determined whether depletion of ATP in normal erythrocytes would recapitulate the phenotype observed with PKD.

**Methods.** We examined ATP levels in homozygous *PKLR*<sup>−/−</sup> and heterozygous *PKLR*<sup>+</sup>/<sup>−</sup> human erythrocytes and used sodium fluoride treatment to inhibit ATP generation in normal human erythrocytes.

**Results.** We demonstrated that ATP levels are reduced in *PKLR*<sup>−/−</sup> (percentage of control erythrocytes, 26%; interquartile range [IQR], 21%–48%) and *PKLR*<sup>+</sup>/<sup>−</sup> erythrocytes (percentage of control erythrocytes, 64%; IQR, 60%–73%) and that there is a correlation between ATP levels in erythrocytes and both inhibition of parasite invasion and enhancement of phagocytosis of erythrocytes infected with ring-stage parasites. Analysis of ATP distribution in parasitized erythrocytes demonstrated that parasites invading PKD erythrocytes respond to low intraerythrocytic ATP levels by means of a parallel increase in parasite-derived ATP via up-regulation of *P. falciparum*-specific pyruvate kinase.

**Conclusion.** These data suggest that reduced erythrocyte ATP levels may contribute to the protection displayed by PKD erythrocytes in vitro and may provide a model system with which to define the molecular basis of protection in inherited PKD.

*Plasmodium falciparum* malaria is a major cause of childhood mortality and has exerted powerful positive selection pressure for genetic variants that confer a survival advantage. The genetic determinants of resistance to malaria are complex and multigenic; however, the most common protective polymorphisms that have been identified involve erythrocyte-specific structural proteins and enzymes [1]. Heterozygosity for disease-associated alleles of certain erythrocyte disorders, including hemoglobinopathies and glucose-6-phosphate dehydrogenase (G6PD) deficiency, confers protection against severe and fatal malaria and may have been retained by positive selection in populations exposed to malaria [2–6]. Various mechanisms have been proposed to explain the protective effect of these red cell variants, including inhibition of parasite invasion and maturation, inhibition of adherence of parasitized erythrocytes to endothelium, and enhanced clearance.
of erythrocytes infected with the ring stage of the parasite [1–3, 7–9].

Pyruvate kinase deficiency (PKD) is the second most common erythrocyte enzyme disorder and is associated with >180 mutations in the PKLR gene [10]. Although its global prevalence has not been well studied, PKD has been reported in all regions of the world where malaria was previously endemic [10, 11]. The prevalence of PKD in Africa is unknown; however, a study from the United States reported that PKD was 2.4 times more common among African Americans than among whites [12]. Pyruvate kinase is a key glycolytic enzyme that catalyzes the transphosphorylation of phosphoenolpyruvate to adenosine diphosphate, yielding pyruvate and ATP. Inhibition of the glycolytic enzyme enolase by sodium fluoride (NaF) [13] has been used to model the physiological effects of PKD in erythrocytes [14]. Enolase catalyzes the transformation of 2-phosphoglycerate to phosphoenolpyruvate and contributes to the production of erythrocyte ATP.

Recently, it was shown that PKD confers protection against Plasmodium chabaudi malaria and babesiosis in vivo and P. falciparum infection in vitro [15–17]. The protective effect against P. falciparum infection is manifested by reduced invasion of PKD erythrocytes by merozoites and enhanced phagocytosis of PKD erythrocytes (ie, erythrocytes from individuals with PKD) parasitized with the ring stage of the parasite [15, 16]. However, the precise biological and biochemical mechanisms underlying protection have yet to be elucidated.

Based on the hypothesis that resistance is dependent on intracellular levels of ATP, the objective of the present study was to examine the relationship between erythrocyte ATP levels and P. falciparum infection in vitro. We demonstrate a correlation between the ATP levels in PKD erythrocytes and NaF-treated normal erythrocytes and the degree of both inhibition of invasion and macrophage uptake of erythrocytes infected with ring-stage parasites. The small proportions of parasites that successfully invade PKD erythrocytes or NaF-treated erythrocytes appear to meet their ATP requirements for intracellular maturation by up-regulating P. falciparum–specific pyruvate kinase (PfPyrK).

**MATERIALS AND METHODS**

**Participants and blood donation.** Patients who were attending the hematology clinics of the Toronto General Hospital and The Hospital for Sick Children, Toronto, Ontario, Canada, from January 2006 through June 2007 and who were identified as having PKD on the basis of their clinical presentation and the results of a pyruvate kinase enzyme assay were eligible for enrollment in the study. After institutional review board approval and written informed consent were received, venous blood samples were drawn from healthy volunteers and from individuals with PKD, G6PD deficiency, and β-thalassemia. Other hemolytic disorders were excluded by hemoglobin electrophoresis and assessment of the G6PD level. Sequence analysis of the 12 coding exons of the PKLR gene was performed to confirm the diagnosis of PKD, as described elsewhere [15]. The research ethics boards of the University Health Network and The Hospital for Sick Children approved the study protocols [15].

**ATP levels in PKD erythrocytes.** Measuring pyruvate kinase is challenging, and enzyme levels may be overestimated as a result of contamination by white blood cells and platelets. Moreover, PKD erythrocytes are cleared more quickly from the circulation in transfusion-dependent cases [18]. Therefore, we estimated pyruvate kinase enzymatic activity by measuring levels of ATP in fresh PKD erythrocytes, using a commercially available ATP kit (FL-ASC; Sigma) based on a luciferin-luciferase bioluminescence assay. ATP levels are expressed as the number of relative luminescence units.

**NaF treatment of normal erythrocytes.** To determine the effect of NaF on the ATP levels of erythrocytes, leukocyte-depleted erythrocytes [7] were washed and suspended in Roswell Park Memorial Institute 1640 medium supplemented with 10 mmol/L glucose and 10 g/L gentamicin (washing medium). Hematocrit was adjusted to 10%, and the preparation was incubated at room temperature with 0, 0.5, 1, or 2 mmol/L NaF [13]. After 24 h, erythrocytes were washed, and ATP was measured as described above.

**Effects of NaF on P. falciparum viability.** To assess any potential direct antiparasitic activity of NaF, 2 methods were used. First, a SYBR-green–based protocol was used to assess parasite survival in the presence of varying concentrations of NaF [19]. Second, the viability of P. falciparum parasites was determined by measuring parasite-specific lactate dehydrogenase levels [20, 21] in the presence of increasing concentrations of NaF (parasite-specific lactate dehydrogenase is released only by viable parasites).

**P. falciparum invasion and maturation assays.** P. falciparum isolates ITG and 3D7 were maintained in continuous mycoplasma-free culture (Roswell Park Memorial Institute 1640 medium containing 20 mmol/L glucose and 2 mmol/L glutamine and supplemented with 6 g/L HEPES, 2 g/L sodium bicarbonate, 10 g/L gentamicin, 10% human serum, and 1.35 mg/L hypoxanthine [pH 7.3]), as described elsewhere [22]. Mature forms were purified (purity, >95%) [23] and used to infect fresh normal, PKD, NaF-treated, G6PD-deficient (G6PDd), and thalassemic erythrocytes (with the latter erythrocytes obtained from individuals with β-thalassemia), as described elsewhere [15]. Invasion and maturation of P. falciparum in NaF-treated erythrocytes for 2 growth cycles were assessed as described elsewhere [15].

**Phagocytosis assay.** Monocytes were isolated from the peripheral blood of healthy human donors, as described elsewhere [24]. NaF-treated and untreated erythrocytes that were para-
Table 1. Characteristics of Participants with PKLR Mutations

<table>
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* Reference ranges are 121–151 g/L for women and 138–172 g/L for men.

* Reference range is 25–85 billion cells/L for adults.

sitized at ring stage were incubated with 50% fresh autologous nonimmune serum at 37°C for 30 min. Erythrocytes were then washed, resuspended to 10% hematocrit, and incubated with adherent macrophages at a target:effector ratio of 40:1. Phagocytosis assays were performed as described elsewhere [25]. All experiments were performed in duplicate and repeated 4 times.

**Compartmental analysis of ATP distribution in parasitized erythrocytes.** Mature parasitized erythrocytes were purified by centrifugation over a Percoll/mannitol gradient (purity, >95%). To assess the total amount of ATP, cells were lysed with Somatic Cell ATP Releasing Reagent (Sigma). For compartmental analysis of ATP, cells were lysed with 0.1% saponin at room temperature for 1 min and were centrifuged at 1300 g for 1 min [26, 27]. The supernatant was collected for analysis of host cytosolic ATP levels, and the pellet was used to assess ATP levels of the parasite. ATP was measured as described above.

**Isolation of parasite RNA and reverse transcription.** Total RNA was extracted from malaria cultures in TRIzol (Invitrogen), purified using the RNeasy Mini Kit (Qiagen), and resuspended in ribonuclease-free water. The SuperScript III kit was used for reverse-transcription reactions, in accordance with the manufacturer’s instructions (Invitrogen), with the use of 4 μg of total RNA.

**Quantification of P. falciparum pyruvate kinase mRNA.** To quantify PfPyrK mRNA, a real-time quantitative polymerase chain reaction (PCR) assay was performed with forward and reverse primers 5′-GACCAAGAACCCAAGACCAA-3′ and 5′-ACCACCTGAGTGTCTCCTG-3′, as described elsewhere [28, 29].

**Statistical analysis.** Data are presented as box-and-whisper plots denoting interquartile and complete ranges, with the horizontal line in each box denoting the median value, unless otherwise noted. We performed comparisons using the 2-tailed Student’s t test or linear regression when the data were normally distributed. Normality was assessed using the Kolmogorov-Smirnov test. For data that were not normally distributed, the Mann-Whitney U statistic was used. All P values have been corrected for multiple comparisons by use of Holms correction.

**RESULTS**

**Decreased ATP levels in PKD erythrocytes and NaF-treated erythrocytes.** We examined the ATP content in PKD erythrocytes from 2 individuals with homozygous G→A loss-of-function mutations at nucleotide (nt) 1269 of the PKLR gene and a third individual homozygous for a G deletion at nt 823 in exon 7, leading to premature termination of the pyruvate kinase open-reading frame. We also identified asymptomatic relatives who were heterozygous for the G→A mutation at nt 1269 (Table 1).

In agreement with other studies [10, 30], we observed that ATP levels were decreased in PKD erythrocytes, compared with control (AA) erythrocytes (Figure 1A). ATP levels correlated with genetic status. Compared with AA erythrocytes, ATP levels were decreased in erythrocytes from heterozygous (percentage of control erythrocytes, 64%; interquartile range [IQR], 60%–73%) and homozygous (percentage of control erythrocytes, 26%; IQR, 21%–48%) individuals.

NaF, an inhibitor of enolase and ATP generation, was used to mimic the ATP depletion observed in PKD erythrocytes [13, 31]. Incubation of normal erythrocytes with 0–2 mmol/L NaF was nontoxic and had no direct antiparasitic activity, as was determined by 2 different measurements of parasite viability (Figure 1B). NaF depleted ATP in normal erythrocytes in a dose-dependent manner (Figure 1C). The ATP level remained low in NaF-treated erythrocytes for at least 4 days after treatment (data not shown).

**NaF treatment of normal erythrocytes simulates the phenotype associated with PKD.** PKD erythrocytes are relatively refractory to invasion by P. falciparum, and PKD erythrocytes infected by ring-stage parasites are more avidly phagocytosed by macrophages than are parasitized normal erythrocytes [15]. To determine whether NaF-induced ATP depletion results in a
Figure 1. Reduced adenosine triphosphate (ATP) levels in pyruvate kinase deficiency (PKD) erythrocytes and sodium fluoride (NaF)–treated normal erythrocytes. A, Measurement of ATP levels in nonparasitized normal (AA) erythrocytes, heterozygous (Htz; \( PKLR^{+/-} \)) erythrocytes, and homozygous (Hmz; \( PKLR^{-/-} \)) erythrocytes. The ATP level was measured in relative fluorescence units and expressed as the percentage of control (AA) erythrocytes. * (8 experiments from 2 heterozygotes) and ** (6 experiments from 3 homozygotes).

B, Chloroquine-susceptible (3D7; \( P<.004 \)) and chloroquine-resistant (ItG; \( P<.002 \)) parasite cultures treated with NaF at different concentrations and assessed for viability by parasite lactate dehydrogenase (pLDH) assays (left) and SYBR-green analysis (right). Data denote the mean value ± standard deviation.

C, Assessment of ATP levels in NaF-treated normal erythrocytes (0–2 mmol/L). Statistical significance between AA and PKD or NaF-treated erythrocytes was determined using the Mann-Whitney U test, with Holm’s correction for multiple comparisons. *\( P<.001 \) (6 experiments for 0.5 mmol/L), **\( P<.001 \) (11 experiments for 1 mmol/L), and ***\( P<.001 \) (11 experiments for 2 mmol/L).
similar phenotype, we investigated whether parasites invade and mature as efficiently in NaF-treated erythrocytes as in control erythrocytes. The results of invasion and replication assays (Figure 2A) showed a progressive and dose-dependent reduction in the invasion of NaF-treated erythrocytes by *Plasmodium falciparum* merozoites, compared with untreated erythrocytes. No significant differences were observed in intracellular maturation at the first cycle, but the second cycle demonstrated a decrease in maturation from ring stage to schizont stage (*P* < .05) (Figure 2A). However, even untreated erythrocytes showed inhibition, and, therefore, the difference observed at the second stage of maturation does not appear to be attributable to the NaF but, rather, to erythrocyte aging in culture, as has been shown in thalassemic erythrocytes [32]. These data demonstrate a reduced invasion by *P. falciparum* in NaF-treated normal erythrocytes that resembles the invasion defect observed in erythrocytes from homozygous patients with PKD [15]. Moreover, they suggest that, similar to PKD, NaF treatment does not dramatically alter the intracellular environment of erythrocytes in such a manner as to inhibit parasite maturation after invasion.

We next examined the phagocytic uptake of *P. falciparum* parasitized, NaF-treated, and control erythrocytes by human monocyte-derived macrophages. Phagocytosis of ring-stage–parasitized NaF-treated erythrocytes was significantly higher than phagocytosis of parasitemia-matched parasitized untreated erythrocytes, whereas macrophage uptake of nonparasitized NaF-treated erythrocytes was not significantly different than that of nonparasitized untreated erythrocytes (Figure 2B). These data show increased macrophage uptake of ring-stage–parasitized NaF-treated erythrocytes.

![Figure 2](image-url)

**Figure 2.** Reduced invasion of sodium fluoride (NaF)–treated erythrocytes by *Plasmodium falciparum*, compared with that noted for control erythrocytes, and more avid phagocytosis of ring-stage–parasitized NaF–treated erythrocytes, compared with ring-stage–parasitized control erythrocytes. *A,* Invasion (left) and maturation (right) of *P. falciparum* in normal untreated erythrocytes (AA; white box) or NaF–treated erythrocytes were assessed over 2 replication cycles. Data are the combined results of at least 4 independent experiments. *P* = .014, **P** < .001, and ***P*** < .05 (linear regression; data normally distributed by Kolmogorov-Smirnov test). *B,* Assessment of phagocytosis of nonparasitized and parasitized ring-stage AA or NaF–treated erythrocytes by human monocyte-derived macrophages. Phagocytosis is expressed as the phagocytic index (ie, the no. of phagocytosed parasitized erythrocytes per macrophage). Data are the combined results of 4 independent experiments. Statistically significant differences between parasitized control AA and parasitized NaF–treated erythrocytes were determined using the Mann-Whitney *U* test, with Holms correction for multiple comparisons. *P* = .081; **P** = .021; ***P*** = .006.
normal erythrocytes that resembles the enhanced clearance of ring-stage–parasitized erythrocytes observed in homozygous and heterozygous PKD erythrocytes [15].

Compartmental analysis of ATP distribution in parasitized PKD erythrocytes. PKD is associated with reduced intraerythrocytic ATP levels [30] that might be expected to negatively affect intracellular parasite growth. However, our data indicate that parasites that do successfully invade PKD [15] or NaF-treated erythrocytes (Figure 2A) mature and replicate. To determine how the parasite responds and adapts to this environment to maintain growth, we used a bioluminescence assay to determine ATP levels and ATP flux in different intracellular compartments under different conditions of infection. We compared the ATP levels of parasitized PKD erythrocytes with those of parasitized AA erythrocytes (Figure 3A), thalassemic and G6PDd erythrocytes (Figure 3B), and NaF-treated AA erythrocytes (figure 3C).

Figure 3A and 3B shows that ATP levels are significantly lower in nonparasitized PKD erythrocytes than in normal erythrocytes. In normal AA erythrocytes, ATP levels decrease after parasite invasion and maturation. In contrast, infection of PKD erythrocytes is associated with a significant increase in ATP levels, compared with what has been noted for their non-parasitized counterparts.

We used differential saponin lysis to assess ATP levels in the host erythrocyte and parasite compartments [27] (Figure 3A). In nonparasitized erythrocytes, ATP was predominantly present in the host cell compartment. In contrast, ATP was evenly distributed between the host (supernatant) and parasite (pellet) compartments in parasitized AA and PKD erythrocytes. Of note, ATP levels in the host and parasite compartments of parasitized PKD erythrocytes were significantly higher than those observed in parasitized AA erythrocyte compartments (Figure 3A). These observations are consistent with previous studies indicating that P. falciparum parasites can generate ATP that supplements the host red cell compartment [27, 33].

These findings suggest that the increase in parasite-generated ATP production after invasion may compensate for low levels of ATP in PKD erythrocytes, thereby facilitating intracellular maturation of the trophozoite. Moreover, parasite-generated ATP can cross the membrane of the parasitophorous vacuole and contribute ATP to the host cell cytoplasm [27, 33].

Increases in ATP levels in PKD and NaF-treated erythrocytes after parasite invasion but not in G6PDd and thalassemic erythrocytes. We next compared ATP levels and flux in parasitized normal, PKD, NaF-treated, and other red blood cell variants, including G6PDd and thalassemic erythrocytes. As mentioned above, ATP levels decreased in parasitized normal erythrocytes. In contrast, after PKD erythrocytes were infected, their ATP levels increased to a level comparable to that noted in parasitized normal erythrocytes (Figure 3B). Similarly, NaF-treated erythrocytes, which initially showed a dose-response decrease in ATP levels, displayed a corresponding increase in ATP after parasite invasion (Figure 3C).

This increase in ATP after infection was specific to PKD and NaF-treated erythrocytes. ATP levels were normal in nonparasitized G6PDd erythrocytes and decreased after infection (Figure 3B). As reported elsewhere [34], ATP levels were significantly lower in nonparasitized thalassemic erythrocytes than in AA erythrocytes; however, the levels decreased after parasite invasion (Figure 3B), similar to what has been noted in AA and G6PDd erythrocytes.

PPyRK gene expression correlates with ATP levels in parasitized erythrocytes. P. falciparum parasites have been shown to possess pyruvate kinase (PPyRK), making it possible for the parasite to utilize erythrocyte glucose to produce ATP [28]. To test the hypothesis that increased ATP levels in parasitized PKD and NaF-treated erythrocytes were the result of parasite-specific ATP generation via up-regulation of PPyRK, we determined expression of PPyRK mRNA in parasitized AA, G6PDd, thalassemic, and NaF-treated erythrocytes by use of reverse-transcription quantitative PCR (RT-qPCR) (Figure 3D). A correlation was observed between ATP levels in parasitized erythrocytes and their PPyRK mRNA expression. As determined by RT-qPCR, PPyRK gene expression increased significantly (8- to 13-fold) in parasitized NaF-treated erythrocytes, compared with control erythrocytes. In contrast, PPyRK expression in parasitized thalassemic and G6PDd erythrocytes was significantly lower than that in parasitized AA erythrocytes. Collectively, these data support the hypothesis that parasites invading PKD and NaF-treated erythrocytes compensate for low intracellular ATP levels by up-regulating parasite-derived ATP generation via PPyRK.

DISCUSSION

The present study provides what is, to our knowledge, the first evidence implicating erythrocyte ATP depletion as the mechanism underlying PKD-associated protection against P. falciparum malaria. ATP is important for parasite invasion and maturation, and defects in the production of erythrocyte ATP may have profound influences on the pathophysiology of malaria [14, 35, 36]. Here we show that ATP levels are reduced in PKD homozygous and heterozygous erythrocytes and that the reduction in ATP correlates with genetic status (Figure 1A) and resistance to parasite invasion [15]. Using NaF to mimic the physiological status of PKD erythrocytes, we demonstrated that NaF treatment resulted in a dose-dependent reduction in ATP in normal erythrocytes (Figure 1C) that correlates with inhibition of merozoite invasion in vitro (Figure 2A). Furthermore, NaF-treated erythrocytes display enhanced phagocytosis when infected with ring-stage parasites, similar to parasitized PKD erythrocytes (Figure 2B). Finally, we used compartmental analysis of ATP distribution to show that the small
Figure 3. Compartmental analysis of adenosine triphosphate (ATP) distribution in parasitized pyruvate kinase–deficient (PKD), sodium fluoride (NaF)–treated, glucose-6-phosphate dehydrogenase–deficient (G6PDd), and “β-thal” erythrocytes (with the latter denoting erythrocytes from individuals with β-thalassemia). ATP levels and flux were examined in parasitized and nonparasitized control (AA) (A), β-thal and G6PDd (B), and NaF-treated AA (C) erythrocytes. In panel A, data are the mean value ± standard deviation of at least 3 experiments and were assessed using the Mann-Whitney U test. *P = .029, for host compartment (supernatant) and parasite (pellet) ATP levels in AA vs PKD erythrocytes. Measurement of ATP levels in normal, G6PDd, and β-thal (B) and NaF-treated erythrocytes (C) was repeated at least 4 times. Statistically significant differences between nonparasitized and parasitized erythrocytes were assessed using the Mann-Whitney U test with Holms correction for multiple comparisons. In panel B, *P = .018, for nonparasitized AA vs nonparasitized β-thal erythrocytes. **P = .002, ***P = .003, †P = .016, and ‡P = .016. In panel C, *P = .057, **P < .05, and ***P < .001. D, Significant up-regulation of P. falciparum pyruvate kinase (PfPyrK) in NaF-treated parasitized erythrocytes. PfPyrK mRNA expression in parasitized normal, G6PDd, β-thal, and NaF-treated erythrocytes was analyzed by reverse-transcription quantitative PCR and expressed as relative fluorescence units. Experiments were repeated 2 times in triplicate. Statistically significant differences were observed for parasitized normal vs parasitized β-thal, parasitized normal vs parasitized G6PDd, and parasitized normal vs parasitized NaF-treated erythrocytes, as assessed by the Mann-Whitney U test with Holms correction for multiple comparisons. *P = .008; **P = .007; ***P = .017; and †P = .03.
Proposed mechanism of pyruvate kinase (PK) deficiency (PKD)–associated protection against parasite infection. PKD is associated with decreased erythrocyte adenosine triphosphate (ATP) levels and increased production of 2,3-diphosphoglycerate (2,3-DPG). Collectively, these may contribute to disruption of the cell-membrane–cytoskeletal protein network, impairing merozoite invasion of the erythrocyte and enhanced clearance of ring-stage–parasitized erythrocytes. Parasite invasion may enhance preexisting membrane damage of the PKD erythrocyte by increasing radical production, hemichrome formation, and band 3 aggregation. These red blood cell changes can lead to higher deposition of complement on the surface of the erythrocyte, increased macrophage uptake, and overexpression of \textit{Plasmodium falciparum}–specific pyruvate kinase. The small proportion of merozoites that successfully invade PKD or sodium fluoride (NaF)–treated erythrocytes appear to facilitate intracellular maturation and survival by up-regulating parasite-specific ATP production, 1,3-DPG, 1,3-diphosphoglycerate; 2-PG, 2-phosphoglycerate; G6P, glucose-6-phosphate; GSH, glutathione; Hb-O₂, oxyhemoglobin; HK, hexokinase; O₂/H₂O₂, superoxide; PEP, phosphoenolpyruvate; PPP, pentose phosphate pathway.

Figure 4. Proposed mechanism of pyruvate kinase (PK) deficiency (PKD)–associated protection against parasite infection. PKD is associated with decreased erythrocyte adenosine triphosphate (ATP) levels and increased production of 2,3-diphosphoglycerate (2,3-DPG). Collectively, these may contribute to disruption of the cell-membrane–cytoskeletal protein network, impairing merozoite invasion of the erythrocyte and enhanced clearance of ring-stage–parasitized erythrocytes. Parasite invasion may enhance preexisting membrane damage of the PKD erythrocyte by increasing radical production, hemichrome formation, and band 3 aggregation. These red blood cell changes can lead to higher deposition of complement on the surface of the erythrocyte, increased macrophage uptake, and overexpression of \textit{Plasmodium falciparum}–specific pyruvate kinase. The small proportion of merozoites that successfully invade PKD or sodium fluoride (NaF)–treated erythrocytes appear to facilitate intracellular maturation and survival by up-regulating parasite-specific ATP production, 1,3-DPG, 1,3-diphosphoglycerate; 2-PG, 2-phosphoglycerate; G6P, glucose-6-phosphate; GSH, glutathione; Hb-O₂, oxyhemoglobin; HK, hexokinase; O₂/H₂O₂, superoxide; PEP, phosphoenolpyruvate; PPP, pentose phosphate pathway.

In the present study, we tested the hypothesis that reduced proportion of parasites that do invade PKD and NaF-treated erythrocytes appear to compensate for low intracellular ATP levels by increasing their own ATP production, thus facilitating normal parasite maturation and replication (Figure 3A, 3B, and 3C). Collectively, these data provide evidence that decreased intracellular ATP levels contribute to PKD-associated resistance to infection in vitro and suggest that NaF-treated erythrocytes may provide a useful model system with which to define the precise biochemical and molecular basis of PKD-associated resistance, as proposed in the present study.

PKD is a heterogeneous syndrome, and the molecular basis for PKD–associated nonspherocytic anemia and its variable phenotype is not well understood. PKD is further complicated by the observation that \textit{PKLR} mutations are not concentrated in known functional domains of the enzyme, and there is a poor correlation between genotype and clinical phenotype [36–39]. The key biochemical abnormalities are decreased levels of erythrocyte ATP and increased levels of metabolic intermediates in the glycolytic pathway, such as 2,3-DPG (2,3-diphosphoglycerate). Elevated levels of 2,3-DPG may inhibit other metabolic pathways, including hexokinase and the pentose phosphate shunt (PPP) [36, 40]. The proposed mechanisms responsible for the resistance of PKD erythrocytes to malaria parasites include low intracellular ATP levels that may impair parasite invasion or growth; accumulation of metabolic intermediates, such as 2,3-DPG, that create an intracellular environment that is unsuitable for parasite metabolic activity and growth; and/or inhibition of G6PD/PPP with consequent impaired reduction of reactive oxygen species by glutathione, rendering infected erythrocytes more susceptible to oxidative membrane damage and early clearance by mononuclear cells (Figure 4) [15, 39–41].

In the present study, we tested the hypothesis that reduced
erythrocyte ATP levels underlie the resistance of PKD erythrocytes to in vitro infection. We found that decreased ATP levels in PKD erythrocytes are associated with impaired parasite invasion and enhanced phagocytosis of ring-stage–parasitized erythrocytes by macrophages. In addition, we extended our studies to examine NaF treatment of normal erythrocytes as a model system to mimic PKD [42] and to further analyze the influence of ATP depletion on parasite invasion and macrophage clearance. NaF-treated erythrocytes displayed a dose-dependent decrease in ATP and simulated the phenotype of PKD erythrocytes against \textit{P. falciparum} infection. Similar to PKD erythrocytes, the small proportion of parasites that did invade NaF-treated erythrocytes appear to mature normally, suggesting that alterations in the intracellular environment associated with the accumulation of glycolytic metabolic intermediates do not dramatically inhibit parasite growth. These observations suggest that NaF-treated erythrocytes may represent a suitable model system for further analysis of PKD-malaria interactions.

The erythrocyte is highly dependent on glucose metabolism for ATP production. Glucose consumption increases by up to 100-fold after parasite invasion [43]. Therefore, it is intriguing that, although parasite invasion is inhibited in PKD and NaF-treated erythrocytes, the parasite is able to mature and replicate once it enters the erythrocyte. Of note, it has been reported that \textit{P. falciparum} parasites can express PfPyrK during infection, allowing parasites to utilize erythrocyte glucose to produce ATP [28]. RT-qPCR analysis of PfPyrK expression in parasitized erythrocytes revealed increased levels of PfPyrK mRNA in parasitized NaF-treated erythrocytes, compared with parasitized normal, thalassemic, and G6PDd parasitized erythrocytes. In contrast to PKD erythrocytes, G6PDd parasitized erythrocytes did not have increased ATP levels, and PfPyrK mRNA levels were not increased. Thalassemic erythrocytes also contain reduced ATP levels that have been attributed to decreased PRPP (5-phosphoribosyl-1-pyrophosphate) synthetase activity and lower intracellular adenosine nucleotide content [34]. We observed decreased levels of ATP in both nonparasitized and parasitized thalassemic erythrocytes, as well as significantly lower expression of PfPyrK, compared with parasitized normal erythrocytes.

Collectively, these findings suggest that a parallel increase in ATP production by the parasite compensates for the decreased ATP levels in PKD erythrocytes, thereby permitting intraerythrocytic maturation. This hypothesis is supported by the observed ATP distribution in the compartmental analysis of parasitized PKD and NaF-treated erythrocytes (Figure 3A) and by the demonstration of increased expression of \textit{P. falciparum} pyruvate kinase in NaF-treated erythrocytes (Figure 3D). We also observed almost equal concentrations of ATP in the host and parasite compartments of parasitized normal and PKD erythrocytes, suggesting that ATP produced by the parasite can cross the parasitophorous vacuole membrane and contribute to ATP levels of the host cell compartment; this contention is supported by other studies [27, 33]. In contrast to PKD erythrocytes that have impaired glycolysis, parasitized normal, G6PDd, and thalassemic erythrocytes have normal pyruvate kinase activity and therefore do not appear to require overexpression of parasite-derived pyruvate kinase to compensate for this enzyme (Figure 3B).

Taken together, the available data support a mechanism of PKD-associated resistance to infection, as depicted in Figure 4. In this model, reduced pyruvate kinase activity causes decreased ATP production and increased production of 2,3-DPG. ATP is required for maintaining membrane integrity via turnover of phosphoryl groups of erythrocyte membrane–associated proteins [44, 45]. Moreover, high levels of 2,3-DPG may influence several metabolic pathways via inhibition of hexokinase, PRPP synthetase (which is important for nicotinamide adenine dinucleotide [NAD]) [46], and G6PD essential for the production of nicotinamide adenine dinucleotide phosphate and for maintaining glutathione in the reduced state. As a consequence, excessive amounts of free radicals may be generated that transform oxyhemoglobin to methemoglobin and, ultimately, to hemichromes, contributing to mechanical destabilization of the PKD erythrocyte membrane [47]. Collectively, the low levels of ATP, together with increased production of 2,3-DPG and reactive oxygen species, may disrupt the cell membrane–cytoskeletal protein network—notably, the spectrin–actin–band 4.1 complex, with consequent band 3 aggregation and impairment of parasite invasion [48, 49]. This model is supported by studies that have (1) implicated band 3 in parasite invasion [48], (2) associated decreased intracellular ATP levels in hereditary ovalocytosis with impaired \textit{P. falciparum} invasion [50], and (3) demonstrated enhanced phagocytosis of ring-stage–parasitized erythrocytes via binding of immunoglobulin G and C3c to damaged erythrocyte membranes [7, 15, 51].

In summary, PKD erythrocytes display protection against \textit{P. falciparum} infection in vitro. Low ATP levels were associated with reduced parasite invasion and enhanced phagocytosis of early-stage–parasitized erythrocytes that may contribute to protection in vivo by reducing overall parasite burden and sequestration in the endothelial beds of vital organs. However, testing this hypothesis will require case-control studies to define the association between \textit{PKLR} polymorphisms and malaria outcome. PKD is a complex disorder, and it will be important for future studies examining the association between PKD and malarial endemicity to consider the erythrocyte phenotype (including intracellular ATP levels) in addition to the genotype,
because not all individuals with PKD display reduced erythrocyte ATP levels [37].

Acknowledgments

We thank Karlee Silver, Kathleen Zhong, and Feng Wang for technical assistance. We also thank Lena Serghides for reviewing the manuscript and Andrea Conroy and Michael Hawkes for statistical analysis.

References