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Direct Monitoring of *In Situ* and *In Vitro* Nitric Oxide Release in the Brain during Malaria Infection with *Plasmodium berghei* N/13/1A

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Abstract

Clinical manifestations of cerebral malaria (CM) involve sequestration of cerebral capillaries with red blood cells, brain hemorrhage, mental disturbance and coma. In addition to numerous cytokines involved in the immune response, nitric oxide (NO) has been shown to be produced in malaria. This tiny molecule with a short half-life acts as a signal in cells or kills pathogens. Despite the importance of NO as a biological mediator, few methods have been described for its assay. Electrochemical detection (ECD) is sensitive and has to be applied at the site of NO production; however, Griess microassay (GMA) is less sensitive and measures NO metabolites (nitrite, nitrate). The ability of the brain to produce NO was investigated here for the first time by *in situ* and *in vitro* NO direct detection in the brains of the terminally-anesthetized *P. berghei* N/13/1A-infected MF1 mice. The ECD assay measures NO with continuous direct monitoring using a NO-selective electrode. The sensitive probe measures the concentration of NO in soft tissues at nanomolar (nM) concentrations. ECD results showed a rise in the brain NO in malarial mice ($P < 0.01$) in both *in situ* and *in vitro* ECD. The increased level of NO in *P. berghei* N/13/1A-infected mice suggests its critical role in host defence and in the brain as a possible target organ. Some key points, including the type of brain tissue, the variation of cell types, and the function of each cell, require further studies to clarify the specific function of the brain as a target organ in CM.

Keywords: brain, cerebral malaria, CM, nitric oxide, NO

Introduction

NO has been shown to have an important role in a great variety of biological functions. Over recent years, few methods have been described that enable reliable *in situ* NO measurement in biological systems. In assay studies of endogenous NO, further uncertainty could result from the method of assay and the samples analyzed [1]. Commonly, NO in malaria has been studied by GMA to measure reactive nitrogen intermediates (RNI) [2], which is an indirect measure and results could be misleading in terms of NO. Electrochemical detection (ECD) is a sensitive assay and measures NO directly, and because of the short biological life of NO, has to be applied at the site of NO production. It is generally agreed that the ideal method for detecting NO should be based on the following characteristics: good selectivity of NO, good sensitivity, fast response

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or recovery times, long-term stability, minimal disturbance, and ease of calibration reagents [3]. The ECD is a continuous direct monitoring of NO release with a highly specific NO-selective electrode. The probe polarographically measures the concentration of gaseous NO in aqueous solutions at nanomolar (nM) concentrations, which appears to be crucial in many areas of biomedical research [4]. Direct ECD of NO was first reported by [5], who modified an earlier electrode, which detected oxygen at a reducing potential [6]. There are few reports using this method *in vivo*, including implantation of an electrochemical probe in a rabbit's inferior vena cava [7], NO direct detection *in vivo* in rat brains during LPS-induced sepsis [8], and in human peripheral blood during hemodialysis of nephropathy patients [4]. The electrochemical technique has high temporal resolution, distinguishes NO from its oxidation products, allows *in vivo* measurement, thus making data available rapidly in real time [6]. Among the many electrode materials, direct detection of NO using prophyrin-modified electrode in micrometer or sub-micrometer tip diameters has been reported with good sensitivity and selectivity [6]. The NO-detection principle for electrochemical probe is common to all such probes, which can be regarded as amperometric electrodes. In each case, a potential is applied to the measuring electrode relative to a reference electrode, and the current, due to the electrochemical oxidation of NO, is monitored according to this reaction:

$$NO + 4OH^- \longrightarrow NO_3 + 2H_2O + 4e^-$$

The reaction happens in the main part of the sensor, which is contained within a gas-permeable Teflon-type membrane. The sensor consists of a working counter electrode combination, where the oxidation of NO is carried out to produce the electric current used to measure NO [3,6]. The objective of this experiment was to detect NO production directly *in situ* and *in vitro* in the brains of terminally-anesthetized *P. berghei* N/13/1A-infected MF1 mice, to compare values with those of control animals, and thereby determine any relationship between death caused by lethal *P. berghei* N/13/1A strain and changes of NO in the brain.

Materials and methods

Animals

The animals used in this experiment were male MF1 mice with body weights of 20.2 ± 0.6 g. The mice were divided into two groups, and malarial animals were inoculated iv with 2×10^7 parasitized red blood cells (PRBC) from a donor *P. berghei* N/13/1A-infected mouse with 66% parasitemia. The control group received an equivalent volume (0.2 ml/mouse) of diluted uninfected RBC (URBC).

Parasitemia

Parasitemia was determined on different days after infection using blood smears stained with Leishman's reagent (Sigma Chemical Co, USA) 2 mg/ml methanol. PRBC were counted in five different fields, each of approximately 200 cells. Results are expressed as the mean percentage (%) of erythrocytes containing Leishman positive bodies. Experiments were licensed under the Animals (Scientific Procedures) UK Act, 1986.

In situ detection of NO in the brain by ECD

After terminal anesthesia, each mouse was placed in a sterotaxis frame (Fig 1) and the skin over the skull was incised. A three mm diameter hole was made at the bregma using a dental drill (FHP Motors, Citenco Ltd, Co, UK) and the electrochemical probe inserted 4 mm deep into the frontal lobe and prefrontal area of the brain. The probe was inserted 1 h before terminal anesthesia on day 4 post-infection, when the parasitemia was 73.6 ± 4.8%. The working electrode was held for a conditioning period depending on each sample (2-5 min) until an electrical current with a low pico-ampere (PA) appeared on the screen. Recording continued until the electrode current decayed to a stable baseline. The output current of the probe correlated linearly with the NO concentration detected at the tip. Each curve represented one brain sample and traces of NO level in the brain in nM using a NO meter with a 200 µm sensor tip (ISO-NO Mark II, World Precision Instruments, USA). Panels show

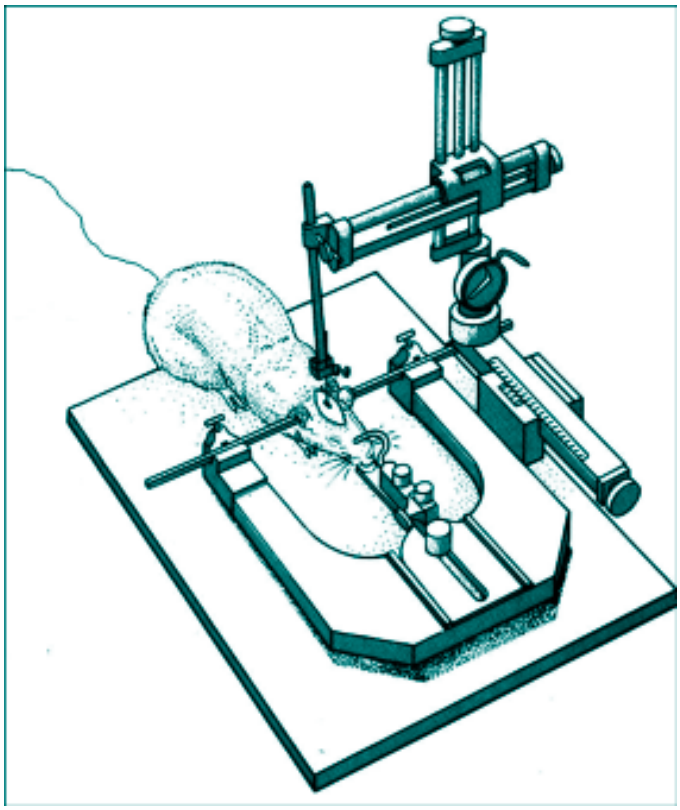

Fig 1 Stereotaxic frame.

the changes in concentration of NO (nM, vertical axis) against time (sec, horizontal axis), displayed by the PC-based data acquisition system, Duo-18 (World Precision Instruments, USA) [3]. The maximum level of NO (nM) for each sample is considered for statistical analysis.

Preparation of brain tissue for *in vitro* ECD

Mice were terminally anesthetized by inhalation of diethyl ether (BDH, England). Following humane killing, brain tissues were removed on days 1, 3, 5 post-infection, placed in 10 ml Krebs-Henseleit solution [1], gassed with 95% O_2 and 5% CO_2, filtered via pore size 22 µm (Millipore Corporation, USA) and stored at 4˚C. Approximately 0.1 g of brain tissues were placed in 1.5 ml microfuge tubes and homogenized using an electrical homogenizer (Model RS541-242, Refer Scientific, UK) with the gradual addition of 1 ml Krebs-Henseleit solution. Homogenates were incubated at room temperature (19-22˚C) for 10 min and then assayed for NO production using ECD [1].

***In vitro* ECD of brain tissue**

An isolated NO meter with a 200 µm sensor tip was calibrated according to the manufacturer's instructions [3]. NO detection *in vitro* was effected according to the method previously described in tissue homogenates [1]. The NO sensor probe was immersed in suspensions of brain homogenates incubated at room temperature (19-22˚C) and NO production measured continuously until each record returned to baseline values. A PC-based data acquisition system (Duo-18, World Precision Instruments, USA) was used to record and display NO concentrations [1,3].

Histology

Histological sections were later taken to define the site of NO detection and where the probe actually ended up. The sections were

obtained across the sagital line between two hemispheres and examined by macroscopical observation.

Results

P. berghei N/13/1A was lethal on day 5 with high parasitemia in outbred MF1 mice. The values for direct monitoring of *in situ* and *in vitro* NO release are presented as mean ± SEM (n = 4-5). The significance of difference was determined using Student's *t*-test. Electrochemical assay showed a rise in brain NO in the malarial group (Fig 2). *In situ* NO elevated in the brain of *P. berghei*-infected mice, when compared with the control group (control 3,071.4 ± 132.6 nM, malaria 3,829.6 ± 131.4 nM, $P < 0.01$) (Fig 3). *In vitro* NO concentration in brain homogenates increased on day 3 (control 1,321.8 ± 199.3 nM, malaria 2,424.5 ± 184.4 nM, $P < 0.01$) and day 5 (control 1,094.5 ± 131.9 nM, malaria 3,107.3 ± 408.2 nM, $P < 0.01$) (Fig 4).

According to histological sections, the probe was located in the frontal lobe and prefrontal area through the bregma.

Discussion

The key role for target organs to produce NO in murine malaria was reported earlier [1,9-10].

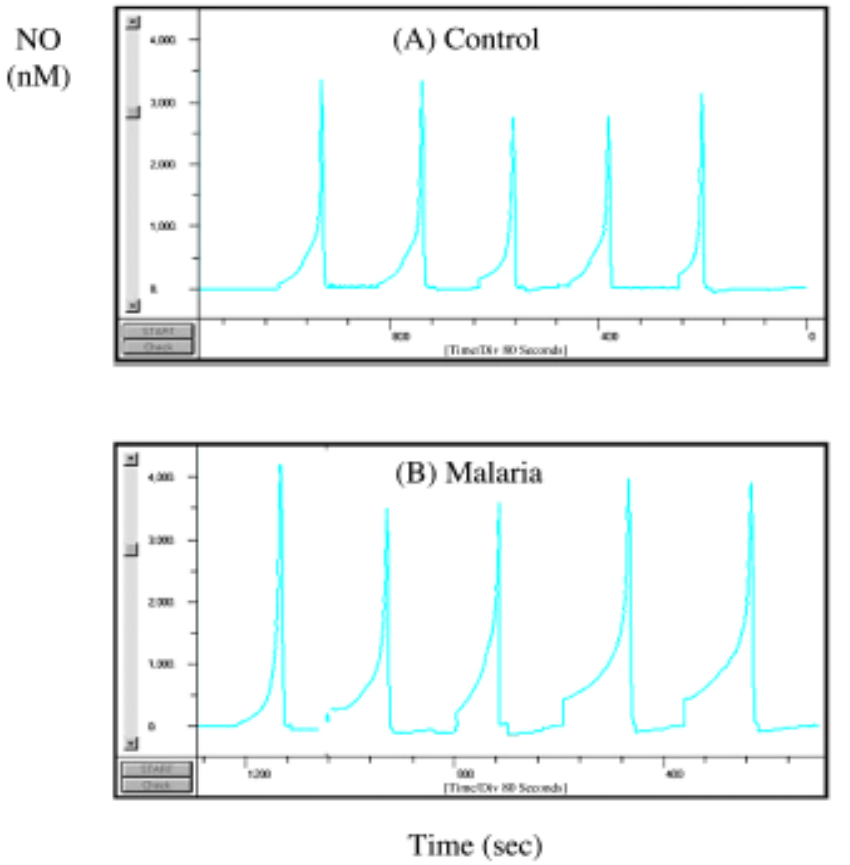

Fig 2 NO production *in situ* in the brain of MF1 mice during *P. berghei* infection. The output of *in situ* direct measurement of NO in MF1 mice of both control (A) and malarial (B) groups (n = 5). Each curve represents one brain sample and traces of NO level in the brain in nanomolars (nM) using a NO meter with a 200 m sensor tip (ISO-NO Mark II, WPI, USA). Panels show the changes in concentration of NO (nM, vertical axis) against time (sec, horizontal axis), displayed by the PC-based data acquisition system, Duo-18 (WPI, USA).

The ability of brain tissue to produce NO in mice infected with murine malaria was investigated here for the first time by *in situ* and *in vitro* direct detection of NO release. Monitoring of NO production in the brain might be more critical than other host tissues. This may be due to the central role of the brain in CNS; therefore, any changes in brain function directly affect other organs. As neuromodulation is one of the functions of NO, changes in NO production in the brain are implicated in its biological activity. The type of brain tissue, the variation of cell types, and the function of each cell, are key points when considering the brain as a possible target organ in severe malaria.

In this experiment, the murine malaria parasite (*P. berghei* N/13/1A) induced increased NO production in the brain when compared with the control group. The high level of NO production in the brain could be related to the brain *per se* and/or might be associated with other tissues. It

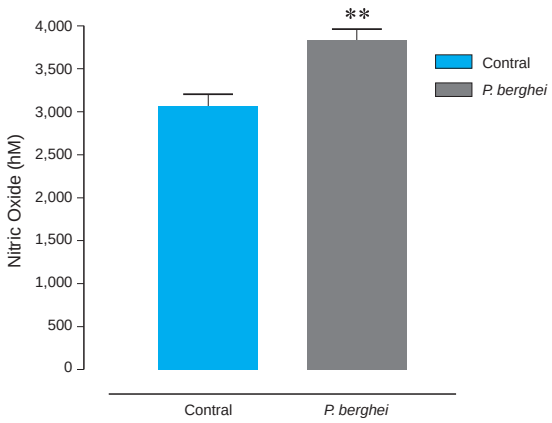

Fig 3 ***In situ*** **NO production in the brain of *P. berghei*-infected mice. Values for *in situ* NO production are presented as mean ± SEM (n = 5) in the brains of control and *P. berghei* N/13/1A-infected MF1 mice. The significance of difference was determined by Student's *t*-test. Statistical significance was established at **$P < 0.01$.**

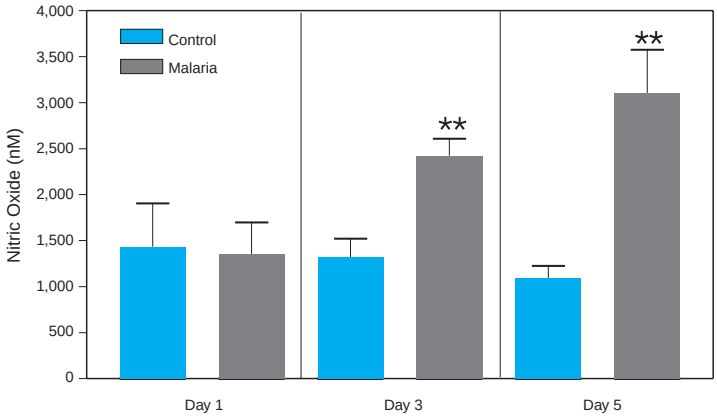

Fig 4 ***In vitro*** **NO detection in the brain of *P. berghei*-infected mice. The production of NO was assayed directly in brain homogenates of MF1 mice infected with *P. berghei* N/13/1A, compared with control groups. The values for NO concentrations (nM) are presented as mean ± SEM (n = 4). The significance of difference (**$P < 0.01$) was determined by Student's *t*-test.**

is suggested that the *Plasmodium* released factors that stimulated immune cells in the circulation to induce greater production of NO in the brain. This might be due to accumulation of diffusible NO from the endothelial cells of blood vessels to surrounding brain tissues.

Commonly, NO in malaria has been studied by assay of measuring RNI production [2], which is an indirect measure of NO. The advantage of ECD is its sensitivity and direct measurement of NO, but it is technically more demanding, expensive and has to be applied at the site of NO

production. In comparison with among methods, ECD is sensitive, but susceptible to interference, whereas GMA is less sensitive, but cheaper. When applied to samples treated with nitrate reductase, Griess measures the RNI or NO metabolites (NO_2^- and NO_3^-) giving an indirect measure of NO production. One key difference between the GMA and ECD is that GMA measures the accumulation of stable RNI, which may originate from non-NO sources, however ECD detects short-lived NO actually released from the L-arginine pathway. In addition, the GMA registers RNI accumulating in the body and the pathway represents an earlier phase of NO involvement, whereas ECD measures currently released NO. Direct NO detection and indirect measurement of RNI may sometimes support each other [4], but not on all occasions [1]. In the current study, the brains of *P. berghei*-infected mice demonstrated NO induction monitored by direct ECD assay. Improvements in the electrochemical probe should allow a broad range of sizes and configurations, allowing change from macroelectrodes, for use in body fluids or suspensions of cultured cells, to ultra-microelectrodes, that could be inserted into individual living cells [6]. Application of such microelectrodes may clarify the responsibility of each cell type for NO production.

The published data are consistent with the hypothesis that NO biosynthesis in the blood and/or spleen *in vivo* helps control *Plasmodium* infections in mice [1]. When parasitemia rises, however, increased production of NO in organs, such as the brain and liver, is associated with pathology rather than recovery [1]. The fact that the brain of malaria-infected mice is involved in NO induction *in situ* and *in vitro* was confirmed for the first time in this experiment, and may be supported by using different strains of parasite in different host species. Perhaps, further studies on CM murine *Plasmodia* versus host species are required to indicate the role of parasite/host combination in NO induction.

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