Influence of NO Synthase Inhibitor L-NAME on Parasitemia and Survival of *Plasmodium berghei* Infected Mice

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Key Words

Eryptosis • Phosphatidylserine • Parasitemia • Malaria • Nitric oxide

circulating erythrocytes and thus delays development of parasitemia during malaria.

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Abstract

Accelerated suicidal death or eryptosis of infected erythrocytes may delay development of parasitemia in malaria. Eryptosis is inhibited by nitric oxide (NO). The present study has been performed to explore, whether inhibition of NO synthase by L-NAME modifies the course of malaria. We show here that L-NAME ($\geq 10 \mu\text{M}$) increased phosphatidylserine exposure of *Plasmodium falciparum* infected human erythrocytes, an effect significantly more marked than in noninfected human erythrocytes. We further show that parasitemia in *Plasmodium berghei* infected mice was significantly decreased (from 50% to 18% of circulating erythrocytes 20 days after infection) by addition of 1 mg/ml L-NAME to the drinking water. According to CFSE labelling L-NAME treatment accelerated the clearance of both, noninfected and infected, erythrocytes from circulating blood, but did not significantly extend the life span of infected animals. In conclusion, treatment with L-NAME shortens the life span of

Introduction

Erythrocyte injury, such as osmotic shock, oxidative stress and energy depletion activates Ca^{2+} permeable cation channels [1-4] with subsequent entry of Ca^{2+} [4], Ca^{2+} -sensitive scrambling of the cell membrane [5, 6] and phosphatidylserine exposure at the cell surface [7-12]. As macrophages are equipped with receptors specific for phosphatidylserine [13], erythrocytes exposing phosphatidylserine at their surface are thus expected to be recognised by the macrophages, engulfed and degraded [14, 15]. Thus, phosphatidylserine exposure or eryptosis [16] represents a suicidal death of erythrocytes with similarities to erythrocyte senescence [16-19] and neocytolysis [20]. Eryptosis is enhanced in several diseases such as iron deficiency [21], phosphate depletion [22], hemolytic uremic syndrome [23], sepsis [24], malaria [25], Wilson's disease [26], sickle cell disease [27-29], thalassemia [28], and glucose-phosphate dehydrogenase deficiency [28].

Moreover, eryptosis is stimulated by methylglyoxal [30], amyloid [31], listeriolysin [32], Bay-5884 [33], curcumin [34], paclitaxel [35], chlorpromazine [36], cyclosporine [37], valinomycin [38], lead [39], mercury [40] and copper [26].

Similar to apoptosis of nucleated cells [14, 15, 41-43], eryptosis is subject to regulation by nitric oxide (NO) [44]. *In vitro* experiments disclosed that NO is a potent inhibitor of eryptosis [44]. *In vivo* experiments indicated that inhibition of NO synthase accelerates the clearance of circulating erythrocytes, i.e. endogeneous formation of NO is required for normal erythrocyte life span [44].

Accelerated eryptosis may confer some protection against a severe course of malaria [10]. Accelerated aging or eryptosis of *Plasmodium*-infected erythrocytes with sickle-cell trait, beta-thalassemia-trait, homozygous Hb-C or glucose-6-phosphate dehydrogenase (G6PD)-deficiency is followed by premature clearance of infected erythrocytes thus blunting the increase of parasitemia [45].

The present study explored whether inhibition of NO synthase would indeed influence the course of malaria in mice infected with the malaria pathogen *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 (registration number PY 2/06). Healthy SV129/J wild type mice (aged 4 months, both male and female) were used for the experiments. Mouse erythrocytes were drawn from animals by retroorbital venipuncture or by incision of the tail vein. Human erythrocytes were drawn from healthy volunteers. 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, N (G)-nitro-L-arginine methyl ester (L-NAME) was added to the Ringer solution at final concentrations varying from 0.1 µM to 100 µM concentrations (Sigma, Taufkirchen, Germany). For incubation with L-NAME the final hematocrit was adjusted to 0.3 %.

For the *in vivo* experiments L-NAME was added to the drinking water (1mg/ml). The oral route of drug administration was chosen, as this route confers less stress to the animals than IP administration. L-NAME is easily dissolved and absorbed by oral administration, palatable and hence there is no problem of bioavailability upon oral administration.

FACS analysis

FACS analysis was performed as described [11]. After incubation in the presence or absence of L-NAME, suspen-

sions from noninfected erythrocytes were stained with annexinV-FLUOS (Roche, Mannheim, Germany), suspensions of *Plasmodium falciparum* infected erythrocytes were stained with annexin V-568 (Roche, Germany) and/or with the DNA/RNA specific dye Syto16 (Molecular Probes) to assess phosphatidylserine exposing and infected erythrocytes, respectively. Annexin V-568 was used for staining infected erythrocytes because it can be detected in FL-2 (emission wavelength 585nm), which facilitates double staining with Syto 16 which is detected in FL-1 (emission wavelength 530nm). For annexin binding, erythrocytes were washed, resuspended in annexin-binding buffer (140 mM NaCl, 10 mM HEPES, 5 mM glucose, 5 mM CaCl₂, pH 7.4), stained with annexin V-568 (dilution 1:50) or annexin V-FLUOS (dilution 1:100), incubated for 20 min at room temperature, and diluted 1:5 with annexin binding buffer. Syto16 (final concentration of 20 nM) was either directly added to the diluted erythrocyte suspension (and incubated for 30 min at 37°C) or co-incubated in the annexin binding buffer. Cells were analyzed by flow cytometry (FACS-Calibur from Becton Dickinson) using FL-1 for Syto16 or annexin V-FLUOS fluorescence intensity and with FL-2 for annexin V-568 fluorescence intensity.

Infection

For infection of human erythrocytes the human pathogen *P. falciparum* strain BinH [46] was grown *in vitro* [47] in banked human erythrocytes (blood group O+). Parasites were cultured as described earlier [48, 49] at a hematocrit of 2 % and a parasitemia 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.

For infection of mice *P. berghei* ANKA-parasitized mouse erythrocytes (1x10⁶) were injected intraperitoneally [50] into mice with free access to L-NAME- free or L-NAME (1 mg/ml)-containing drinking water from the 10th day of infection. Parasitemia was determined daily by Syto-16 staining in FACS analysis

In vitro growth assays of P. falciparum infecting human erythrocytes

The *Plasmodium falciparum* BinH strain was cultured and synchronized to the ring stage by sorbitol treatment as described previously [2]. For the *in vitro* growth assay, synchronized parasitized erythrocytes were aliquoted in 96-well plates (200 µl aliquots, 1% hematocrit, 0.2 – 0.5 % parasitemia) and grown for 48 h in the presence or absence of L-NAME (0.1 µM - 100 µM L-NAME). The parasitemia was assessed at time 0 and after 48 h. Parasitemia was defined by the percentage of erythrocytes stained with the DNA/RNA specific fluorescence dye Syto16. To estimate the DNA/RNA amplification, the culture was ring stage-synchronized, and re-synchronized after 6 hours of culture (to narrow the developmental parasite stage), aliquoted (200 µl aliquots, 2% hematocrit and 10% parasitemia) and cultured for further 16 h in the presence or absence of L-NAME (0.1 µM – 100 µM L-NAME). Thereafter, the DNA/RNA amount of the parasitized erythrocytes was determined by Syto16 fluorescence as a measure of intraerythrocytic parasite copies.

Fig. 1. Effects of NO-synthase inhibitor L-NAME on phosphatidylserine exposure of infected and noninfected erythrocytes and on intraerythrocytic parasite growth: A. Dot blots of annexin binding and Syto16 fluorescence of human erythrocytes in noninfected cells (upper panels) and in cells infected with *Plasmodium falciparum* (lower panels) following a 48 hours incubation in the absence (left panels), or presence (right panels) of 10 μ M L-NAME. B. Arithmetic means $\pm$ SEM (n = 6) of annexin binding of infected (closed symbols) and noninfected (open symbols) erythrocytes as a function of L-NAME concentration. * indicates significant difference (p<0.05) from control, # indicates significant difference to noninfected erythrocytes. C. Intraerythrocytic DNA amplification in erythrocytes as a function of L-NAME concentration (arithmetic means $\pm$ SEM, n = 8). D. *In vitro* growth (48 h) of *Plasmodium falciparum* in human erythrocytes as a function of L-NAME concentration (arithmetic means $\pm$ SEM, n = 8). The parasitemia at time 0 was 0.2%.

Measurement of *in vivo* clearance of fluorescence-labelled erythrocytes

Fluorescence-labelled erythrocytes were obtained by staining erythrocytes with carboxyfluorescein diacetate, succinimidyl ester (CFSE) from Molecular Probes (Leiden, The Netherlands). The labelling solution was prepared by addition of adequate amounts of a CFSE stock solution (10 mM in DMSO) to phosphate-buffered saline (PBS) to yield a final concentration of 5 μ M. Then, the cells were incubated with labelling solution for 30 min at 37°C. The cells were pelleted at 2000 rpm for 5 min, washed and resuspended in fresh, prewarmed PBS. The fluorescence-labelled erythrocytes were injected into the tail veins of healthy wild type SV 129 mice. At indicated time points, blood was taken from the mice and CFSE dependent fluorescence intensity of the erythrocytes was measured in the fluorescence channel FL-1. The percentage of CFSE-positive erythrocytes was calculated in % of the whole cell population.

Determination of reticulocyte numbers

For determination of relative reticulocyte numbers, 5 μ l of whole blood were added to 1 ml Retic-COUNT (Thiazole orange) reagent from Becton Dickinson. Samples were stained for 30 min at room temperature in the dark. Then, FACS analysis of stained samples was performed according to the manufacturer's instructions. Forward scatter (FSC), side scatter (SSC), and thiazole orange-fluorescence intensity (in the fluorescence channel FL-1) of the blood cells were measured on a FACS-Calibur from Becton Dickinson. Finally, the numbers of Retic-COUNT positive reticulocytes were determined and relative reticulocyte numbers were expressed as percentages of the total, gated erythrocyte populations. Gating of erythrocytes was achieved by analysis of FSC vs. SSC dot plots using the CellQuestTM software.

Analysis of blood cell numbers

Erythrocyte density, packed cell volume, mean erythrocyte volume, white blood cell counts, and hemoglobin content

were determined using an electronic hematology particle counter (type MDM 905) from Medical Diagnostics Marx (Butzbach, Germany), equipped with a photometric unit for hemoglobin determinations.

Measurement of systolic blood pressure

Systolic arterial blood pressure was determined by the tail-cuff method (model 179; IITC Life Sciences, Woodland Hills, CA) while the animals were fed the control diet and after 4 days of L-NAME (1 mg/ml) in drinking water. As reviewed recently, [51], the tail-cuff arterial blood pressure determination requires certain precautions to reduce the stress of the animals, including appropriate training of the mice over multiple days and adequate prewarming to dilate the tail artery. The animals were placed in a heating chamber at an ambient temperature of 30°C for 15 min, and for each animal 10–20 blood pressure traces were recorded in one session. The readings from 3 days were then averaged to obtain a mean blood pressure for the control mice. All recordings and data analysis were done using a computerized data acquisition system and software (PowerLab 400 and Chart 4; ADInstruments). All measurements were done by one individual between 2 and 4 PM.

Statistics

Data are expressed as arithmetic means $\pm$ SEM and statistical analysis was made by two-tailed unpaired t-test, chi square test or ANOVA using Turkey's test as post hoc test, where appropriate, p<0.05 was considered as statistically significant.

Results

To elucidate, whether inhibition of NO synthase or infection with *Plasmodium falciparum* modifies the percentage of cells exposing phosphatidylserine at the cell surface, we determined annexin binding in FACS analysis. The percentage of annexin-positive erythrocytes in

Fig. 2. Effects of L-NAME on forward scatter of infected and noninfected erythrocytes: A: Dot blots of forward scatter and syto16 fluorescence of human erythrocytes in noninfected cells (upper panels) and in cells infected with *Plasmodium falciparum* (lower panels) following a 48 hours incubation in the absence (left panels), or presence (right panels) of 10 μM L-NAME. B: Arithmetic means $\pm$ SEM (n = 6) of forward scatter of infected (closed symbols) and noninfected (open symbols) erythrocytes before and after treatment with L-NAME, as a function of L-NAME concentration (arithmetic means $\pm$ SEM, n = 6). * indicates significant difference (p<0.05) from control, # indicates significant difference from infected erythrocytes.

freshly drawn human blood was low (1.7 ± 0.2 %, n = 8). A 24 h incubation in Ringer solution increased the percentage of annexin binding cells to 4.5 ± 0.2 % (n = 8), a 48 h incubation in Ringer solution increased the percentage of annexin binding noninfected erythrocytes to 17 ± 1 % (n = 6). As illustrated in Fig. 1, infection significantly enhanced the percentage of annexin binding erythrocytes. Moreover, the presence of L-NAME significantly increased the percentage of annexin binding erythrocytes (Fig. 1B). L-NAME did not significantly modify the DNA/

Fig. 3. Clearance of erythrocytes from circulating blood of mice: Disappearance of CFSE-labelled erythrocytes from circulating blood following injection into the tail vein of untreated and L-NAME treated mice (1mg/ml in drinking water). A: Arithmetic means $\pm$ SEM (n = 8) of the percentage of CFSE-labelled noninfected erythrocytes in circulating blood of control mice (open symbols) and L-NAME treated mice (closed symbols). * indicates significant difference (p<0.05) from control mice. B: Abundance of CFSE-labelled noninfected erythrocytes in spleen of untreated (open bar) and L-NAME treated (closed bar) mice. Arithmetic means $\pm$ SEM (n = 8). * indicates significant difference (p<0.05) from control mice. C: Arithmetic means $\pm$ SEM (n = 6) of the percentage of CFSE-labelled infected erythrocytes in control mice (open symbols) and L-NAME treated mice (closed symbols). * indicates significant difference (p<0.05) from control mice. D: Abundance of CFSE-labelled infected erythrocytes in spleen of untreated (open bar) and L-NAME treated (closed bar) mice. Arithmetic means $\pm$ SEM (n = 6). * indicates significant difference (p<0.05) from control mice.

RNA content or the *in vitro* percentage of infected erythrocytes. Thus, L-NAME did apparently not influence the intraerythrocytic growth of the pathogen and the *in vitro* infection of erythrocytes.

The effect of L-NAME on phosphatidylserine exposure was paralleled by a decrease of forward scatter of both infected and noninfected erythrocytes (Fig. 2). In those experiments, there was no significant change of parasitemia following a 48 h treatment with 10 μM of L-NAME.

Fig. 4. Parasitemia and survival of *Plasmodium berghei* infected mice. A: Original histograms of parasitemia in untreated animals (upper panels) and animals treated from day 10 until day 20 with L-NAME (1mg/ml in drinking water, lower panels) 10 (left panels) and 20 (right panels) days after infection with *Plasmodium berghei*. B: Arithmetic means $\pm$ SEM (n = 15-21) of parasitemia in mice without treatment (open circles) or treated with L-NAME (closed circles) as a function of days after infection with *Plasmodium berghei*. C: Survival of mice without treatment (open circles) or with L-NAME treatment (closed circles) as a function of days after infection with *Plasmodium berghei*.

	control	10 days	20 days	30 days
Erythrocytes	11.12 $\pm$ 0.55	10.09 $\pm$ 0.28	8.51 $\pm$ 0.15*	8.98 $\pm$ 0.3*
Hematocrit	55.22 $\pm$ 2.33	50.6 $\pm$ 1.63	39.1 $\pm$ 0.94*	39.85 $\pm$ 1.35*
MCV	49.12 $\pm$ 0.53	46.2 $\pm$ 0.89*	45.93 $\pm$ 0.36*	44.33 $\pm$ 0.27*
RTC	1.31 $\pm$ 0.20	1.32 $\pm$ 0.13	2.21 $\pm$ 0.31*	5.58 $\pm$ 0.65*
HBG	22.55 $\pm$ 1.69	20.75 $\pm$ 0.53	20.33 $\pm$ 0.29	21.68 $\pm$ 0.55
WBC	11.27 $\pm$ 1.64	11.15 $\pm$ 0.55	12.55 $\pm$ 0.8	11.88 $\pm$ 1.4

Table 1. Erythrocyte number (10⁶/μl), packed cell volume (hematocrit, %), mean corpuscular volume (fl), reticulocyte count (RTC, %), hemoglobin (HBG, g/100 ml) and white blood cell count (WBC 1000/μl) in mice prior to (control) and following a 10, 20 or 30 day treatment with 100 μM L-NAME (all values arithmetic means $\pm$ SEM, n = 6). Erythrocyte and leukocyte counts were determined using MDM 905 (Medical Diagnostics Marx, Butzbach, Germany). Data are provided for untreated (control) and L-NAME treated mice. * indicates significant difference from the control animals.

Further experiments were performed to explore whether the enhanced phosphatidylserine exposure leads to enhanced clearance of erythrocytes from circulating blood *in vivo*. To this end erythrocytes have been labelled with the dye CFSE and were injected into the tail vein of untreated and L-NAME treated mice. As illustrated in Fig. 3A, clearance of erythrocytes was significantly faster in L-NAME treated mice than in untreated animals. Conversely, the number of labelled erythrocytes recovered in the spleen was significantly enhanced by L-NAME treatment (Fig. 3B).

As illustrated in Fig. 3C, only a small portion of labelled infected erythrocytes were cleared within 120 minutes in untreated mice, but a significantly larger percentage of labelled infected erythrocytes disappeared within

120 minutes after injection in L-NAME treated mice. Conversely, the number of labelled erythrocytes recovered in the spleen was significantly enhanced by L-NAME treatment (Fig. 3D).

Further experiments were performed to determine whether the effect of L-NAME on phosphatidylserine exposure and survival of circulating erythrocytes would affect the course of malaria. To this end, wild type mice were infected with *Plasmodium berghei* and the parasitemia monitored daily. As illustrated in Fig. 4A-B, parasitized erythrocytes appeared in the circulating blood approximately 8 days after infection. In untreated animals, the percentage of parasitized erythrocytes increased gradually up to 53% within the following two weeks. The addition of L-NAME (1 mg/ml) to the drinking water sig-

nificantly blunted the increase of parasitemia (up to 21%). Thus, L-NAME treatment indeed significantly modified the increase of parasitemia.

As illustrated in Fig 4C, the favourable influence of L-NAME on parasitemia was, however, not paralleled by significantly enhanced survival of infected animals ($p = 0.43$, chi square test).

To explore the effect of L-NAME treatment on blood parameters, the blood count was analysed in non-infected mice treated with L-NAME (1 mg/ml) in the drinking water. As listed in Table 1, L-NAME treatment was followed by significant decrease of erythrocyte number, haematocrit and mean corpuscular volume after 20 and 30 days of treatment. Apparently, accelerated loss of erythrocytes was compensated by increased formation of erythrocytes which is apparent from the significantly increased percentage of reticulocytes.

Further experiments were performed to test for the hypertensive effect of L-NAME treatment in mice. To this end, wild type mice were treated with L-NAME (1 mg/ml) in drinking water and the blood pressure was measured before and after L-NAME treatment. Systolic blood pressure was significantly ($P < 0.01$) higher after four days of L-NAME treatment (114 ± 3 mmHg, $n = 6$) than in control mice (90 ± 3 mmHg, $n = 6$).

Discussion

As reported previously [44], NO is a potent inhibitor of eryptosis. Thus, inhibition of NO synthase is expected to favour or accelerate eryptosis. Accordingly, the NO synthase inhibitor L-NAME triggers Ca^{2+} sensitive cell membrane scrambling with subsequent exposure of phosphatidylserine at the erythrocyte surface. Macrophages are equipped with receptors specific for phosphatidylserine [13] and thus engulf and degrade erythrocytes exposing phosphatidylserine at their surface. Accordingly, treatment of mice with L-NAME accelerated the clearance of erythrocytes from circulating blood. The majority of labelled cells cleared from circulating blood accumulated in the spleen.

The antieryptotic effect of NO donors was mimicked by di-buteryl-cGMP [44], suggesting the involvement of guanylate cyclase, cGMP and protein kinase G, a well known signalling pathway of NO action [52-54]. cGMP stimulates the Ca^{2+} ATPase [55-58], which contributes to the regulation of erythrocyte Ca^{2+} concentration [59, 60]. However, NO donors did not appreciably decrease cytosolic Ca^{2+} activity [44] and may

thus be effective through other signalling pathways. In nucleated cells the antiapoptotic effect of NO is in part due to inhibition of caspases [41, 61]. However, caspases are apparently not involved in the triggering of eryptosis following increase of cytosolic Ca^{2+} activity [11]. Presumably, NO is effective through nitrosylation [62-64] of enzymes necessary for induction of cell membrane scrambling [44].

Infection with *Plasmodium* similarly triggers eryptosis [25]. The intraerythrocytic pathogen imposes oxidative stress to the host cell [2, 47, 50, 65] leading to cation channel activation [2, 47, 50] and Ca^{2+} entry [2]. If the stimulation of eryptosis remains unabated, it may proceed too fast for the pathogen to allow intraerythrocytic maturation. Thus, inhibition of NO formation provides some protection against an increase of parasitemia.

Despite its favorable influence on the development of parasitemia, L-NAME treatment did not significantly decrease or delay the death rate of *Plasmodium*-infected mice. Chronic administration of N-nitro-L-arginine methyl ester (L-NAME), an artificial inhibitor of NO synthase (NOS), raises blood pressure in animals [66-68]. Chronic administration of L-NAME induces a proinflammatory phenotype in the arterial wall [69]. Recent studies indicate the involvement of the renin-angiotensin system and the sympathetic nervous system in the mechanism to elevate blood pressure in this model [70]. The hypertension, peripheral ischemia and organ damage following impaired NO formation presumably outweighs the advantage of decreasing parasitemia. Thus, due to those severe side effects, inhibition of NO synthase is apparently not a therapeutic option in the treatment of malaria.

In conclusion, L-NAME treatment delays the development of parasitemia. The observations support the view that accelerated suicidal death of infected erythrocytes is a host mechanism counteracting infection with the intracellular pathogen. However, the blunted parasitemia does not provide protection against a lethal course of malaria.

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