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Glutathione peroxidase contributes with heme oxygenase-1 to redox balance in mouse brain during the course of cerebral malaria

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ABSTRACT

Oxidative stress has been attributed both a key pathogenic and rescuing role in cerebral malaria (CM). In a *Plasmodium berghei* ANKA murine model of CM, host redox signaling and functioning were examined during the course of neurological damage. Host antioxidant defenses were early altered at the transcriptional level indicated by the gradually diminished expression of superoxide dismutase-1 (*sod-1*), *sod-2*, *sod-3* and catalase genes. During severe disease, this led to the dysfunctional activity of superoxide dismutase and catalase enzymes in damaged brain regions. Vitagene associated markers (heat shock protein 70 and thioredoxin-1) also showed a decaying expression pattern that paralleled reduced expression of the transcription factors Parkinson disease 7, Forkhead box O 3 and X-box binding protein 1 with a role in preserving brain redox status. However, the oxidative stress markers reactive oxygen/nitrogen species were not accumulated in the brains of CM mice and redox proteomics and immunohistochemistry failed to detect quantitative or qualitative differences in protein carbonylation. Thus, the loss of antioxidant capacity was compensated for in all cerebral regions by progressive upregulation of heme oxygenase-1, and in specific regions by early glutathione peroxidase-1 induction. This study shows for the first time a scenario of cooperative glutathione peroxidase and heme oxygenase-1 upregulation to suppress superoxide dismutase, catalase, heat shock protein-70 and thioredoxin-1 downregulation effects in experimental CM, counteracting oxidative damage and maintaining redox equilibrium. Our findings reconcile the apparent inconsistency between the lack of oxidative metabolite build up and reported protective effect of antioxidant therapy against CM.

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1. Introduction

Malaria caused by *Plasmodium falciparum* has the highest rates of mortality in children because of its most life-threatening complication cerebral malaria (CM). This is an acute neurological condition characterized by seizures, metabolic acidosis, hypoglycaemia and coma [1]. CM has a mortality rate of 10–14% and causes around 600,000 annual deaths among young children predominantly in sub-Saharan Africa and Southeast Asia [2]. Moreover, child survivors often show long-standing neurocognitive sequelae, particularly in Africa [1,3].

Prior studies have attributed an important role in CM development to an altered redox equilibrium. Some authors have suggested that reactive oxygen species (ROS), produced by both host and parasite, accumulate during the development of this acute neurological condition promoting oxidative stress [4–6]. The increased production of free radicals (e.g. superoxide anion and hydroxyl radical) has been observed in activated leucocytes, in endothelial cells during the adhesion of parasitized red blood cells (pRBC) and, most strikingly, during hemoglobin digestion in the parasite food vacuole [4,7–9]. This, in turn, causes protein oxidation in the parasite and host membranes [10,11]. Indeed, enhanced host ROS production during the course of CM is thought to play both a beneficial and pathological role depending on the amount and place of release. Beneficial ROS effects contest parasite growth whereas prolonged ROS exposure could cause cell damage in the host brain [4,5]. In addition, several antioxidant treatments have shown protection against experimental cerebral malaria (ECM) and prevention of persistent cognitive damage [6,12,13], suggesting the failure of host antioxidant mechanism during CM. Notwithstanding, some studies have failed to observe ROS accumulation and oxidative stress in brain during CM [14], questioning the role played by these molecules in the pathogenesis of this disease [8].

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Intra and extracellular antioxidant defenses exist in the host to prevent oxidative stress, whereby the superoxide radical is reduced by superoxide dismutase (SOD) to hydrogen peroxide, which is reduced to water by both catalase (CAT), a peroxisomal enzyme with a central function in brain cells, and glutathione peroxidase (GPX) [15]. Moreover, brain cells overcome oxidative stress through additional antioxidant pathways such as those involving heat shock proteins (HSP), heme oxygenase (HO), and thioredoxin (TRX) [16].

In the present study, we examined the state of these host antioxidant defenses and their role in oxidative damage during the course of the disease in a mouse ECM model. Here we report the dysfunction of several host antioxidant systems associated with the reduced expression of transcription factors involved in preserving brain host redox status. Oxidative stress markers failed to accumulate in brain regions in parallel with the compensatory induction of two major antioxidant enzymes. Our results reconcile the controversial idea of a lack of oxidative stress build up with the known protective effect of antioxidant therapy against CM.

2. Materials and methods

2.1. Induction of malaria in mice and disease assessment

All experiments involving animals were conducted at the Universidad Complutense de Madrid in accordance with national and international guidelines for animal care. The study protocol was approved by the Animal Experimentation Committee of the Complutense University at its meeting on February 11th 2011.

We used 44 five-weeks-old male C57BL/6 mice, as a CM susceptible strain and 20 five-weeks-old male BALB/c mice, as a non-susceptible CM model. The animals were purchased from Harlan Ibérica (Barcelona, Spain) and housed under standard conditions and supplied with food and water ad libitum. In 32 C57BL/6 mice, CM was induced by intraperitoneal injection of 5×10^6 *P. berghei* ANKA pRBC, as previously described [17]. In a separate experiment 10 BALB/c mice were injected with a similar number of *P. berghei* ANKA pRBC. Uninfected mice were used as controls in both experiments. Our choice of using non-injected animals was based on the national and international regulations regarding animal experimentation implemented at our University by the Committee of Animal Experimentation aimed at minimizing animal suffering. According to previous findings and reports [17,18], injected and non-injected control mice show identical behavior and histological phenotype during the experimental course, with no signs of disease or distress.

The infected C57BL/6 mice developing CM were monitored daily by inspection of clinical signs and classified into 4 clinical stages using the method of clustering animals by neurological symptoms as previously described [17]. BALB/c mice were sacrificed when reaching the same range of parasitemia than C57BL/6 mice. After their sacrifice by cervical dislocation, the olfactory bulb, frontal cortex, hippocampus, thalamus–hypothalamus, cerebellum and brainstem were immediately removed.

2.2. Mouse antioxidant system and transcription factor expression assays

Antioxidant molecules and transcription factors (TF) mRNA levels in olfactory bulb, frontal cortex, hippocampus, thalamus–hypothalamus, cerebellum and brainstem were determined by quantitative reverse transcriptase PCR (qRT-PCR) as previously described [18]. Specific primers and probes for *sod-1*, *sod-2*, *sod-3*, *gpx-1*, *cat*, *trx-1*, *ho-1*, *hsp70*, Parkinson disease 7 (*park7*), Forkhead box O 1 (*foxo1*), *foxo3*, X-box binding protein 1 (*xbp1*) and the house keeping *β-actin* genes (Assays-on-Demand™ Gene Expression products, TaqMan MGB probes, Applied Biosystems, Warrington, UK) were used. The specificity of the primers and probes used was verified by basic local alignment search tool (BLAST) analysis, comparing the mouse genes selected with the *P. berghei* genome and no significant similarity was found. The *β-actin* gene served as an endogenous control to check for any slight variation in the initial concentration, the total RNA quality and the conversion efficiency of the reverse transcription reaction.

N-fold changes were calculated by expressing the amount of mRNA for each molecule present in each mouse vs. the mean amount of mRNA for the molecule detected in control mice.

2.3. CAT, SOD and GPX enzyme activity

100 μg of total brainstem and cerebellum protein extract from the different groups of animals were loaded onto a 15% or 8% native acrylamide gel (for SOD and GPX or CAT activity, respectively) in Tris–glycine running buffer and run at 120 V. Enzyme activities were visualized as described previously [19–21] as clear bands against a dark background. In all assays, protein loading was determined by Coomassie Brilliant Blue staining. Bands were quantified using Quantity-One 1-D analysis software (Bio-Rad Laboratories Inc., Munich, Germany). Relative optical density was calculated as the normalized enzyme activity in each mouse relative to the mean value obtained in control mice.

2.4. Reactive oxygen species/reactive nitrogen species (RNS)

Cerebella and brainstems from animals at stage IV of CM were homogenized in cold phosphate buffer and total ROS/RNS contents were determined using the OxiSelect™ In Vitro ROS/RNS Assay Kit (Cell Biolabs, Inc., San Diego, USA) following the manufacturer's instructions.

2.5. Protein carbonylation

2.5.1. Oxyblot analysis

Protein from control and CM cerebella was extracted and derivatized before 2D electrophoresis as previously described [22]. Derivatized proteins were precipitated using trichloroacetic acid (TCA)-acetone and resuspended in a buffer containing 7 M urea, 2 M thiourea, 4% 3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate (CHAPS), 0.5% MEGA-10, and 10 mM dithioerythritol.

Proteins were cup-loaded onto immobilized pH gradient (IPG) strips (pH 3–11 NL, 18 cm) that had been previously hydrated overnight in a buffer containing 7 M urea, 2 M thiourea, 4% CHAPS, 1.2% DeStreak and 0.5% ampholytes, pH 3–11. First-dimensional separation was by isoelectric focusing (IEF) using the IPGphor 3 IEF system (GE Healthcare, Buckinghamshire, UK) at 20 °C. The voltage was gradually ramped: 3 h 300 V, 6 h 300–1000 V, 3 h 1000–8000 V, and a final step-n-hold at 8000 V for the next 7 h. The run was terminated after ~70,000 V h. The focused strips were equilibrated in equilibration solution (50 mM Tris 8.8, 6 M urea, 30% glycerol, 2% sodium lauryl sulfate (SDS)) containing 1% dithiothreitol reducing agent for 10 min, and transferred to 4% iodoacetamide equilibration solution for a further 10 min. The second-dimension SDS-PAGE was run on homogeneous 12.5% T and 2.6% C casted polyacrylamide gels. Electrophoresis was carried out at 4 °C, 2 W overnight using two Hoeffer units. For each experimental condition (CM or control), 4 gels were run in parallel under identical 2D electrophoresis conditions. Two gels were stained with Sypro Ruby to visualize total proteins and the other two gels were used for subsequent carbonyl modified protein detection. These two gels prepared for CM and control animals were transferred to polyvinylidene fluoride (PVDF) membranes and transfer efficiency was confirmed by: (i) gel staining with Sypro Tangerine before transfer and (ii) membrane staining with Sypro Ruby. Any potential interference of Sypro Tangerine during immunodetection was previously discarded. Carbonyl groups in the transferred membranes containing derivatized proteins were immunodetected as previously described [22] using the anti-2,4-dinitrophenylhydrazone (DNP) primary antibody (1/10,000, Sigma, Saint Louis, USA). Gel and immunoblot images were analyzed using Quantity-One 1-D and PDQuest analysis software (BioRad Laboratories Inc., Munich, Germany). The total amount

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