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Transcriptional profiling of *Giardia intestinalis* in response to oxidative stress

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ABSTRACT

Giardia intestinalis is a microaerophilic parasite that infects the human upper small intestine, an environment that is fairly aerobic with reactive oxygen species being produced to fight off the parasite. It is quite perplexing how *Giardia*, lacking conventional eukaryotic antioxidant machinery (e.g. catalase, superoxide dismutase and glutathione peroxidase), can cope with the oxidative stress in this environment. We used transcriptomics (RNA sequencing and quantitative PCR) to study giardial gene expression changes in response to oxygen (O_2; 1 h) and hydrogen peroxide (H_2O_2; 150 μM, 500 μM and 1 mM for 1 h). The results showed phenotypic and transcriptional differences between *Giardia* isolates of different genotypes (WB, assemblage A and GS, assemblage B), with GS being more tolerant to H_2O_2 and exhibiting higher basic transcript levels of antioxidant genes (e.g. NADH oxidase lateral transfer candidate, peroxiredoxin 1 (Prx1) and thioredoxin (Trx)-like proteins). Cysteine is a major antioxidant in *Giardia* and its role in oxidative defense could be highlighted here by the up-regulation of gene transcripts encoding the cysteine-rich variable surface proteins (VSPs) and high cysteine membrane proteins (HCMPs). Genes in the thioredoxin system (Prx1, Trx and Trx reductase) occupied a central role in the gene expression response to oxidative stress, together with genes encoding metabolic (NADPH-producing enzymes, glutathione and glycerol biosynthetic enzymes) and O_2-consuming nitric oxide detoxification enzymes (e.g. nitroreductase, flavohemoprotein and a flavodiiron protein). This study reveals the intricate network of genes associated with the oxidative stress response in *Giardia*, and provides a stepping-stone towards future studies at the protein level.

1. Introduction

Giardia intestinalis is a unicellular, microaerophilic intestinal protozoan parasite that infects humans and other mammals (Thompson, 2000). Giardiasis, the disease caused by *Giardia* infection, is spread globally with 280 million cases reported annually (Lane and Lloyd, 2002) with a major impact on malnourished children in the developing world (Savioli et al., 2006; Silva et al., 2009). *Giardia* cysts, the infective form of the parasite, are ingested via the fecal-oral route and hatch in the small intestine, releasing actively dividing trophozoites (Ortega and Adam, 1997). Trophozoites attach to enterocytes in the upper small intestine, multiply and induce diarrhea and other associated symptoms (Ortega and Adam, 1997).

The small intestinal environment infected by *Giardia* is fairly aerobic and oxygen (O_2) concentrations fluctuate, peaking at 60 μM (Dawson et al., 1965; Atkinson, 1980; Sheridan et al., 1990; He et al., 1999; Mastronicola et al., 2014). Reactive oxygen species (ROS) are produced at low levels to promote signaling and intestinal homeostasis but at high levels to fight off invading pathogens (Spooner and Yilmaz, 2011; Jones et al., 2012; Neish and Jones, 2014). *Giardia* is able to consume O_2, however this activity ceases within 90 min at O_2 concentrations above 50 μM (Lloyd et al., 2000). The ability of *Giardia* to consume O_2 (i.e. respire) (Weinbach et al., 1980) implies that O_2 can serve as a terminal electron acceptor (Brown et al., 1998). Upon O_2 reduction, free oxygen radicals ($O_2^{\cdot-}$) and hydrogen peroxide (H_2O_2) are produced, and both can react with endogenous iron (Fe^{2+}) to produce highly toxic hydroxyl radicals ($^{\cdot}HO$) (Brown et al., 1998). In addition, an enzyme known as DT diaphorase (i.e. NAD(P)H:menadione oxidoreductase) has been shown to increase susceptibility of *Giardia* to O_2 by heightening the levels of intracellular H_2O_2 (Li and Wang, 2006). Under aerobic conditions glucose metabolism remains fermentative (Müller, 1988); however, pyruvate catabolism undergoes different pathways depending on O_2 pressure (Paget et al., 1990, 1993). At O_2 concentrations up to 46 μM, acetate is produced upon pyruvate decarboxylation (Paget et al., 1993). Electrons are passed

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to a pyruvate: ferredoxin oxidoreductase (PFOR) (Townson et al., 1996), an electron carrier ferredoxin (FdI) (Townson et al., 1994) and a final electron acceptor $NADP^+$. Both PFOR and FdI are O_2-labile, affecting the regeneration of an intracellular pool of NADPH and oxidised ferredoxin under high O_2 tension (Brown et al., 1998). The above aspects of *Giardia* metabolism imply that O_2 and its metabolic products will have detrimental effects on *Giardia* viability. Indeed, a body of evidence shows that oxidative stress in *Giardia* affects plasma membrane permeability, cell volume, homeostasis of the thiol pool and induces cell cycle blockage leading to trophozoite death (Lloyd et al., 2000, 2004; Paget et al., 2004; Ghosh et al., 2009). Given the aforementioned complexity of the small intestine in terms of the sheer oxidative stress therein, the ability of an amitochondriate organism such as *Giardia*, lacking all conventional eukaryotic O_2 detoxification systems (i.e. superoxide dismutase, catalase and glutathione peroxidase) (Brown et al., 1995), to thrive in the hostile small intestinal environment has been an enigmatic aspect of the parasite's biology.

Over the past years, many efforts have been made towards unraveling the antioxidant defense system in *Giardia*. A NADH oxidase has been suggested to be the primary player in defense against oxidative stress, owing to its ability to convert O_2 to water (Brown et al., 1995). *Giardia* NADH oxidase protects PFOR and FdI from oxidative damage and might function similar to mitochondrial cytochrome oxidase, promoting removal of excess H^+ (Brown et al., 1996a, 1998). Another suggested function is that it maintains an intracellular redox ratio in *Giardia* by regenerating NAD^+ (Brown et al., 1998). A membrane-associated NADH peroxidase has been also reported in *Giardia* with the ability to decompose H_2O_2, a member of ROS (Brown et al., 1995). The detailed function of this enzyme requires further investigation but it has been suggested to complement NADH oxidase (Brown et al., 1998). Recently, a flavodiiron protein (FDP) has been identified and characterised in *Giardia* (Di Matteo et al., 2008; Vicente et al., 2009a). In FDP, electrons are shuttled from a flavin mononucleotide (FMN) to a non-heme di-iron (Fe–Fe) active site, reducing equivalents derived from NADPH. FDP is capable of scavenging both O_2 and nitric oxide (NO); however, *Giardia* FDP has been shown to have a higher affinity for O_2 (Di Matteo et al., 2008). Therefore, FDP in *Giardia* provides a further means for reducing O_2 into water. Nevertheless, FDP is H_2O_2-sensitive and is degraded shortly after exposure to concentrations above 100 μM (Mastronicola et al., 2011).

In *Giardia,* reduced thiols and the thioredoxin system, including thioredoxin (Trx), thioredoxin reductase (TrxR) and NADPH, play an important role in maintaining a reduced intracellular environment, and thus defense against oxidative stress (Brown et al., 1998). Cysteine is the major low molecular weight thiol in *Giardia* (Brown et al., 1993) which has been shown, either alone or in combination with ascorbic acid (i.e. another antioxidant), to extend viability of *Giardia* for a longer period under oxygen stress (Gillin and Diamond, 1981a,b). The cysteine-rich variable surface proteins (VSPs) and high cysteine membrane proteins (HCMPs) might also play protective roles in defence against exogenous oxidative stress, basically due to their surface location and amino acid composition. To maintain an optimal intracellular thiol-disulphide ratio, *Giardia* has a NADPH-dependent Trx-like disulphide reductase (Brown et al., 1996b). This enzyme is capable of reducing cystine, preserving the intracellular levels of cysteine. Electrons are transferred from NADPH to their respective thiol-containing substrate and this helps to regenerate $NADP^+$ levels (Brown et al., 1996b, 1998). Interestingly, *Giardia* genome mining shows the presence of many hypothetical proteins (HPs) with Trx-like domains (www.giardiadb.www), indicating that the Trx system in *Giardia* might include a wider array of Trxs which are yet to be characterised. The Trx system and low molecular weight thiols including cysteine, thioglycolate, sulphite and CoA (Brown et al., 1993) are believed to be the major thiol cycling system in *Giardia* (Brown et al., 1998), and are equally important as NADH oxidase in defence against oxidative stress. *Giardia* also has peroxiredoxins (Prxs) and a recent characterisation of their function has shown their involvement in detoxifying O_2, H_2O_2, alkyl-hydroperoxides, and a compound called peroxynitrite ($ONOO^-$) produced from the reaction of $O_2^{\cdot-}$ with NO (Mastronicola et al., 2014). Prxs are cysteine-dependent peroxidases, in which a cysteine-sulfhydryl group (SH) is oxidised (e.g. by H_2O_2) to sulfenic acid (S–OH), which reacts with another SH, generating a disulfide bond. Trxs react with the generated disulfide bond to revert Prxs to their original reduced state (Hall et al., 2011; Poole et al., 2011). Overall, *Giardia* Prxs could be considered as wide range detoxifiers, which help protect the parasite against both oxidative and nitrosative stress.

To date, our understanding of the oxidative stress response in *Giardia* is still incomplete as to whether this response varies between isolates. Therefore, herein we used RNA sequencing (RNA Seq) technology to unveil the genes associated with gene expression changes upon oxidative stress in the reference isolates WB and GS that belong to the two major human genotypes (WB assemblage A and GS assemblage B). We show phenotypic differences between the isolates and identify changes in gene expression levels in response to O_2 and H_2O_2 including a nitroreductase (Fd-NR1), HCMPs, heat shock proteins (HSPs), Trx-like HPs, metabolic enzymes and many more.

2. Materials and methods

2.1. Parasite growth

The *G. intestinalis* reference isolates WB clone C6 (assemblage A) and GS (assemblage B) were used in this study. Both isolates were grown in 10 ml flat plastic tubes (Thermo Fisher Nunc, MA, USA) and 50 ml Falcon tubes filled with TYDK medium supplemented with bile (0.25 g/L) and 10% heat inactivated bovine serum (Life Technologies, CA, USA) (Keister, 1983). All materials used in the TYDK medium were purchased from Sigma-Aldrich (MO, USA). Cultures were incubated at 37 °C for 72 h until reaching peak densities and then used in the experiments. Trophozoites of each isolate (1×10^7) were also grown in 75 cm^2 tissue culture flasks filled with 30 ml of TYDK medium (vented cap, 37 °C, up to 20 h). Flasks were placed in a plastic bag with anaerobic environment-generating sachets (AnaeroGen, Oxoid Limited, Hampshire, UK) and were later used in experiments (see Section 2.3).

2.2. Experimental design

H_2O_2 is a major component of ROS and it was used as an oxidative stress-inducing agent in our experiments. The aim was to sequence the *Giardia* transcriptome upon exposure to a H_2O_2 concentration that did not affect parasite viability. Concentrations ranging from 100 μM to 3 mM H_2O_2 were tested and all incubations were performed under anaerobic conditions (Oxoid jar with AnaeroGen sachet, Oxoid Limited) to avoid intracellular ROS generation as a result of exposure to oxygen (Lloyd et al., 2000) and exacerbating the effects of H_2O_2 treatment on trophozoite viability. Freshly prepared medium was used in each experiment to eliminate any variation in results due to the age of the media.

Based on the data obtained from previous experiments (Ringqvist et al., 2011; Ma'ayeh and Brook-Carter, 2012; Ferella et al., 2014), *Giardia* trophozoites in DMEM, incubated alone or with intestinal epithelial cells (IECs) in a tissue culture incubator (5% CO_2, 95% air), exhibit a transcriptional profile associated with pronounced oxidative stress. Nevertheless, whether the induction of oxidative stress is related to the interaction medium, ROS produced by IECs or exposure to O_2 in the interaction environment,