Redox Biology 7 (2016) 21–29

Contents lists available at ScienceDirect

Redox Biology

journal homepage: www.elsevier.com/locate/redox

Research Paper

The antimalarial drug primaquine targets Fe–S cluster proteins and yeast respiratory growth

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ARTICLE INFO

Article history:
Received 13 October 2015
Received in revised form
22 October 2015
Accepted 22 October 2015
Available online 26 November 2015

Keywords:
Mitochondria
Malaria
Aconitase
Sod2
Oxidative stress
Yeast model
Primaquine

ABSTRACT

Malaria is a major health burden in tropical and subtropical countries. The antimalarial drug primaquine is extremely useful for killing the transmissible gametocyte forms of *Plasmodium falciparum* and the hepatic quiescent forms of *P. vivax*. Yet its mechanism of action is still poorly understood. In this study, we used the yeast *Saccharomyces cerevisiae* model to help uncover the mode of action of primaquine. We found that the growth inhibitory effect of primaquine was restricted to cells that relied on respiratory function to proliferate and that deletion of *SOD2* encoding the mitochondrial superoxide dismutase severely increased its effect, which can be countered by the overexpression of *AIM32* and *MCR1* encoding mitochondrial enzymes involved in the response to oxidative stress. This indicated that ROS produced by respiratory activity had a key role in primaquine-induced growth defect. We observed that Δ*sod2* cells treated with primaquine displayed a severely decreased activity of aconitase that contains a Fe–S cluster notoriously sensitive to oxidative damage. We also showed that *in vitro* exposure to primaquine impaired the activity of purified aconitase and accelerated the turnover of the Fe–S cluster of the essential protein Rli1. It is suggested that ROS-labile Fe–S groups are the primary targets of primaquine. Aconitase activity is known to be essential at certain life-cycle stages of the malaria parasite. Thus primaquine-induced damage of its labile Fe–S cluster – and of other ROS-sensitive enzymes – could inhibit parasite development.

1. Introduction

Malaria is a mosquito borne infectious disease that causes flu-like symptoms and can lead to organ failure and death. In 2013, an estimated 198 million cases of malaria were registered by the World Health Organisation (WHO, 2014).

The disease is caused by a unicellular eukaryotic parasite of the genus *Plasmodium*. Among the five species known to infect humans, *P. falciparum* is the most dangerous and *P. falciparum* and *P. vivax* are the most frequently encountered worldwide. *Plasmodium* parasites have a complex life cycle during which they adopt different forms [1]. In the context of malaria control and elimination, an increasing effort is focused on the transmissible blood stage forms of the parasite known as gametocytes and on the quiescent liver stage forms of *P. vivax* and *P. ovale* that cause relapses, known as hypnozoites. Targeting these parasite reservoirs currently relies solely on drugs. Primaquine is the only approved drug effective against hypnozoites and is also an efficient transmission-blocking agent through its gametocytocide activity (for review [2]).

Primaquine (PQ)[1] is an 8-aminoquinoline developed in the 1940's that displays antimalarial activity at low micromolar concentrations against hypnozoites [3] and gametocytes [4–6]. Despite this remarkable activity, its precise mechanism of action is still poorly understood. Ultrastructural and functional studies suggest that PQ treatment affects the parasite mitochondrion [7–10]. In humans, PQ is transformed into several metabolites mainly by the cytochrome P450 (CYP450) 2D6 and by the monoamine oxidase A [11]. Some PQ phenolic metabolites have been described to be the active compounds against *Plasmodium* parasites and are

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http://dx.doi.org/10.1016/j.redox.2015.10.008

most likely to act through an oxidative killing mechanism [12–14]. PQ was also shown to induce oxidative stress in mammalian cells [15,16]. In addition, PQ treatment provokes severe side effects in people deficient in erythrocytic enzymes controlling oxidative stress, such as the glucose-6-phosphate dehydrogenase (G6PD) and the NADH-methemoglobin reductase [17,18].

Here we explored the mechanism of action of PQ in the yeast *S. cerevisiae*. Yeast has well-known advantages for such studies, providing valuable genetic and biochemical tools. Deletion of non-essential genes is straightforward and collections of deletion mutants are available. In addition, yeast can survive in the absence of respiration by using the fermentation process as an energy source. Therefore the detrimental effect of a compound, such as PQ, can be tested in cells lacking enzymes involved in antioxidant defence with or without active respiratory function. Using this approach, we identified genes involved in PQ sensitivity. The data support key roles for oxidative damage and respiratory function in the action of PQ. We then tested the consequence of PQ exposure on Fe–S cluster containing enzymes known to be susceptible to oxidative damage.

[1] List of abbreviations: PQ, primaquine; SOD, superoxide dismutase; SO, superoxide; ROS, reactive oxygen species; OD, optical density; wt, wildtype strain; Fe–S, Iron–Sulphur.

2. Materials and methods

2.1. Chemicals

Primaquine, sodium L-ascorbate, DL-isocitric acid, L-malic acid, n-acetyl-L-cysteine, mitochondrial aconitase from porcine heart and *cis*-aconitate were purchased from Sigma Aldrich.

2.2. S. cerevisiae strains and culture media

S. cerevisiae strains from the series BY4742 (*MAT*α; *his3*Δ; *leu2*Δ; *lys2*Δ; *ura3*Δ), including this wt (wildtype) and derived isogenic deletion strains, were from Euroscarf (Frankfurt, Germany). The wt strain CWWT (*MAT*α *ade2-1 his3- leu2-3,112 trp1-1 ura3-1*) is derived from W303-1B.

The following growth media were used: YPD (1% yeast extract, 2% peptone, 2% glucose), YPEth (1% yeast extract, 2% peptone, 2% ethanol), YPG (1% yeast extract, 2% peptone, 3% glycerol), YPGal (1% yeast extract, 2% peptone, 0.2% glucose, 2% galactose), YP10 (1% yeast extract, 2% peptone, 10% glucose), and CSM (0.7% yeast nitrogen base, 2% glucose, 2% agar and 0.8 g/l of a complete supplement mixture minus uracil or minus leucine, supplied by Bio 101 (san Diego, CA, USA)).

2.3. Primaquine sensitivity test

PQ sensitivity was assessed by monitoring the inhibition of yeast cell proliferation. Yeasts were grown in 5 ml culture medium with increasing concentrations of primaquine. Cultures were inoculated from two-day-old cultures on YPG to an $OD_{600\ nm}$ of 0.1 and incubated at 28 °C. Cell density measured as $OD_{600\ nm}$ was estimated when the control cultures had reached stationary phase, one or three days after inoculation, depending on the culture medium. PQ sensitivity is presented for each strain and culture condition as the percentage of growth relative to control, *i.e.* untreated by PQ.

2.4. Generation of rho° mutants

Rho° mutants (devoid of mitochondrial DNA) were generated by growing cells on YPD medium containing 40 g/L of ethidium bromide for three rounds of culture.

2.5. Assessment of rho$^-$ or rho° production

The mutant Δ*sod2* was grown in YPD at 1 mM PQ. Three rounds of 24 h culture were performed. Cells were then diluted and spread on YPD plates to obtain approx. 200 cells per plate. After three days growth, the colonies were replica-plated to YPG agar. The number of colonies able to grow on this respiratory medium was recorded. This was compared to data obtained with untreated cultures.

2.6. Isolation of multicopy suppressors of primaquine sensitivity

The PQ-sensitive Δ*sod2* mutant was transformed with a high-copy wild type genomic library made in the *URA3* 2μ vector pFL44L [19]. Approximatively 20,000 Ura$^+$ clones were selected and replica-plated onto YPG containing 500 μM PQ. Clones with increased resistance to PQ appearing after 4–5 days were analysed. The plasmid-borne resistance to PQ was rechecked. The chromosomal fragments present on the plasmids were then identified by sequencing.

2.7. Gene cloning on high copy number plasmid for gene overexpression

SOD2, MCR1, SOD1 and *LYS7* genes with their own promoter region were cloned in the multicopy vector pFL44-URA3 (*SOD2* and *MCR1*), YEp352-URA3 (*SOD1*) or YEp351-LEU2 (*LYS7*). The fragment encompassing the *SOD2* gene (open reading frame (ORF) and native promoter) was digested with *BamH*I and *Xma*I enzymes from a vector isolated from the high copy number library containing the *SOD2* gene and then ligated between the *BamH*I-*Xma*I sites of the pFL44 vector. Fragments encompassing the *SOD1* and *LYS7* genes were digested with *EcoR*I and *Xba*I enzymes (*SOD1*) or *Sal*I and *Sac*I enzymes (*LYS7*) from the pRS405-SOD1 vector and pRS403-LYS7 vectors respectively. Those two vectors were kindly supplied by P. Piper, University of Sheffield, UK. The fragments were then ligated between the corresponding sites in the relevant destination vectors (see above). The *MCR1* gene fragment was amplified from yeast genomic DNA with the *Sal*I_MCR1_F (CATAGTCGACAATGCAAACTCTCCCACCAG) and MCR1_R (CTGCCAAGAAGACGTTGGTT) primers, digested with *Sal*I and *Sph*I enzymes and then ligated between the *Sal*I-*Sph*I sites of the pFL44 vector.

2.8. Aconitase and fumarase measurement using cell extracts

The aconitase and fumarase activities were determined spectrophotometrically by monitoring the formation of cis-aconitate and fumarate at 240 nm and 25 °C [20–22]. Briefly, cell extracts were prepared from 2.0×10^8 cells (OD_{600} ~20) grown on YPGal. Lysis was performed at 4 °C in 10 mM MES buffer, pH6 containing 0.6 mM $MnCl_2$ and deprived of oxygen (by bubbling with nitrogen gas) with 0.5 mm glass beads (v/v), by vortexing for 30 s followed by incubation on ice for 30 s, repeating the process seven times. Cell debris were removed by centrifugation at 13,000 rpm for 5 min, and the resulting supernatant was aliquoted and frozen immediately in liquid nitrogen and kept at −80 °C (for a maximum of one month). Samples were thawed just before the assay [23]. Protein concentration was determined spectrophotometrically with the Bio-Rad protein assay kit, according to the manufacturer's instructions.

For the aconitase activity, the assay mixture contained 50 mM potassium phosphate buffer, pH 7.4, 30 mM sodium isocitrate, 0.6 mM $MnCl_2$, and 150–250 μg of protein for a final volume of 1 ml. For the fumarase activity, the assay mixture contained