Effect of Anandamide in *Plasmodium Berghei*-infected Mice

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Key Words
Malaria • Cell volume • Phosphatidylserine • Red blood cells • Eryptosis • Cell death

Abstract
Eryptosis, the suicidal death of erythrocytes, is characterized by exposure of phosphatidylserine at the erythrocyte surface and cell shrinkage. Triggers of eryptosis include anandamide. Enhanced eryptosis of infected human erythrocytes is expected to delay the development of parasitaemia during infection with *Plasmodium*, the parasite causing malaria. The present experiments aimed to test, whether anandamide influences eryptosis, parasite growth and/or host survival during *in vitro or in vivo* infection with *Plasmodia*. Human erythrocytes were infected *in vitro* with *P. falciparum*, and mice *in vivo* with *P. berghei*. Parasitemia was determined with Syto16. Phosphatidylserine-exposing erythrocytes were identified by analysing annexin V-binding in FACS analysis. *In vitro* infection of human erythrocytes was followed by a significant increase in annexin V-binding, an effect slightly enhanced by anandamide (≥50 µM), which significantly reduced intraerythrocytic DNA/RNA content and *in vitro* parasitaemia. *In vivo* administration of anandamide (5 mg/kg b.w. subcutaneously) blunted the parasitaemia (from 36.9% to 24.2% of circulating erythrocytes 21 days after infection) and significantly enhanced the survival of *P. berghei*-infected mice (from 0% to 67% 26 days after infection). The percentage of phosphatidylserine-exposing erythrocytes was significantly increased in anandamide-treated infected mice compared to non-treated infected mice. In conclusion, anandamide stimulated eryptosis of infected erythrocytes thus counteracting parasitaemia and a lethal course of the disease.

Introduction
Suicidal death of erythrocytes or eryptosis is characterized by cell shrinkage and cell membrane scrambling leading to exposure of phosphatidylserine at the cell surface [1-6]. The cell membrane scrambling is stimulated by increased cytosolic Ca2+ activity [1, 3, 4, 7] and ceramide [8]. Ca2+ enters erythrocytes via Ca2+-perme-
able cation channels, which have been shown to be activated by osmotic shock, oxidative stress and energy depletion [7, 9-12]. In addition, Ca²⁺ activates Ca²⁺-sensitive K⁺ channels [13, 14] leading to KCl exit followed by osmotically obliged water [15]. Thus, Ca²⁺ stimulates suicidal erythrocyte death not only in nucleated cells [16], but as well in human erythrocytes [17].

The phosphatidylserine-exposing erythrocytes are recognized by receptors specific for phosphatidylserine on macrophages [18, 19], which engulf and degrade phosphatidylserine-exposing cells [20, 21]. Thus, apoptotic erythrocytes are rapidly cleared from circulating blood.

Eryptosis is stimulated by infection with *Plasmodium falciparum* [22, 23]. *Plasmodia* induce oxidative stress to host erythrocytes leading to activation of the Ca²⁺-permeable cation channels resulting in Ca²⁺ entry [24] and subsequent cell membrane scrambling [25].

Rapid clearance of infected erythrocytes during malaria could prevent intraerythrocytic development of trophozoites with the ability to intoxicate macrophages [26, 27]. Thus, at least in theory, accelerated eryptosis could protect against a severe course of malaria [28]. Along those lines, sickle-cell-trait, beta-thalassemia-trait, homozygous Hb-C and G6PD-deficiency lead to premature suicidal erythrocyte death upon infection with *Plasmodium* thus leading to accelerated clearance of ring stage-infected erythrocytes [6, 29-33].

Eryptosis could be stimulated by anandamide [34], a cannabinoid receptor agonist with pleotropic actions [35-37]. The present study explored whether anandamide augments phosphatidylserine exposure of *Plasmodium falciparum*-infected human erythrocytes and influences parasitaemia and survival following murine infection with *Plasmodium berghei*.

**Materials and Methods**

*Animals, cells and solutions*

Animal experiments were performed according to the German animal protection law and approved by the local authorities. Healthy SV129/J wild type mice (aged 4 months, both male and female) were used for the experiments. From the 8th day after infection murine erythrocytes were drawn from animals by incision of the tail vein. Human erythrocytes were osmotically obliged water [15]. Thus, Ca²⁺ stimulates suicidal erythrocyte death not only in nucleated cells [16], but as well in human erythrocytes [17].

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Experiments were performed at 37°C in Ringer solution containing (in mM) 125 NaCl, 5 KCl, 1 MgSO₄, 32 N-2-hydroxyethylpiperazine-N-2-ethanesulfonic acid (HEPES)/NaOH (pH 7.4), 5 glucose, 1 CaCl₂. Anandamide was added to the NaCl Ringer at final concentrations varying from 1 µM to 100 µM (Sigma, Schnelldorf, Germany).

**Determination of phosphatidylserine exposure**

FACS analysis was performed as described [7, 38]. After incubation in the presence or absence of anandamide, suspensions of noninfected erythrocytes were stained with annexin V-FLUOS (Roche, Mannheim, Germany), suspensions of *Plasmodium falciparum*-infected erythrocytes were stained with annexin V-APC (BD Biosciences Pharmingen, Heidelberg, Germany) and/or with the DNA/RNA-specific dye Syto16 (Molecular Probes, Göttingen, Germany) to identify phosphatidylserine-exposing and infected erythrocytes, respectively. For annexin V-binding, erythrocytes were washed, resuspended in annexin V-binding buffer (Ringer solution containing 5 mM CaCl₂, pH 7.4; the additional Ca²⁺ is required for binding of annexin V), stained with annexin V-APC (dilution 1:20) or annexin V-FLUOS (dilution 1:100), incubated for 20 min at room temperature, and diluted 1:5 with annexin V-binding buffer. Syto16 (final concentration of 20 nM) was added directly to the diluted erythrocyte suspension or co-incubated in the annexin V-binding buffer. Cells were analyzed by flow cytometry (FACS-Calibur, Becton Dickinson, Heidelberg, Germany) in FL-1 for Syto16 or annexin V-FLUOS fluorescence intensity (detected at 530 nm) and in FL-4 for annexin V-APC fluorescence intensity (detected at 660 nm).

**In vitro culture of Plasmodium falciparum**

For infection of human erythrocytes the human, but not murine pathogenic *Plasmodium falciparum* strain BinH [39] was grown in human erythrocytes in vitro [40, 41]. Parasites were cultured as described earlier [42, 43] at a haematocrit of 2% and a parasitaemia of 2-10% in RPMI 1640 medium supplemented with Albumax II (0.5%; Gibco, Karlsruhe, Germany) in an atmosphere of 90% N₂, 5% CO₂, 5% O₂.

**In vivo proliferation of Plasmodium berghei**

For infection of mice, the murine, but not human pathogenic *Plasmodium berghei* strain ANKA-parasitized murine erythrocytes (1x10⁷) were injected intraperitoneally [44] into wildtype mice. Where indicated, anandamide (5 mg/kg b.w.) was administered subcutaneously from the eighth day of infection. Blood was collected from the mice from the day 8 after infection by incision of the tail. Parasitaemia was determined by Syto16 staining in FACS analysis.

**In vitro growth assays of Plasmodium falciparum-infected human erythrocytes**

The *Plasmodium falciparum* BinH strain was cultured and synchronized to the ring stage by sorbitol treatment as described previously [24]. For the *in vitro* growth assay, synchronized parasitized erythrocytes were aliquoted in 96-well plates (200 µl aliquots, 1% haematocrit, 0.5 - 2% parasitaemia) and grown for 48 h in the presence or absence of anandamide (1 µM - 100 µM). The parasitaemia was assessed 0 h and 48 h later by flow cytometry. Parasitaemia was defined by the percentage of erythrocytes stained with the DNA/RNA specific fluorescent dye Syto16.

To estimate DNA/RNA amplification, the culture was ring stage-synchronized, and re-synchronized after 6 h of culture (to narrow the developmental parasite stage), aliquoted (200 µl
aliquots, 2% haematocrit and 10% parasitaemia) and cultured for further 16 h in the presence or absence of anandamide (1 µM - 100 µM). Thereafter, the DNA/RNA amount of the parasitized erythrocytes was determined by Syto16 fluorescence as a measure of intraerythrocytic parasite copies.

**Statistics**

Data are expressed as arithmetic means ± SEM, and statistical analysis was made by t-test or ANOVA using Tukey’s test as post hoc test, as appropriate. p<0.05 was considered as statistically significant.

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Results

The percentage of phosphatidylserine-exposing erythrocytes was determined from annexin V-binding in FACS analysis. Infection with *Plasmodium falciparum* significantly enhanced the percentage of annexin V-binding erythrocytes retrieved from mice infected with *Plasmodium berghei* on day 10 after infection and untreated (left bars) or treated with (right bars) anandamide. *** indicates significant difference (p<0.001) from non-infected erythrocytes, ### indicates significant difference (p<0.001) from control.

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**Fig. 3.** Parasitaemia and survival of *Plasmodium berghei*-infected mice. A: Original graphs of parasitaemia-dependent Syto16 fluorescence in untreated animals (upper panels) and animals treated from day 8 until day 21 with 5 mg/kg b.w. of anandamide s.c. (lower panels) 10 (left panels) and 20 (right panels) days after infection with *Plasmodium berghei*. B: Arithmetic means ± SEM of parasitemia in mice without treatment (open circles, n=10) or with 5 mg/kg b.w. of anandamide s.c. (closed circles, n=6) as a function of days after infection with *Plasmodium berghei*. *, **, *** indicate significant difference (p<0.05, p<0.01, p<0.001) from the untreated animals. The mice were infected by injection of 1x10⁶ *Plasmodium berghei* ANKA-parasitized murine erythrocytes intraperitoneally. C: Survival of mice without treatment (open circles) or with 5 mg/kg b.w. of anandamide s.c. (closed circles) as a function of days after infection with *Plasmodium berghei*.

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**Fig. 4.** In vivo effect of anandamide on phosphatidylserine exposure of infected and noninfected erythrocytes. Arithmetic means ± SEM (n = 6-10) of the percentage of annexin V-binding in infected (open bars) and non-infected (closed bars) erythrocytes retrieved from mice infected with *Plasmodium berghei* on day 10 after infection and untreated (left bars) or treated with (right bars) anandamide. *** indicates significant difference (p<0.001) from non-infected erythrocytes, ### indicates significant difference (p<0.001) from control.
increased gradually (Fig. 3B). The percentage of parasitized erythrocytes amounted to 36.9 ± 4.0% in animals without anandamide treatment on day 21 after infection. In anandamide-treated animals the parasitaemia increased only to 24.2 ± 1.9% 21 days after infection (Fig. 3B).

Anandamide treatment further increased the survival of *Plasmodium berghei*-infected mice. All nontreated animals died within 26 days after the infection, but 67% of the anandamide-treated animals survived the infection for 26 days (Fig. 3C).

Additional experiments were performed to determine whether stimulation of eryptosis accounts for the milder course of malaria in the anandamide-treated mice. As a result, on day 8 after infection, infected erythrocytes exposed significantly more phosphatidylserine than non-infected erythrocytes. More importantly, treatment of the mice with anandamide significantly increased the phosphatidylserine exposure of infected erythrocytes but did not appreciably modify phosphatidylserine exposure of non-infected erythrocytes (Fig. 4).

**Discussion**

The present observations reveal a novel therapeutic effect of anandamide. During infection with *Plasmodium berghei* anandamide blunted the increase in parasitaemia and, more importantly, substantially enhanced the survival of infected mice. Without treatment, infection of the mice with *Plasmodium berghei* was followed by an invariably lethal course [44]. In contrast, most of the anandamide-treated mice survived the first 26 days of *Plasmodium berghei* infection.

The effect of anandamide could be either due to a direct toxic effect of the drug on the pathogen or due to accelerated host erythrocyte death leading to premature removal of the pathogen within its host cell. A toxic action on the pathogen is reflected by a decrease of the intraerythrocytic DNA/RNA content. The scrambling effect of anandamide may further decrease the life span of infected erythrocytes in circulating blood. Anandamide is known to trigger eryptosis [45]. It stimulates scrambling of the cell membrane and thus fosters the phagocytosis of the affected erythrocyte [20, 21]. Phosphatidylserine exposure is a wellknown hallmark of eryptosis [17].

The effect of anandamide on eryptosis of human erythrocytes was only mild. Possibly the substance was bound to protein and thus, the free concentration lower than in previous experiments, when the erythrocytes were incubated in protein free Ringer solution. Moreover, the oxidative stress induced by *Plasmodium falciparum* may have partially destroyed the substance. The effect on eryptosis was more pronounced in murine erythrocytes. The possibility must be considered that anandamide has distinct actions in between human and mouse erythrocytes. Accordingly, enhanced eryptosis may play a role in the host defence against *Plasmodia* in murine but less so in human erythrocytes.

A variety of substances and conditions has been shown to stimulate phosphatidylserine scrambling of erythrocytes [46-62] including chlorpromazine [63], lead [64] or azathioprine [65]. Moreover, eryptosis contributes to or even accounts for several anemic conditions, such as sickle-cell anemia [66, 67], beta-thalassemia [6], glucose-6-phosphate dehydrogenase (G6PD)-deficiency [6], phosphate depletion [68], iron deficiency [69], Hemolytic Uremic Syndrome [70], sepsis [71], malaria [28, 72-75] and Wilson’s disease [51]. Several of those substances or diseases could confer some protection against a severe course of malaria. As a matter of fact, iron deficiency [22] and treatment with chlorpromazine [49], lead [23] or azathioprine [76] have recently been shown to accelerate eryptosis of infected erythrocytes and to be protective against a severe course of malaria.

In conclusion, anandamide accelerates eryptosis of infected erythrocytes. Stimulation of eryptosis of infected erythrocytes blunts the parasitaemia and leads to a favourable course of the disease.

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