Molecular & Biochemical Parasitology 171 (2010) 17–24

Contents lists available at ScienceDirect

Molecular & Biochemical Parasitology

The flavin inhibitor diphenyleneiodonium renders *Trichomonas vaginalis* resistant to metronidazole, inhibits thioredoxin reductase and flavin reductase, and shuts off hydrogenosomal enzymatic pathways

David Leitsch[a,*], Daniel Kolarich[b,1], Michael Duchêne[a]

[a] Department of Specific Prophylaxis and Tropical Medicine at the Center for Physiology, Pathophysiology and Immunology, Medical University of Vienna, A-1090 Vienna, Austria
[b] Department of Chemistry, University of Natural Resources and Applied Life Sciences, A-1190 Vienna, Austria

ARTICLE INFO

Article history:
Received 6 October 2009
Received in revised form 10 January 2010
Accepted 12 January 2010
Available online 20 January 2010

Keywords:
Trichomonas vaginalis
Metronidazole
Drug resistance
Flavin metabolism
Diphenyleneiodonium

ABSTRACT

Infections with the microaerophilic protozoan parasite *Trichomonas vaginalis* are commonly treated with metronidazole, a 5-nitroimidazole drug. Metronidazole is selectively toxic to microaerophiles and anaerobes because reduction at the drug's nitro group, which is a precondition for toxicity, occurs only quantitatively in these organisms. In our previous work we identified the flavin enzyme thioredoxin reductase as an electron donor to 5-nitroimidazole drugs in *T. vaginalis* and observed that highly metronidazole-resistant cell lines lack thioredoxin reductase and flavin reductase activities. In this study we added the flavin inhibitor diphenyleneiodonium (DPI) to *T. vaginalis* cultures in order to test our hypothesis that metronidazole reduction is catalyzed by flavin enzymes, *e.g.* thioredoxin reductase, and intracellular free flavins.

Indeed, within hours, DPI rendered *T. vaginalis* insensitive to metronidazole concentrations as high as 1 mM and prevented the formation of metronidazole adducts with proteins. Thioredoxin reductase activity was absent from DPI-treated cells and flavin reductase activity was sharply decreased. In addition, DPI-treated cells also upregulated the expression of antioxidant enzymes, *i.e.* thioredoxin peroxidases and superoxide dismutases, and displayed a fundamentally altered metabolism caused by inactivation of pyruvate:ferredoxin oxidoreductase (PFOR) and concomitant upregulation of lactate dehydrogenase (LDH) activity. Thus, the disruption of the cellular flavin metabolism by DPI mediated metabolic steps which are similar to that of cells with metronidazole resistance induced *in vitro*. Finally, we present direct evidence that the increased expression of antioxidant enzymes is dispensable for acquiring resistance to metronidazole.

1. Introduction

Trichomonas vaginalis is a microaerophilic protozoan parasite that causes vaginitis and urethritis (trichomoniasis). In fact, trichomoniasis is the most frequent non-viral sexually transmitted human disease with more than 170 million cases reported per year [1,2]. Treatment of *T. vaginalis* infections is mainly based on metronidazole, also the drug of choice in the treatment of infections with other microaerophilic protozoan parasites such as *Entamoeba histolytica* and *Giardia intestinalis* [3]. Metronidazole is a nitroimidazole, *i.e.* one of a group of drugs which require reduction at their nitro group in order to exert toxicity [4], either *via* the single electron transfer reduction product, a nitroimidazole nitroradical anion, or *via* further reduced reactive intermediates, *i.e.* a nitrosoimidazole or hydroxylamineimidazole [5]. Reduction of nitroimidazoles can occur in microaerophilic/anaerobic as well as in aerobic organisms [6,7], but the interference of oxygen results in the quick reoxidation of the nitroradical anion to the parent compound, a redox cycling effect also termed "futile cycle" [8]. Under microaerophilic and anaerobic conditions, however, redox cycling does hardly or not at all occur, resulting in cell death due to reactive nitroimidazole intermediates. Nitroimidazoles with very low midpoint redox potentials, mainly 5-nitroimidazoles such as metronidazole, seem to act somewhat differently from 2-nitroimidazoles with higher midpoint redox potentials [9]. However, covalent adduct formation with proteins and non-protein-thiols was observed with 5-nitroimidazoles and 2-nitroimidazoles alike [10,11].

Abbreviations: DPI, diphenyleneiodonium; 2DE, two-dimensional gel electrophoresis; PFOR, pyruvate:ferredoxin oxidoreductase; hyd MDH, hydrogenosomal malate dehydrogenase; LDH, lactate dehydrogenase.

* Corresponding author. Tel.: +43 1 4277 64871; fax: +43 1 4277 64899.
E-mail address: david.leitsch@meduniwien.ac.at (D. Leitsch).
[1] Present address: Core of Biomolecular Frontiers, Macquarie University, Sydney 2109, Australia.

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doi:10.1016/j.molbiopara.2010.01.001

Since metronidazole has a very low midpoint redox potential (−486 mV) [12], the high susceptibility of microaerophilic parasites to metronidazole has also been ascribed to the abundance of factors with extraordinary low redox potentials, *e.g.* ferredoxin, an iron–sulfur cluster electron carrier protein [13]. In these organisms ferredoxin is mainly reduced by pyruvate:ferredoxin oxidoreductase (PFOR) during the oxidative decarboxylation of pyruvate [3]. Reduced ferredoxin, in turn, can then reduce nitroimidazoles, including metronidazole [14–18]. This scenario received strong support from research on trichomonads, *i.e. T. vaginalis* and the cattle parasite *Tritrichomonas foetus*, that display high-level resistance to metronidazole generated *in vitro* [19–24]. These metronidazole-resistant cell lines lacked ferredoxin, PFOR, and other enzymes that localize to the hydrogenosome [25], a mitochondrion-related organelle [26] typically found in trichomonads and first discovered in *T. foetus* [27]. Another hydrogenosomal enzyme which is involved in a pathway resulting in the reduction of ferredoxin, hydrogenosomal malate dehydrogenase, was also found to be widely missing in highly metronidazole-resistant trichomonads [19,24]. The above-mentioned findings led to a firm establishment of the model of hydrogenosomal metronidazole activation in *T. vaginalis*. However, in the past years this view has been seriously challenged by observations made in several laboratories. In *T. foetus* [28] as well as in *T. vaginalis* [11], depletion of iron from the growth medium with the iron chelating agent bipyridyl leads to the total or near-to-total loss of PFOR and hydrogenosomal malate dehydrogenase expression. Nevertheless, thus treated cells remain fully susceptible to metronidazole [11,29]. Also a genetic knock-out of ferredoxin 1 in *T. vaginalis*, leading to a concomitant decrease of PFOR activity to only 5% of the original level, had no effect on metronidazole susceptibility [30].

In contrast, the completion of the *T. vaginalis* genome project [26] allowed the recent discovery of several, presumably cytosolic, nitroreductases which could play a role in metronidazole reduction [31]. Furthermore, our previous work revealed an alternative pathway of metronidazole reduction by the flavin enzyme thioredoxin reductase in *E. histolytica* and *T. vaginalis* [10,11]. Accordingly, a *T. vaginalis* cell line with high-level metronidazole resistance generated *in vitro* almost entirely lacked thioredoxin reductase activity, obviously due to the lack of FAD cofactor, and was not able to reduce free flavins [11]. We consequently ascribed metronidazole resistance in *T. vaginalis* to a severe impairment of flavin-mediated redox reactions and further hypothesized that the loss of hydrogenosomal enzymes in metronidazole-resistant trichomonads could be a consequence of metronidazole resistance rather than its cause.

In the present study we tested these hypotheses by inhibiting the flavin-linked metabolism in *T. vaginalis* with diphenyleneiodonium (DPI), a flavin inhibitor that reacts *via* a radical mechanism and forms covalent adducts with flavins [32]. We assayed thus treated cells for metronidazole susceptibility and relevant physiological alterations.

2. Materials and methods

2.1. Chemicals

Diphenyleneiodonium chloride (DPI), NADPH, NADH and methylviologen were obtained from Sigma–Aldrich. Dithionitrobenzene (DTNB) was obtained from Merck Chemicals.

2.2. Cell culture

T. vaginalis C1 (ATCC 30001) were normally grown at 37 °C in 40 ml culture flasks (BD Biosciences) completely filled with TYM medium [33]. When anaerobic conditions were to be attained, cells were incubated in vented, half filled culture flasks in an anaerobic jar in the presence of Anaerocult™ A (Merck Chemicals), resulting in an atmosphere with 0% oxygen and 18% CO_2. For microaerobic conditions the same procedure was applied with Anaerocult™ C, resulting in an atmosphere with 5% oxygen and 8% CO_2. Aerobic conditions were attained by incubating cells in vented, half filled culture flasks in an aerated incubator. In contrast to our previous study [11], we used a granulated yeast extract from Merck Chemicals instead of BBL™ yeast extract from BD Biosciences. The growth medium was supplemented with 25 μg ml^{-1} ammonium iron(III) citrate when cells were to be used for PFOR, hydrogenosomal malate dehydrogenase, and lactate dehydrogenase enzyme assays.

2.3. Preparation of cell lysates for two-dimensional gel electrophoresis (2DE) and image analysis

Cells were harvested at room temperature by centrifugation at 700 × *g* for 5 min and washed twice with PBS to remove residual serum components. Preparation of cell lysates for two-dimensional electrophoresis (2DE) and two-dimensional gel electrophoresis were performed as described previously [34]. About 500–1000 μg of protein were applied per gel and per run. After staining, gels were scanned with an Epson 1680 Pro scanner and spots were quantitatively and qualitatively analysed with the Melanie™ 2D-gel analysis software (GeneBio).

2.4. Enzyme assays with hydrogenosome-free extracts

Hydrogenosome-free extracts were obtained after taking up cells in SM buffer [250 mM sucrose, 10 mM MOPS (3-morpholinopropane-1-sulfonic acid), pH 7.2] and subsequent disruption in a Dounce homogenizer. Afterwards, the extracts were centrifuged at 20,000 × *g* for 10 min in order to remove the hydrogenosomes. Thioredoxin reducing activity of cell extracts was measured by determining reduction of DTNB (OD_{412}) upon addition of recombinant *T. vaginalis* thioredoxin (30 μg ml^{-1}). Recombinant thioredoxin was produced as described before [11]. All measurements were performed under aerobic conditions at 37 °C in a Perkin Elmer Lambda UV/VID spectrometer. Extracts were used at a concentration of 40 μg of protein per reaction (100 mM Tris/HCl pH 7.5, 1 mM EDTA, 1 mM DTNB, 0.5 mM NADPH). Lactate dehydrogenase was measured as described [35] but by applying cell extracts at a concentration of 5 μg protein per reaction (100 mM Tris/HCl pH 7.5, 0.2 mM NADH, 1 mM EDTA, 2 mM pyruvate). Flavin reductase activity was measured with cell extracts (20 μg of protein per reaction) at λ = 340 nm by determining the consumption of NADPH (0.2 mM) upon addition of FMN (10 μM).

2.5. Assays with hydrogenosome-enriched fractions

After harvest, cells were resuspended in SMD buffer [250 mM sucrose, 10 mM MOPS (3-morpholinopropane-1-sulfonic acid) pH 7.2 and 10 mM dithiothreitol] and disrupted in a Dounce homogenizer. The hydrogenosome-enriched fractions were obtained by centrifugation at 20,000 × *g* for 20 min. The resulting pellets were resuspended and subsequently used for the enzyme assays. Measurement of PFOR activity was performed as described [27] by applying hydrogenosome-enriched extract at a concentration of 10 μg of protein per reaction. However, only 10 mM methylviologen instead of 20 mM was used in the assay. Hydrogenosomal malate dehydrogenase activity was measured according to [36] but the reaction buffer contained 1 mM NAD^+ and was buffered in 100 mM Tris pH 7.5. All measurements were performed at 37 °C in a Perkin Elmer Lambda UV/VID spectrometer.