On the pathophysiology of severe falciparum malaria with special reference to red cell deformability
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Nitric oxides in plasma, urine and cerebrospinal fluid in patients with severe falciparum malaria


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Nitric oxide in plasma, urine, and cerebrospinal fluid in patients with severe fascioliasis.
Introduction

Severe falciparum malaria remains an important cause of mortality in the tropical world, with an annual global mortality of 1-2 million people and a mortality rate as high as 15% to 30%, despite effective anti-malarial treatment. It has been proposed that nitric oxide (NO) plays a central role in the pathogenesis of severe, and in particular cerebral, malaria. NO is an important mediator of many homeostatic processes and host defence mechanisms and may also have a function as neurotransmitter. The production of NO is increased by pro-inflammatory cytokines such as TNFα and interleukin-1, that reach high levels in severe falciparum. Excessive synthesis of NO by cerebrovascular endothelial cells might alter neurotransmission and thus contribute to the pathogenesis of cerebral malaria. On the other hand NO is thought to play a role in parasite killing. NO also reduces the induction of endothelium bound adhesive molecules such as ICAM-1, which are thought to play an important role in the intracerebral sequestration of parasitized erythrocytes.

Study of NO production in vivo is difficult because of its very short plasma-halftime of less than 10 seconds. In plasma nitric oxide is almost immediately oxidized to nitrite and subsequently converted to nitrate and this seems to be the most important fate of the molecule. As a consequence, all clinical information about the possible role of NO in falciparum malaria derives from the measurements of plasma and urine levels of nitrites and nitrates. These studies have produced sometimes contradictory results. In the present study we measured nitrate and nitrite (NOx) levels in patients with severe falciparum malaria, both in plasma and urine, in order to provide a better estimate of total body NOx production. In addition we measured NOx levels in cerebrospinal fluid (CSF) in patients with cerebral malaria and compared these with NOx values obtained in CSF from controls.

Patients and methods

Consecutive adult patients admitted to Mae Sot Hospital, Tak province, Thailand, with acute falciparum malaria were included in this study, provided that written informed consent was obtained from the patients or their attendant relatives. Malaria transmission is low in this area with a seasonal peak during the rainy season which starts in late spring. Disease severity was classified according to standard criteria. Exclusion criteria were: age below 14 years, pregnancy and previous antimalarial drug treatment within 24 hours of admission. Previous quinine treatment was checked in a baseline blood sample by a rapid quinine dipstick method in all patients. A full clinical examination was performed on admission and all details were recorded on a standard form. Patients were randomly assigned to treatment with either intravenous quinine dihydrochloride or intravenous artesunate in a comparative study, the results of which will be published elsewhere. Full supportive care...
was given as described previously. If necessary patients were transferred to an intensive care unit for mechanical ventilation, peritoneal dialysis, or hemodynamic support and monitoring. This investigation was part of studies approved by the Ethical and Scientific Review Sub-committee of the Ministry of Public Health, Thailand.

**Laboratory methods**

Baseline blood samples were taken for full blood count, glucose, lactate and routine biochemistry. Thick and thin films from peripheral blood were taken on admission and stained with Field's stain for parasite counting. Blood samples for plasma NOx and creatinine measurements were taken 12 hourly. Urine was collected in 12 hourly samples for the time of admission for NOx and creatinine measurements. Chlorhexidine was added to the containers to prevent bacterial growth. In cases of cerebral malaria a lumbar puncture was performed, and routine laboratory measurements were done to exclude other causes of altered consciousness. Five patients undergoing a lumbar puncture for anaesthesia served as a control group for this part of the study. All samples were immediately stored at -20°C for later analysis.

NOx levels were assessed by ion-pair chromatography. In order to precipitate proteins in plasma or CSF, to 100 mL sample 500 mL H₂O, 100 mL 0.35 M ZnSO₄ and 100 mL 0.75 M NaOH was added. Urines were treated in the same way after 1 to 4 dilution in water. The precipitates of protein were removed by 10 min. centrifugation at 1500 g. Standards were processed in the same way as the samples. A volume of 20 mL of the deproteinized sample was injected on a 100 x 3.0 mm Chromsphere c18 column (Chrompack, Bergen op Zoom, The Netherlands). Separation was achieved by ion-pair chromatography with 0.01 mol/L n-octylamine as eluent. The pH of the eluent was brought to 6 with sulphuric acid and then adjusted to pH 6.5 with 2 mmol/L ammonium acetate. Detection was at 215 nm. The flowrate was 0.65 mL/min. The coefficient of variation of duplicate determinations was below 2%. Plasma NOx levels were corrected for kidney function by taking the ratio of NOx concentration and plasma creatinine concentration. Similarly total urinary NOx values were corrected for renal function by taking the ratio of NOx and total urinary creatinine values in the same sample. Fractional NOx excretion (the fraction of filtered NOx that is excreted) was calculated with the formula: total NOx in urine (mmol/12h) / [NOx] in plasma (mmol/L) divided by total creatinine in urine (mmol/12h) / [creatinine] in plasma (mmol/L).

In one patient who died two hours after urine collections were started, the total amount of NOx excretion in the urine in the first twelve hours was obtained by extrapolation of the excretion in the first 2 hours after admission.
Nitric oxides in severe falciparum malaria

Statistical methods

Statistical analyses were carried out using SPSS 6.1 statistical programmes (SPSS Corporation, Benelux). Normally distributed data were analysed using Student’s t-test. The Mann Whitney test was used to compare non-normally distributed variables. Correlations were assessed by the method of Pearson for normally distributed variables, and the method of Spearman for the remainder. Wilcoxon matched pairs rank test was used to compare changes in NOx levels between two time points.

Results

Twenty-five consecutive patients with severe falciparum malaria, eleven of whom had cerebral malaria, were studied. Seven patients died. Of this group one patient who was included initially because of severe anaemia accompanying her malaria, was excluded subsequently when it became apparent that her anaemia resulted from thalassaemia.

Figure 1. Plasma NOx-levels over time corrected for kidney function in patients with severe falciparum malaria in survivors and fatal cases. The 2 patients with elevated plasma [NOx] in the group with fatal cases both suffered from severe kidney insufficiency, with a calculated creatinine clearance of respectively 4.0 ml/min (*) and 8.0 ml/min (**) at admission. Despite correction of plasma [NOx] for kidney function by taking the ratio with plasma creatinine, this measure is still likely to be an overestimation of NOx production since fractional NOx excretion (FeNOx) declines with deteriorating kidney function \(^1\). In these patients FeNOx at admission was respectively 1.3% (*) and 10.5% (**) vs. a mean FeNOx of 15.5% for all patients.
<table>
<thead>
<tr>
<th>Variable</th>
<th>Survivors $^1$ (n=17)</th>
<th>Fatal cases $^1$ (n=7)</th>
<th>Significance of difference (p)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age</td>
<td>26±8</td>
<td>28±14</td>
<td>n.s.</td>
</tr>
<tr>
<td>Pulse rate (per min)</td>
<td>114±11</td>
<td>109±15</td>
<td>n.s.</td>
</tr>
<tr>
<td>Blood pressure (mmHg)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>systolic</td>
<td>107±12</td>
<td>104±14</td>
<td>n.s.</td>
</tr>
<tr>
<td>diastolic</td>
<td>61±13</td>
<td>63±20</td>
<td>n.s.</td>
</tr>
<tr>
<td>Temperature (°C)</td>
<td>38.8±1.1</td>
<td>38.2±1.4</td>
<td></td>
</tr>
<tr>
<td>Median coma score</td>
<td>15</td>
<td>8</td>
<td>p=0.004 $^2$</td>
</tr>
<tr>
<td>Packed cell volume (%)</td>
<td>34±11</td>
<td>31±9</td>
<td>n.s.</td>
</tr>
<tr>
<td>Parasitaemia (%)</td>
<td>6±6</td>
<td>10±11</td>
<td>n.s.</td>
</tr>
<tr>
<td>Plasma Creatinine (µmol/l)</td>
<td>81±18</td>
<td>148±97</td>
<td>p=0.01</td>
</tr>
<tr>
<td>Plasma Lactate (mmol/l)</td>
<td>4.4±2.4</td>
<td>13.1±9.2</td>
<td>p=0.019</td>
</tr>
<tr>
<td>Plasma Glucose (mmol/l)</td>
<td>9.5±6.3</td>
<td>8.8±3.5</td>
<td>n.s.</td>
</tr>
<tr>
<td>Plasma NOx (µmol/l)</td>
<td>33.6</td>
<td>117.4</td>
<td>n.s.</td>
</tr>
<tr>
<td>median (range)</td>
<td>(15.2-151.1)</td>
<td>(20.2-935.0)</td>
<td></td>
</tr>
<tr>
<td>Plasma NOx / creatinine $^2_{\text{plasma}}$ (µmol/l / µmol/l)</td>
<td>0.51</td>
<td>0.63</td>
<td>n.s.</td>
</tr>
<tr>
<td>median (range)</td>
<td>(0.16-1.78)</td>
<td>(0.26-3.08)</td>
<td></td>
</tr>
<tr>
<td>Urine NOx (µmol)</td>
<td>422.9</td>
<td>85.3</td>
<td>p=0.03 $^2$</td>
</tr>
<tr>
<td>in first 12h after admission</td>
<td>(204.3-2311.5)</td>
<td>(22.4-561.8)</td>
<td></td>
</tr>
<tr>
<td>median (range)</td>
<td>(n=14)</td>
<td>(n=7)</td>
<td></td>
</tr>
<tr>
<td>Urine NOx / creatinine $^2_{\text{urine}}$ (µmol / mmol)</td>
<td>82.8</td>
<td>47.1</td>
<td>n.s.</td>
</tr>
<tr>
<td>median (range)</td>
<td>(27.7-360.7)</td>
<td>(38.5-179.3)</td>
<td></td>
</tr>
<tr>
<td>CSF NOx (µmol/l)</td>
<td>4.8</td>
<td>13.8</td>
<td>n.s.</td>
</tr>
<tr>
<td>median (range)</td>
<td>(2.5-7.7)</td>
<td>(1.5-61.4)</td>
<td></td>
</tr>
</tbody>
</table>

Table 1. Admission clinical and laboratory variables in patients with severe malaria.  
$^1$ Values expressed as mean ± standard deviation  
$^2$ Comparisons by Mann-Whitney U test; all other comparisons by Student’s t-test

Complete sequential 12 hour urine-samples were collected in 21 of these patients. Clinical and laboratory findings are summarized in table 1. There was no significant difference between fatal cases and survivors in the NOx levels in plasma or CSF, although values tended to be lower in survivors. This tendency disappeared when plasma nitrate levels were corrected for renal function by dividing plasma NOx by plasma creatinine. The total amount of NOx excreted in the first 12-hours following admission was greater in survivors than in the fatal cases (p=0.03, Wilcoxon rank test). This difference also disappeared when the NOx levels in the urine were corrected for renal function by dividing them by the total creatinine level in the same sample. The mean fractional clearance of NOx in the survivors was 17%, compared to 12% in the fatal cases (n.s.). Admission parasitaemia did not correlate significantly with either plasma, urine or CSF-nitrate levels.

Plasma NOx levels were lower 48 hours after admission (median value 0.63 µM/µM vs.
Nitric oxides in severe falciparum malaria

Figure 2. Urinary NOx-levels over time corrected for kidney function in patient with severe falciparum malaria in survivors and fatal cases. The sharp increase in urinary NOx in one patients in the fatal group (*) could be attributed to a urinary tract infection.

0.38 μM/μM, p=0.007, by Wilcoxon matched pairs rank test), also if the data were not corrected for kidney function (median values 38.4 μmol/l vs. 32.4 μmol/l, p=0.04). The two patients with elevated plasma [NOx] in the group with fatal cases both had renal failure, with an estimated creatinine clearance on admission of <5.0 ml/min and 8.0 ml/min respectively (fig.1). Correction of plasma [NOx] for renal function by taking the ratio with plasma creatinine, may still overestimate NOx production since fractional NOx excretion (FeNOx) declines with deteriorating kidney function. In these patients admission FeNOx values were 1.3% and 10.5% respectively vs. a mean FeNOx of 15.5% for all patients.

Urinary NOx tended to fall following admission (fig. 2). One patient who died showed a sharp increase in urinary NOx, but this was attributed to a coincident urinary tract infection.

There was a significant correlation between nitrate levels in the CSF and the plasma. (Spearman correlation coefficient= 0.59, p=0.045). We did not find a significant correlation between nitrate levels in the CSF and the Glasgow coma scale, nor with coma recovery time or survival. CSF nitrate levels did not differ significantly between healthy controls (n=5) and patients with severe malaria (n=11), with median values of respectively 2.4 μmol/l (range: 0 - 8.9 μmol/l) and 4.8 μmol/l (range 1.5 μmol/l - 61.4 μmol/l).
Discussion

In this study we could not show a correlation between either plasma NOx-levels or the total amount of NOx excreted in the urine, and disease severity or outcome in patients with severe falciparum malaria, after correction of the values for renal function. So overall NO production does not seem to be a predictor of disease outcome or severity in non-immune adult patients. This study group differs from those in several recent studies performed on malarial patients in highly endemic areas. In these latter studies the highest serum NOx levels were found in healthy controls or patients with asymptomatic parasitaemia from which data it was concluded that high NOx levels might be associated with malarial tolerance rather than with disease severity 14,15,21.

Patients with renal failure showed very high plasma NOx levels with only little NOx excreted in the urine. Because NOx excretion is dependent on renal function and severe malaria is associated with renal impairment, severe malaria can be associated with higher levels of plasma NOx. As fractional NOx secretion falls as renal function deteriorates correction of plasma NOx levels by taking the ratio with plasma creatinine may still overestimate NOx production in patients with renal insufficiency 14.

Nutritional status may also confound the interpretation of plasma NOx levels. In our study, bias from exogenous nutritional sources of NOx can also not be excluded, but most patients had probably eaten very little before admission. As gastric or parenteral feeding is never started immediately, patients with severe malaria such as those reported here almost invariably suffer from a period of starvation during the first days after admission. This period of starvation was associated with a decline in plasma NOx levels. As the plasma halftime of nitrates is approximately 8 hours when kidney function is normal, a much sharper drop in NOx levels would be expected if levels on admission were only elevated because of NOx derived from food taken before admission 24,25. It is likely that a decline in endogenous NO production after treatment contributed to the decline in plasma NOx levels.

NOx levels in the CSF were all much lower than blood levels, suggesting that NO production in the central nervous system is much lower than in the blood compartment. There was a clear correlation between plasma and CSF levels of NOx. This can be interpreted either as reflecting free diffusion of nitrates and nitrates across the blood-brain barrier, or that cerebral NO production is a reflection of NO production elsewhere in the body. Moreover, NOx levels in CSF did not correlate with the coma-depth in our patients. This is in accordance with our findings in Ghanaian children 26. These findings do not support the hypothesis that high levels of intra-cerebral NO production impair neurotransmission in the brain in severe falciparum malaria 3,14. This hypothesis was formulated without data on NOx levels in the CSF. CSF levels of NOx, however, might not be a good indicator of the local NO-
concentrations at the cerebral tissue level. For instance in Alzheimer disease, increased activity of iNOS can be measured in post-mortem obtained brain microvessels, without evidence of increased NOx levels in the CSF. Similarly, increased microvascular iNOS in the brain could be increased in cerebral malaria, thus influencing nearby synapses, without increased NOx levels in the CSF, but this hypothesis will be difficult to proof in vivo.

In conclusion there is no correlation between NOx levels in plasma, urine or CSF and disease severity in patients with severe falciparum malaria in this study. Plasma NOx levels are dependant on renal function. High NOx levels in CSF did not correlate with coma depth in cerebral malaria and the CSF-levels were much lower than those found in the blood compartment. These findings do not support a pivotal role for nitrate oxide in the pathogenesis of cerebral malaria. However, local overproduction of NO might not be reflected in total NOx production or CSF levels of NOx. The role of NO in severe falciparum malaria remains therefore to be elucidated.

References
Chapter 8


