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ORIGINAL RESEARCH

Proteomic analysis revealed alterations of the *Plasmodium falciparum* metabolism following salicylhydroxamic acid exposure

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Objectives: Although human respiratory metabolism is characterized by the mitochondrial electron transport chain, some organisms present a "branched respiratory chain." This branched pathway includes both a classical and an alternative respiratory chain. The latter involves an alternative oxidase. Though the *Plasmodium falciparum* alternative oxidase is not yet identified, a specific inhibitor of this enzyme, salicylhydroxamic acid (SHAM), showed a drug effect on *P. falciparum* respiratory function using oxygen consumption measurements. The present study aimed to highlight the metabolic pathways that are affected in *P. falciparum* following SHAM exposure.

Design: A proteomic approach was used to analyze the *P. falciparum* proteome and determine the metabolic pathways altered following SHAM treatment. To evaluate the SHAM effect on parasite growth, the phenotypic alterations of *P. falciparum* after SHAM or/and hyperoxia exposure were observed.

Results: After SHAM exposure, 26 proteins were significantly deregulated using a fluorescent two dimensional-differential gel electrophoresis. Among these deregulated proteins, some were particularly involved in energetic metabolism. And the combinatory effect of SHAM/hyperoxia seems deleterious for the growth of *P. falciparum*.

Conclusion: Our results indicated that SHAM appears to activate glycolysis and decrease stress defense systems. These data provide a better understanding of parasite biology.

Keywords: *Plasmodium falciparum*, salicylhydroxamic acid, hyperoxia, glycolysis, proteomic

Background

The malaria parasite *Plasmodium falciparum* possesses a mitochondrion with specific structural and physiological features.[1–4] Unlike most other eukaryotes, malarial mitochondrial electron transport chain (mETC) activity appears to be primarily involved in de novo pyrimidine biosynthesis through the activity of dihydroorotate dehydrogenase rather than through an adenosine triphosphate (ATP) source.[4–6] Intraerythrocytic stages of malaria parasites have been considered for a long time to rely primarily on anaerobic glycolysis for ATP production. However, recently five subunits of the F_1 subcomplex and four F_0 subunits of the mitochondrial F_0F_1 ATP synthase have been reported in the *P. falciparum* genome, supporting the fact that oxidative phosphorylation could be functional.[7] As parasite respiratory metabolism is distinct from human respiration, diverse inhibitors of the major complexes of the mETC have been used in chemotherapy[8] and validated the importance of the mitochondrion in the growth of the asexual intraerythrocytic stage of *P. falciparum*.[9,10] Moreover,

the metabolic changes of the mitochondrion contribute to environmental adaptations of parasites during its life cycle.[11] Effectively, in vivo, *P. falciparum* life cycle involves two hosts: the mosquito and humans during which the parasite is subjected to varying oxygen levels (from 5% oxygen in human venous blood to 13% oxygen in the human lungs and 21% oxygen in mosquito salivary glands).

Study of the rotenone drug effect underlined the divergence of the first component (complex I) of the *P. falciparum* mETC.[12–14] The eukaryotic complex I is classically composed of a rotenone-sensitive nicotinamide adenine dinucleotide hydride (NADH) dehydrogenase. In contrast, *P. falciparum* encodes a rotenone-insensitive alternative complex I (type II NADH dehydrogenase, PfNDH2). This complex I is comparable to type II NADH dehydrogenase found in plants. Not surprisingly, parasite mitochondria have an endosymbiotic origin probably resulting from algal symbiont into a protoeukaryote.[15–17] Moreover, other mitochondrial drugs such as atovaquone inhibit electron transfer at the level of the bc1 complex (complex III) by interfering with the ubiquinol oxidation site of cytochrome b. The drug effect is lethal for the parasite, presumably by interruption of essential links to de novo pyrimidine biosynthesis and to the collapse of the mitochondrial transmembrane potential.[18,19] This drug is already currently used in endemic regions in combination with proguanil (Malarone®).[20] Also, the combination of atovaquone and salicylhydroxamic acid (SHAM), a specific inhibitor of alternative oxidase (AOX), inhibits parasite growth synergistically.[21,22] The AOX, which is absent from mammals, has been most extensively studied in higher plants. The plant respiratory pathway includes an alternative respiratory chain, cyanide-resistant, and the classical mETC, cyanide-sensitive. The AOX does not appear to contribute directly to mitochondrial transmembrane potential or the energy balance of the cell.[23,24] However, it can contribute indirectly by accepting electrons from enzymes that provide electrons to coenzyme Q for the preservation of tricarboxylic acid cycle (TCA) turnover and the balance of carbon metabolism and electron transport. From the mitochondrial transmembrane potential and oxygen consumption measurements in presence of *P. falciparum* mETC inhibitors, the evidence of an alternative respiratory pathway in which electrons are transferred directly from coenzyme Q to oxygen was underlined.[25] This cyanide-resistant alternative respiratory pathway was inhibited by two inhibitors, propyl gallate and SHAM, which are specific inhibitors of AOX activity in other organisms.[26–28] The AOX gene has been characterized in several organisms such as plants, yeast, bacterium, and notably from other parasites, *Trypanosoma brucei* and *Cryptosporidium parvum*.[28–30] Nevertheless, until now, no gene encoding AOX has been detected in the *P. falciparum* genome but 60% of the predicted genes could not be assigned to orthologous functions.

In the present work, a two dimensional-differential gel electrophoresis (2D-DIGE) approach, combined with mass spectrometry (MS) analysis, was used to define the consequences of SHAM on the *P. falciparum* proteome. Studies were previously reported that the hyperoxia induces a cycle delay on *P. falciparum* of 4 hours with a biological adaptation of the parasite to next cycle.[11,31] The deleterious effect of this drug on the in vitro parasite growth in hyperoxia, the involved metabolic pathways, and its mode of action on glycolysis are discussed, supporting the hypothesis that the energetic metabolism may indeed provide an attractive chemotherapeutic target.

Methods

SHAM or/and hyperoxia exposure of *P. falciparum* cultures

The in vitro antimalarial activity of SHAM was determined using the isotopic semi microtest method as previously described by Desjardins et al.[32] Drug concentrations were from 0.5 to 600 μM. The IC_{50}, ie, the drug concentration corresponding to 50% of the uptake of [^{3}H] hypoxanthine by the parasite in drug-free control wells, was determined by nonlinear regression analysis of log dose-response curves.

The 3D7 *P. falciparum* strain was cultured in human A+ erythrocytes, as previously described.[33] Cultures were tightly synchronized (with 4 hour intervals) using combined D-sorbitol and CS columns on a VarioMACS (Miltenyi Biotec, Paris, France) according to standard procedures.[34,35]

To evaluate the effect of SHAM, hyperoxia, and the combinatory effect, cultures were adapted to hyperoxic conditions (21% oxygen, 5% carbon dioxide, 74% nitrogen gas mixture) and regularly synchronized during six *P. falciparum* parasites' cycles before the addition of SHAM. The effects of SHAM alone and combined SHAM/hyperoxia were observed at 0, 24, 32, 48, and 78 hours (0 hours corresponding to SHAM addition at the ring stage). Viability, parasitemia, and morphology were monitored daily by examining blood smears stained with RAL 555 (RAL Diagnostics, Martillac, France) from SHAM addition at the ring stage.

To test the SHAM effects on *P. falciparum* proteome (3% parasitemia and 6% hematocrit), the drug was added to 25 mL culture medium at IC_{50} and the parasites were further

incubated for 12 hours and harvested. For each proteomic experiment, four biological replicates were performed.

Protein extraction

After SHAM pressure, parasitized erythrocytes (late ring stages aged 16–20 hours) were washed three times in phosphate buffered saline (PBS) medium (Invitrogen, Cergy Pontoise, France) and lysed in cold water-saponin (0.1%, Sigma, St Louis, MO) for 10 minutes. The lysate was then centrifuged at 1500 *g* for 5 minutes. The supernatant was discarded and the pellet containing free parasites was recovered by washing in cold PBS medium followed by a centrifugation step (1500 *g* for 5 minutes). The free parasites were washed until the supernatant became colorless. The pellet was then suspended in 4% (w/v) CHAPS (Sigma) and disrupted by ultrasonication (Vibracell 72412; Bioblock Scientific, Illkirch, France) 60 seconds on ice at maximum amplitude. The lysate was then centrifuged at 16,100 *g* for 15 minutes. The supernatant was further precipitated with 100% acetone (Sigma). The protein concentration for each sample was estimated using the BioRad Lowry-based DC assay (BioRad Laboratories, Hercules, CA), according to the manufacturer's instructions. Total proteins were suspended in standard cell lysis buffer (7 M urea, 2 M thiourea, 4% CHAPS, 30 mM Tris base, pH 8.5 [Sigma]) to obtain a protein concentration adjusted to 2.5 µg/µL. Protein samples were minimally labeled with CyDye according to the manufacturer's protocols (GE Healthcare, Piscataway, NJ).[36]

Briefly, protein extracts (50 µg) were labeled with 400 pmol of CyDye, freshly dissolved in anhydrous dimethyl formamide (Sigma) and incubated on ice for 30 minutes in the dark. The reaction was quenched with 1 µL of free lysine (10 nM, Sigma) by incubating for 10 minutes on ice. An equal volume of 2× sample buffer (8 M urea, 2 M thiourea, 4% (w/v) CHAPS, 10 mM Dithiothreitol (DTT) and 1% (v/v) IPG buffer 3–10 [GE Healthcare]) was added to the CyDye-labeled sample. The mixture of labeled proteins was then separated by 2D-DIGE.

2D electrophoresis of parasite proteins, image analysis, and in-gel digestion

Isoelectric focusing was performed on 18 cm pH 3–10 linear IPG strips (GE Healthcare). Destreak buffer containing 1% (v/v) IPG buffer pH 3–10 was used for overnight rehydration of IPG strips. The samples were applied at the acidic end of the IPG strip using a cup-loading technique. Isoelectric focusing was carried out on an Ettan IPGphor II (GE Healthcare) electrophoresis unit at 20°C for a total of 45 kVh (ramp to 300 V in 3 hours, ramp to 1000 V in 6 hours, ramp to 8000 V in 3 hours, hold at 8000 V for 4 hours). IPG strips were equilibrated in a equilibration buffer containing 50 mM Tris hydrochloride, pH 8.6, 6 M urea, 2% sodium dodecyl sulfate (SDS) and 30% glycerol supplemented with 1% (w/v) DTT for 15 minutes at room temperature, followed by protein alkylation (carbamidomethylation) in the same equilibration buffer containing 2.5% (w/v) iodoacetamide instead of DTT for 15 minutes at room temperature. IPG strips were then placed on the top of 10% uniform polyacrylamide gels. Strips were overlaid with 0.5% agarose in 1x running buffer containing bromophenol blue, and the proteins were further separated by SDS-polyacrylamide gel electrophoresis (10 W per gel) at 20°C in the Ettan DALTsix Electrophoresis System (GE Healthcare). After electrophoresis, the gels with CyDye-labeled proteins were directly imaged using a Typhoon™ Trio image scanner (GE Healthcare, Buckinghamshire, UK).

After 2D-DIGE, gels were scanned on the Typhoon™ Trio image scanner at different excitation wavelengths (Cy3, 580 BP 30/green [532 nm]; Cy5, 670 BP 30/red [633 nm]; Cy2, 520 BP 40/blue [488 nm]). The intensity was adjusted to ensure that the maximum volume of each image was within 60,000–80,000 U. Analysis of 2D-DIGE was performed using DeCyder 6.5 software (GE Healthcare), according to the manufacturer's recommendations. Briefly, the differential in-gel analysis module was used to detect the intra-gel spots and the biological variation analysis module to match different gels using the in-gel standard. The paired *t*-test was used for statistical analysis of the data. Protein spots that were expressed differentially between two experimental conditions ($|\text{ratio}| \geq 1.5$, $P \leq 0.05$ *t*-test) were marked with master gel numbers. Based on DeCyder 6.5, analysis spots of interest were excised from gels using a Shimadzu Xcise automated gel processing platform (Shimadzu Biotech, Kyoto, Japan). Excised spots were prepared as described previously and then stored at –20°C before analysis by MS.[37]

MS analysis

The samples were analyzed by nanoscale capillary liquid chromatography-tandem MS (nano LC-MS/MS). Purification and analysis were performed on a C18 capillary column using a CapLC system (Waters, Milford, MA) coupled to a hybrid quadrupole orthogonal acceleration time-of-flight tandem mass spectrometer (Q-TOF Ultima; Waters). Chromatographic separation was conducted on a reversed-phase capillary column (Atlantis™ dC18, 3 µm, 75 µm × 150 mm Nano Ease™; Waters) with a 180–200 nL/minute flow. The gradient profile consisted of a linear

gradient from 95% A (water, 0.1% formic acid) to 60% B (80% acetonitrile, 0.1% formic acid) in 60 minutes followed by a linear gradient by 95% B in 10 minutes. Mass data acquisitions were piloted by MassLynx 4.0 software (Micromass Ltd, Manchester, UK) using automatic switching between MS and MS/MS modes. The internal parameters of Q-TOF were set as follows. The electrospray capillary voltage was set to 3.2 kV, the cone voltage was set to 30 V, and the source temperature was set to 80°C. The MS survey scan was m/z 400–1,300 with a scan time of 1 second and an interscan time of 0.1 second. When the intensity of a peak rose above a threshold of 15 counts, tandem mass spectra were acquired. Normalized collision energies for peptide fragmentation were set using the charge-state recognition files for +2 and +3 peptide ions. The scan range for MS/MS acquisition was from m/z 50 to 1500 with a scan time of 1 second and an interscan time of 0.1 second. Fragmentation was performed using argon as the collision gas and with the collision energy profile optimized for various mass ranges and charges of precursor ions. Mass data collected during a nano LC-MS/MS analysis were processed using ProteinLynx Global Server 2.2 software (Waters) with the following parameters: no background subtraction, smooth 3/2 Savitzky Golay, and no deisotoping to generate peak lists in the Micromass pkl format. Pkl files were then fed into a local search engine Mascot Daemon v2.2.2 (Matrix Science, London, UK). The data were searched against the *Homo sapiens* (218,356 sequences) and *P. falciparum* (13,110 sequences) National Center for Biotechnology Information nonredundant (NCBInr) protein databases (March 15, 2010). Search parameters allowed for one missed tryptic cleavage site, the carbamidomethylation of cysteine, and the possible oxidation of methionine; precursor and product ion mass error tolerance was <0.2 Da. All identified proteins had a Mascot score greater than 34 and 43 for *P. falciparum* and *H. sapiens*, respectively, corresponding to statistically significant identification ($P < 0.05$).

Results

SHAM and hyperoxia combinatory effect on the asexual blood cycle of *P. falciparum*

To study the deleterious effect of SHAM on asexual blood stages of *P. falciparum* in hyperoxia, 21% oxygen-adapted cultures of 3D7 were exposed to SHAM IC_{50}. The parasitemia and percentages of the different stages of parasites were evaluated by blood smears at 0, 24, 32, 48, and 78 hours (0 hours corresponding to SHAM addition at the ring stage). In in vitro culture under hyperoxic conditions, the 3D7 *P. falciparum* strain had a life cycle of 49 hours against 45 hours in normal conditions (Figure 1B). Parasite exposure to 21% oxygen and SHAM pressure resulted in morphologic alterations involving cellular degeneration during the first 24 hours (Figures 1B and 1C). These results suggest that SHAM addition to 3D7 *P. falciparum* strain under oxidative stress had lethal effects from the beginning of the parasitic cycle and that the life cycle does not continue beyond the ring stage. A previous study demonstrated that hyperoxia induces a delay of *P. falciparum* cycle of 4 hours with an energetic metabolism adaptation crucial to parasite survival.[11] Consequently, the SHAM/hyperoxia combinatory effect appeared to result in cellular death, suggesting that the adaptive pathway for hyperoxia was disturbed under SHAM pressure.

Effect of SHAM on *P. falciparum* asexual blood cycle

To establish the experimental design for the proteome study, the SHAM IC_{50} and the action timing of the SHAM should be determined.

Consistent with previously published results,[21,38] SHAM inhibited growth of the 3D7 strain with an average IC_{50} of 246 +/–4 μM.

To determine the SHAM effect on asexual blood stages of *P. falciparum*, the drug was added at the IC_{50} concentration on synchronous cultures of 3D7 at the ring stage. The parasitemia and parasitic stage percentages were monitored during two life cycles by blood smears at 0, 24, 32, 48, and 78 hours in three independent experiments (0 hours corresponding to SHAM addition at the ring stage). The parasitemia of SHAM-treated cultures was low at 32 hours compared to untreated parasites (Figures 1A and 1D, 0.6% parasitemia vs 0.25%, respectively). At the end of 48 hours under SHAM exposure, 54% of the parasites were degenerated but the parasites not affected by SHAM continued their cycle without phenotypic changes and lengthening of the parasitic cycle. Thus, the SHAM perturbed the parasite growth between the 24th and 32nd hours of the cycle at IC_{50} concentration.

SHAM-alteration to the *P. falciparum* proteome

To study the SHAM effect on *P. falciparum*, a proteomic approach was performed in synchronized parasites exposed to SHAM IC_{50}. To identify altered *P. falciparum* proteins, 2D-DIGE experiments coupled to MS were performed. Four independent cultures of untreated and SHAM-treated *P. falciparum* were included in this analysis. After protein separation by 2D-DIGE using pH 3–10 IPG strips and

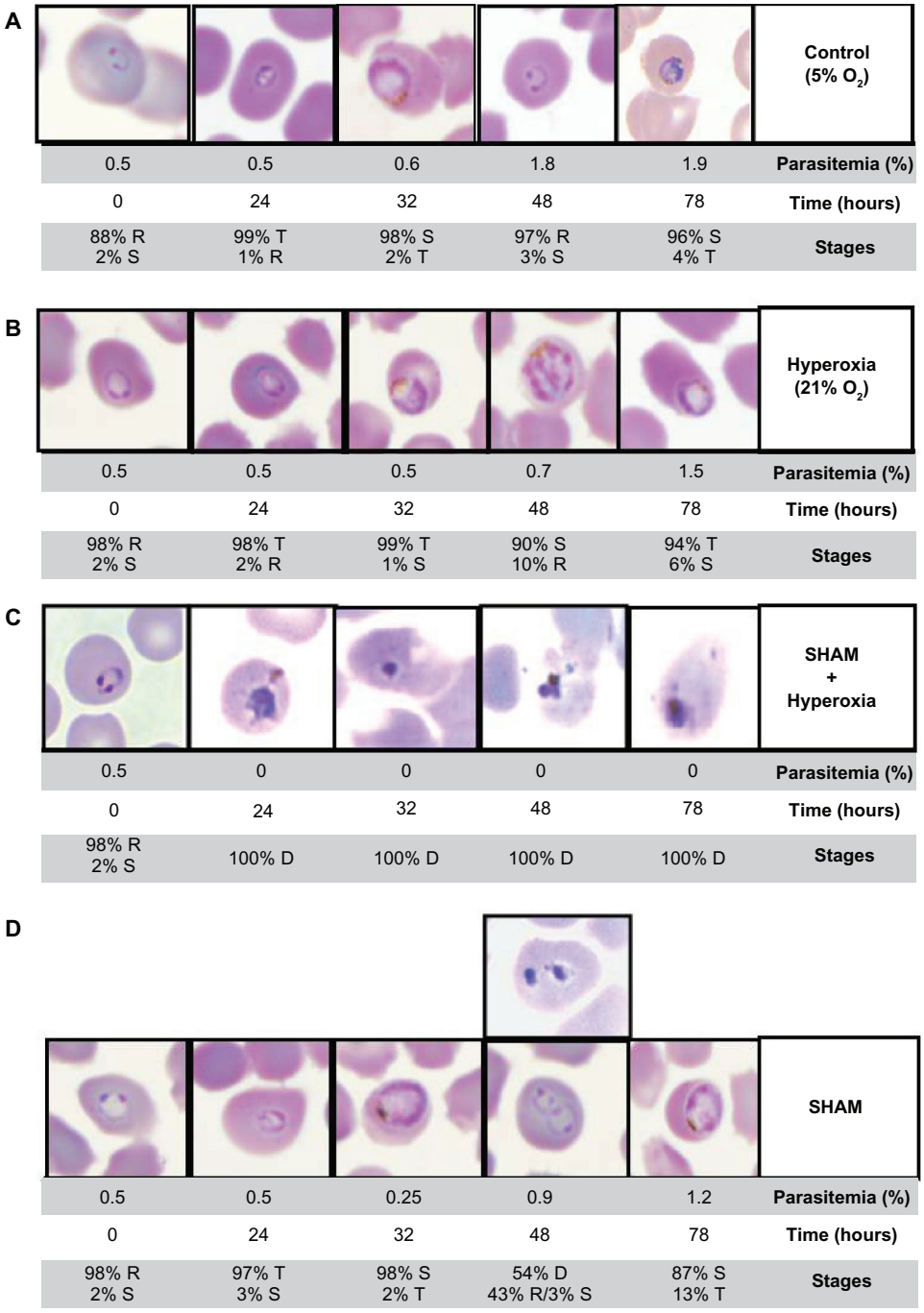

Figure 1 Morphologic alterations of *Plasmodium falciparum* following salicylhydroxamic acid (SHAM) pressure. Phenotypic changes of *P. falciparum* asexual blood stages were observed during two parasite cycles under (**A**) normoxia, (**B**) hyperoxia (ie, 21% oxygen), (**C**) SHAM/hyperoxia conditions, and (**D**) SHAM pressure. The parasitemia and parasitic stages were evaluated by blood smears at 0, 24, 32, 48, and 78 hours. After SHAM addition following hyperoxic exposure, significant morphological alterations were observed during the two cycles of parasites. The different intraerythrocytic stages and their corresponding percentages are indicated as follows: ring (R), trophozoites (T), schizonts (S), and parasite's death (D).

homogeneous 10% SDS-polyacrylamide gel electrophoresis, each gel was individually imaged, and all gel images were analyzed using DeCyder 6.5 software. Among 1998 matched protein spots, 18 spots were differentially modulated (|fold change| $\geq$ 1.5, $P \leq 0.05$ *t*-test) between the control and SHAM treatment (seven and eleven spots were up- and down-modulated, respectively; Figure 2). The corresponding protein spots were excised from gels, analyzed by LC-MS/MS, and searched against *P. falciparum* and *H. sapiens* databases (NCBInr). Eight protein spots not fulfilling the selection criteria were still selected and so submitted to MS analysis. Their *P* values were significant or very close to significance with

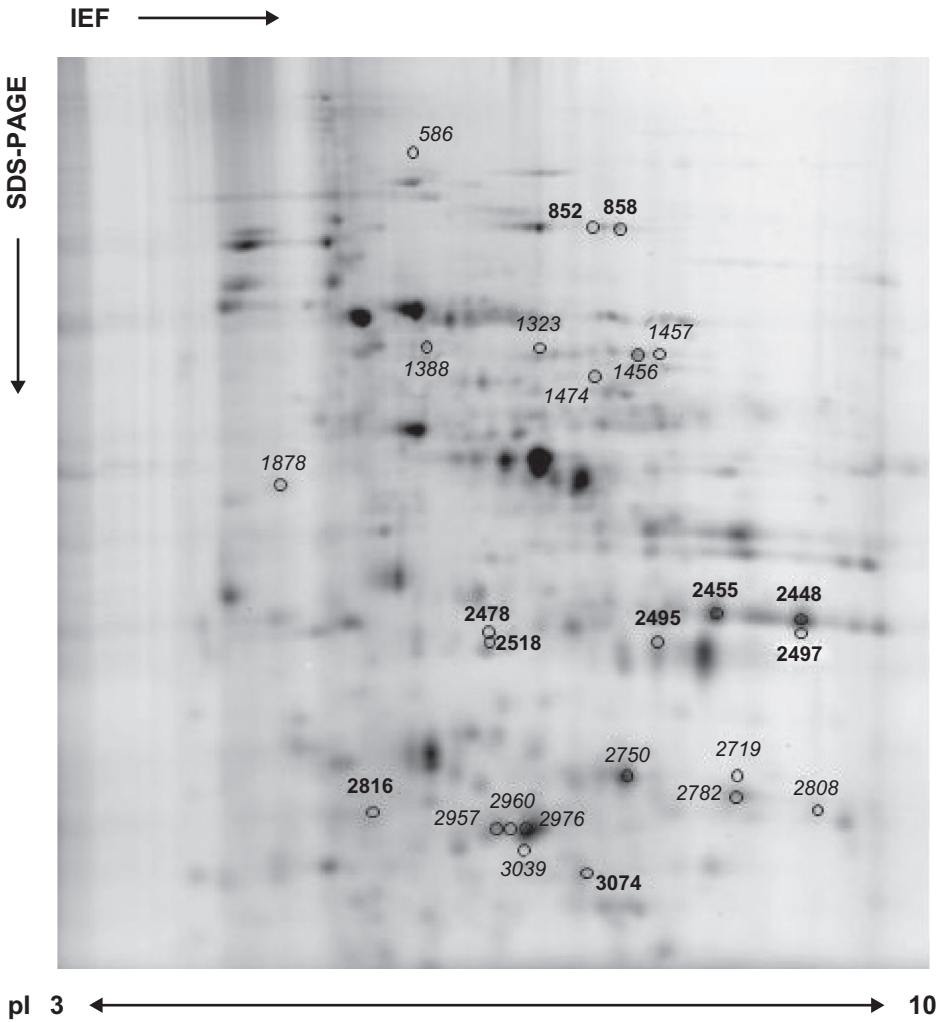

Figure 2 Salicylhydroxamic acid (SHAM) effect on *Plasmodium falciparum* 3D7 strain proteome. Representative data from a two dimensional-differential gel electrophoresis experiment using a 10% homogenous sodium dodecyl sulfate polyacrylamide gel with pH range 3–10 are shown. The proteins from untreated *P. falciparum* parasites or treated parasites with 250 μM SHAM were labeled with Cy3 and Cy5, respectively. As determined by DeCyder 6.5 software, protein spots that were up- and down-expressed on *P. falciparum* under SHAM treatment ($|FC| \geq 1.5$, $P \leq 0.05$ *t*-test) were marked with master numbers (Table 1). Bold and italicized numbers correspond, respectively, to identified proteins from *P. falciparum* and *Homo sapiens*.

|FC| closed from 1.5 (Table 1). With this last selection, a total of 26 protein spots were identified by MS that correspond to 10 *P. falciparum* and 16 *H. sapiens* specific proteins (Table 1). Among these eight last selected proteins, six correspond to isoforms supporting the abundance variation of significant deregulated proteins. Thus, some proteins were detected in more than one spot (eg, four for glyceraldehyde-3-phosphate dehydrogenase, three for catalase), suggesting the presence of different deregulated isoforms. So, the number of distinct proteins identified was five for *P. falciparum* and nine for *H. sapiens* (Table 1). The importation of human proteins into the parasite during erythrocytic cycle is a well-known phenomenon, as previously described in other studies.[39] The deregulated protein spots need to be confirmed on other wild *P. falciparum* strains or isolates.

To determine the metabolic pathways perturbed under SHAM pressure, the identified proteins were classified using the NCBI Clusters of Orthologous Groups of proteins database (Table 1). Among the functional categories, some proteins were predicted to play a role in glycolysis, chaperone-assisted protein folding, and redox metabolism. Among the antioxidant proteins, human catalase, carbonic anhydrase I, and carbonic anhydrase II were altered in SHAM-treated *P. falciparum*.

Discussion

SHAM and hyperoxia exposure affects *P. falciparum* growth

Previous studies provided evidence for the existence of a *P. falciparum* AOX[40] and supported the ability of SHAM

Table 1 Proteins identified from the two dimensional-differential gel electrophoresis analysis in salicylhydroxamic acid-treated *Plasmodium falciparum*

gi number	Gene identification	Protein name	MW (kDa)	pI	Master spot number	Significance (Mascot score)	Average ratio	*t*-test
Plasmodium falciparum								
Glycolysis								
gi|124810131	PF14_0598	glyceraldehyde-3-phosphate dehydrogenase	37.06	7.59	2448*	155	1.58	0.070
					2455	67	1.64	0.024
					2495*	47	1.66	0.057
					2497*	178	1.36	0.019
gi|124513266	PF13_0141	L-lactate dehydrogenase	34.33	7.12	2478*	146	1.96	0.056
					2518	175	1.72	0.049
gi|124802328	PF10_0155	enolase	49.02	6.21	2816	57	1.86	0.028
gi|124804024	PF11_0208	phosphoglycerate mutase	29.89	8.31	3074	48	–2.55	0.013
Translation								
gi|124809712	PF14_0486	elongation factor 2	94.54	6.36	852	346	1.50	0.039
			94.54	6.36	858*	279	1.42	0.051
Homo sapiens								
Redox metabolism								
gi|4502517		carbonic anhydrase I	28.91	6.59	2957	136	–2.26	0.013
					2960	160	–1.63	0.037
					2976	241	–3.72	0.031
gi|4557395		carbonic anhydrase II	29.28	6.87	3039	135	–2.26	**0.029**
Chaperone assisted protein folding								
gi|14124984		chaperonin containing TCP1, subunit 3 (gamma)	60.93	6.10	1323	96	1.49	0.0043
gi|48762932		chaperonin containing TCP1, subunit 8 (theta)	60.15	5.42	1388*	218	1.46	**0.0067**
Hemoglobin digestion								
gi|4557014		catalase	59.94	6.90	1456	191	–3.75	0.033
					1457	219	–3.63	0.029
					1474*	185	–3.29	0.055
gi|189054178		beta-globin	66.15	7.62	2719	267	–2.68	0.0032
					2750	244	–2.15	0.0071
					2782	154	–4.32	0.0067
					2808	112	–3.93	0.0021
Pentose phosphate cycle								
gi|5803187		transaldolase I	37.68	6.36	2478*	83	1.96	0.056
DNA replication								
gi|15029922		RNH1 protein	50.10	4.83	1878	114	1.58	0.024
Integral membrane proteins								
gi|62088410		spectrin, beta, erythrocytic variant	269.04	5.23	586	532	1.52	0.010

Notes: The spot number corresponds to the same numbers as indicated in Figure 2. The Mascot gi number of the spots, their gene identification (gene corresponding as found in PlasmoDB), their name, the theoretical MW, and pI values, as well as the corresponding Mascot score are listed for MS/MS analysis (scores greater than 34 for *Plasmodium falciparum* and 43 for *Homo sapiens* are considered as significant [$P < 0.05$]). Paired average volume ratio (experiment salicylhydroxamic acid versus control) and *P* values (*t*-test) were obtained using DeCyder 6.5 software. *Protein spots do not fulfill both criteria of selection, but which were nevertheless submitted to MS analysis thanks to their *P* values or FC significant.

Abbreviations: MW, molecular weight; pI, isoelectric point, MS, mass spectrometry.

to potentiate the activity of atovaquone, a complex III inhibitor, in in vitro chemosusceptibility assays.[21] Other studies have examined the effects of cyanide, a complex IV inhibitor, on *P. falciparum* respiratory metabolism using a polarographic assay.[38] In the presence of high concentrations of cyanide, 25% of the parasite oxygen consumption was residual, suggesting that the parasite exploits a cyanide-resistant alternative respiratory pathway. This residual oxygen consumption was totally inhibited by SHAM (coenzyme Q analog), a specific inhibitor of AOX enzymatic activity involved in this alternative pathway.[13]

Our results indicate that SHAM (IC_{50} 250 μM) induced the death of effectively 54% of the parasites but in hyperoxic conditions and to the same IC_{50}, parasite death is 100%. Therefore, in hyperoxic conditions, SHAM exposure has a deleterious effect on parasite survival. SHAM seems to inhibit the metabolic adaptation involved in hyperoxia, which is essential to parasite survival.

Consequently, as described in plants,[23,24] SHAM action seems to prevent the decrease in the levels of reactive oxygen species (ROS), which are exceedingly produced in hyperoxia by the mETC.[42] Because the AOX activity reduces oxygen, its activity should be dependent on the oxygen concentration and also on the coenzyme Q concentration and its redox state.[23] This alternative pathway could provide a regulatory function for excess electrons when the mETC is saturated (as during hyperoxia) and thus, decrease ATP production (unpublished data). Additionally, this "electron leak" mechanism may improve potential repression of the TCA cycle by elevated levels of NADH and ATP.[24]

SHAM alters the *P. falciparum* proteome

To investigate the metabolic pathways altered by the activity of SHAM, a comparative study on the *P. falciparum* proteome was performed using 2D-DIGE coupled to MS for identification. The experimental design (parasite stage, drug dose, incubation time) was chosen based on our preliminary results. In our study, SHAM was exposed at the IC_{50} concentration to ring stage parasites for 12 hours.

Most SHAM-altered proteins are involved in glycolysis and redox metabolism. The major implication of glycolytic enzymes (glyceraldehyde-3-phosphate dehydrogenase, L-lactate dehydrogenase, enolase, and phosphoglycerate mutase) suggests the need to sustain the major energy dependence of the parasite on glycolysis. Although the function of the *P. falciparum* mitochondrion remains unclear, its contribution to ATP production is considered to be minimal in asexual stages. *P. falciparum* relies principally on anaerobic glycolysis for energy production.[4] Therefore, the parasites are dependent on glycolysis in a microaerophilic environment while in hyperoxia conditions; our previous results showed that the respiratory metabolism is favored at the expense of glycolysis.[11] This observation could explain the lethal effect of SHAM in hyperoxic conditions. The NADH generated during glycolysis are reoxidized through glycerol-3-phosphate in the cytosol and by PfNDH2 in the mitochondria.[43,44] In plants, the alternative respiratory pathway involves an alternative NADH dehydrogenase, coenzyme Q, and AOX.[23] Mitochondrial AOX exists under two forms, covalent and noncovalent homodimers.[45] The oxidized covalent form of the enzyme is inactive, whereas reducing the disulfide bond generated by NADH activates the enzyme, possibly mediated by a glutathione/thioredoxin coupling system.[46] Interestingly, glycolysis activation under SHAM pressure could be the result of a metabolic adaptation in a microaerophilic environment. The end products of *P. falciparum* glycolysis are pyruvate, lactate, and glycerol. Firstly, pyruvate is an allosteric activator of AOX counterbalancing the AOX inhibition by SHAM. Under conditions of partial respiration, accumulation of pyruvate would result in increased NADH, which could potentially activate AOX.[2] Secondly, lactate, via L-lactate dehydrogenase, may be a substrate for the mETC under stress conditions.[43] Finally, glycerol production by the glycerol-3-phosphate permitted the reoxidation of NADH and the activity of the mETC[47] (Figure 3). In a microaerophilic environment, the activation of glycolysis is concordant with the role of glycolytic metabolism in the reoxidation of NADH,[48] an essential cofactor of the alternative respiratory pathway in *T. brucei*[26] and *C. parvum*.[44] Indeed, *T. brucei brucei* survives using an anaerobic pathway while *T. brucei brucei* AOX is inhibited.[26,41] Collectively, these results showed that SHAM, via its action on glycolysis metabolism, appears to play a role in NAD/NADH balance. The mechanism of regulation

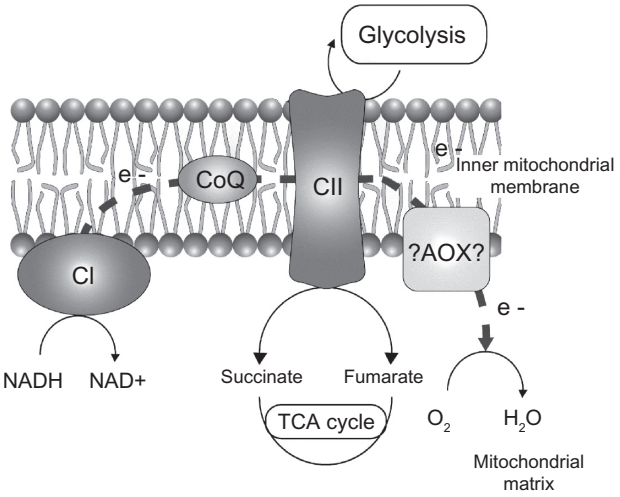

Figure 3 A schematic representation of the *Plasmodium falciparum* metabolic pathways perturbed under salicylhydroxamic acid treatment. Glycolysis and mitochondrial respiratory chain pathways based on the Ginsburg website are represented.[50]

of AOX activity involved several compounds, including those in redox states (NAD/NADH) and allosteric effectors (pyruvate).[49] Thus, the existence of an AOX function in *P. falciparum* could be beneficial for the survival of the parasite in hyperoxic environments as in the salivary glands of mosquitoes or perhaps in the human lungs.

SHAM treatment leads to an inhibition of antioxidant proteins. Among the proteins identified, some originated from the human erythrocyte host and correspond to host proteins imported by the parasite or proteins arising from host–parasite interaction. Consequently, cytosolic catalase and carbonic anhydrase I were down-represented under SHAM pressure. While *P. falciparum* lacks the antioxidant enzyme catalase, host catalase was imported into the food vacuole[39,50] and accomplished hydrogen peroxide detoxification.[51] Thus, the down-importation of catalase could be attributed to a SHAM effect against oxidative damage by diminishing hydrogen peroxide production. Carbonic anhydrase has been identified as a major intracellular peroxidation target in erythrocytes and could be attributed to oxidative stress induced by SHAM in the parasitized erythrocytes.[52]

In our study, a number of identified proteins (enolase, catalase, glyceraldehyde-3-phosphate dehydrogenase, carbonic anhydrase) have been previously found as potential glutaredoxin target proteins.[53] In higher plants, the reduction of AOX in its active form involved a glutathione/thioredoxin coupling system, suggesting a SHAM effect on feedback regulation of AOX activity as described in higher plants.[24,46]

The other deregulated proteins were particularly involved in protein processing (chaperonin-containing T-complex 1), as well as translation (elongation factor 2), consequences of a general stress state of the parasite. Chaperone-assisted protein folding was described to contribute to protein folding, assembly, and translocation, inducing expression of proteins in these pathways under several types of cellular stress.[54]

Conclusion

Although the AOX gene was not characterized yet in the *P. falciparum*, SHAM, an AOX specific inhibitor, disturbed the in vitro growth of *P. falciparum* in hyperoxic conditions. Additionally, recent studies sustained the existence of the alternative respiratory pathway in *P. falciparum,* illustrated by the identification of alternative NADH dehydrogenase, mostly described in some detail for plants.[13,55] Despite the fact that the *P. falciparum* AOX gene was not yet identified, the metabolic pathways involved in SHAM response illustrates the existence of a similar AOX function. The failure of a comparative sequence analysis is mainly due to the extreme adenine-thymine bias (80%) of the parasite genome or the high gene variability between other species and *P. falciparum*.[56] Nevertheless, an AOX-like function could play a role in oxidative stress defense and could be a major benefit to *P. falciparum*, which appears to be sensitive to hyperoxic conditions. Indeed, the respiratory metabolism was the predominant source of ROS on *P. falciparum,* and partial inhibition of this metabolism led to a decrease of ROS. This hypothesis is in agreement with a publication reporting the AOX protective role in preventing ROS production.[23]

Several studies on the use of mETC inhibitors underlined the crucial role of respiratory metabolism to parasite survival.[9] However, much work remains to scrutinize the specific biochemical pathways of the *P. falciparum* mitochondria.[8] This unique particularity of the alternative respiratory pathway could be a promising target for the development of a new group of antimalarial drugs.

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Disclosure

The authors report no conflicts of interest in this work.

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