Enzymatic detoxification of O$_2$ and NO in the human parasite, \textit{Giardia intestinalis}: A mini review

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\textit{Giardia intestinalis} is the etiological agent of giardiasis, a common human intestinal disease with 280 million cases per year. \textit{Giardiasis} is typically treated with the broad-range antibiotic metronidazole; however, the emergence of drug-resistant strains calls for the development of new anti-parasitic drugs. Very little is known regarding the molecular mechanisms adopted by \textit{Giardia} to cope with the oxidative/nitrosative environmental stress, encountered by the parasite during colonization of the human intestine. \textit{Giardia} is particularly sensitive to oxidative stress, as it lacks some of the most common ROS-detoxifying enzymes and it is endowed with O$_2$-labile key metabolic enzymes. Surprisingly, it colonizes a fairly aerobic (up to 50 µM O$_2$) tract of the human gut (the upper part of the small intestine). Accordingly, survival of the parasite relies on antioxidant systems, though, as yet, the only two H$_2$O-forming and O$_2$-consuming enzymes described in \textit{Giardia} are NADH oxidase and flavodiiron protein (FDP). Nitric oxide (NO) is an antimicrobial agent produced, together with ROS, by the host immune system to fight pathogens. \textit{In vitro} NO-stress has been reported to have cytostatic, rather than cytotoxic, effects on \textit{Giardia}. This effect leads to the suggestion that \textit{Giardia} is endowed with defense mechanisms against NO and, very recently, the NO-detoxifying flavohemoglobin from it has been characterized.

\textbf{Keywords:} Flavodiiron protein, flavohemoglobin, \textit{Giardia intestinalis}, NADH-oxidase, stress response

\section*{Introduction}

\textit{Giardia intestinalis} is an amitochondriate, microaerophilic, protozoan parasite that causes giardiasis, a common human intestinal disease with 280 millions symptomatic cases/year worldwide\cite{1}. \textit{Giardia} is also a potential zoonotic pathogen with an important economic impact, as it is capable of infecting productive animals\cite{2}. \textit{G. intestinalis} exists in two forms, the cyst and the trophozoite. The dormant, infective cysts are encapsulated in a hard wall and represent a survival strategy for the pathogen outside the host\cite{3}. The vegetative, disease-causing trophozoite is a pear-shaped cell characterized by the presence of two nuclei and a peculiar ventral adhesive disk used to attach to the intestinal epithelium. \textit{Giardiasis} is typically treated with the broad-range antibiotic metronidazole; however, the emergence of drug-resistant strains and the severe collateral effects of long-term treatments call for the development of new drugs.

Despite the anaerobic, essentially fermentative metabolism\cite{4}, \textit{Giardia} trophozoites colonize the human small intestine, where up to 50 µM O$_2$ has been detected\cite{5}. The parasite is, therefore, likely to be subjected to oxidative stress \textit{in vivo}. Accordingly, survival of \textit{Giardia} in the host should rely on efficient defence mechanisms against O$_2$ itself, as well as ‘reactive oxygen (ROS) and nitrogen species’ (RNS). In aerobic organisms, ROS detoxification is typically accomplished (among other enzymes) by superoxide dismutase, catalase and glutathione peroxidase. Surprisingly, none of these common antioxidant enzymes has been detected in \textit{G. intestinalis}\cite{6,7} and the only two H$_2$O-forming enzymes described to be involved in O$_2$ scavenging are NADH oxidase\cite{8} and flavodiiron protein (FDP$_{GI}$)\cite{9}.

Nitric oxide (NO) and its reaction product with superoxide anion (O$_2^-$), peroxynitrite (ONOO$^-$) are well known antimicrobial agents produced along with ROS by the host immune system to control infections caused by pathogens. Little is known regarding the mechanisms of NO-detoxification in \textit{Giardia}. NO inhibits growth, encystation and excystation of

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entamoeba histolytica were reported to afford protection from oxidative stress conditions are reviewed—the newly discovered antioxidant enzymes likely to promote parasite survival in the host are representing potential targets for new anti-parasitic drugs.

**O₂ Metabolism**

The protozoan parasite, *G. intestinalis* is amitochondriate. It thus lacks the Krebs cycle and oxidative phosphorylation pathways, thereby relying on glycolysis for its energy metabolism. *Giardia* displays a significant sensitivity to O₂, that is attributed to: (i) the expression of O₂-labile key metabolic enzymes, such as, pyruvate:ferredoxin oxidoreductase (PFOR); (ii) ROS produced by reaction of O₂ with NAD(P)H:menadione oxidoreductase (DT-diaphorase); and (iii) the lack of some of the conventional ROS scavenging enzymes, such as, catalase, superoxide dismutase and glutathione peroxidase. Somewhat paradoxically, this parasite colonizes the mucosa of duodenum and jejunum, where up to 50 µM O₂ was reported in the lumen. Consistently, *Giardia* is capable of effectively reducing O₂ to H₂O with a rate of 3-6 µM O₂ per min per 10⁶ trophozoites at 37°C. This O₂-consuming activity is inhibited by flavoantagonists, thiol inhibitors and metal chelators, and it was, therefore, originally attributed to a flavohemoglobin. Very recently, it has been shown that *Giardia* displays a significant sensitivity to O₂, which contains a Fe-Cys redox centre. Rubredoxin is reduced, in turn, by the NADH:rubredoxin oxidoreductase (NRO). Electrons derived from NADH are shuttled via FMN to the FDP diiron center and serve to catalyze the full reduction of O₂ (or NO) to H₂O (or N₂O) with apparent *Kₘ* values of 16 and 4.2 µM, respectively. For a decade, NADH oxidase was, therefore, suggested and universally accepted to be the enzyme responsible for *Giardia*’s atypical “respiration”, and was postulated to prevent potential generation of O₂•⁻ or H₂O₂ by acting as a terminal oxidase.

Flavodiiron proteins (FDPs) are modular flavoproteins identified in the late 90s by Wasserfallen and co-workers in several strict or facultative anaerobic bacteria and archaea. Interestingly, FDPs have also been found in a few protozoan parasites, namely *G. intestinalis, T. vaginalis* and *E. histolytica*. These enzymes share a common core of about 400 residues with two distinct domains: the C-terminal domain with a flavodoxin-like fold and a flavin mononucleotide (FMN) cofactor; the N-terminal domain with a flavodoxin-like fold and a flavin mononucleotide. The recombinant FDP from *Giardia* has also been identified in *Giardia* as an efficient, H₂O-forming O₂-reductase.

**O₂-Detoxifying Enzymes: NADH Oxidase and FDP**

NADH oxidases are flavoenzymes endowed with a conserved catalytic cysteine and are widespread in both prokaryotic and eukaryotic kingdoms. In prokaryotes, they catalyze the direct one-, two- or four-electron reduction of O₂ at the expense of NADH; thus, producing O₂•⁻, H₂O₂ or H₂O, respectively. The H₂O-forming NADH oxidases were reported to afford protection from oxidative stress in bacteria and were also detected in the microaerophilic protozoa *Trichomonas vaginalis*, *Entamoeba histolytica* and *G. intestinalis*.

The H₂O-producing NADH oxidase activity (1.2-2.5 units per mg protein) was first described in *Giardia* extracts in 1995 by Brown and co-workers. They were subsequently able to purify the NADH oxidase (46 kDa) from *Giardia* trophozoites, thereby showing that this enzyme efficiently catalyzes the full reduction of O₂ to H₂O, without production of O₂•⁻ or H₂O₂. The NADH oxidase is inhibited by mercurials, flavoantagonists and heavy metals (Cu²⁺ and Zn²⁺) and can oxidize both NADPH and NADH with *Kₘ* values of 16 and 4.2 µM, respectively. For a decade, NADH oxidase was, therefore, suggested and universally accepted to be the enzyme responsible for *Giardia*’s atypical “respiration”, and was postulated to prevent potential generation of O₂•⁻ or H₂O₂ by acting as a terminal oxidase.

**O₂-Forming NADH Oxidases**

In this paper, the enzymatic mechanisms of *Giardia* resistance to oxidative/nitrosative stress conditions are reviewed—the newly discovered antioxidant enzymes likely to promote parasite survival in the host are representing potential targets for new anti-parasitic drugs.
been shown to be essentially unreactive towards NO\(^9\). Therefore, we proposed that FDP\(_{Gi}\), acting alternatively/ synergically with NADH oxidase, plays a crucial role in vivo protecting \textit{Giardia} trophozoites from O\(_2\) toxicity. In 2000, Lloyd \textit{et al.}\(^{14}\) reported that, following exposure to H\(_2\)O\(_2\), \textit{Giardia} trophozoites undergo a severe O\(_2\)-consumption impairment. In this regard, we have recently shown that a short-term exposure of \textit{Giardia} trophozoites to H\(_2\)O\(_2\) (≥ 100 \textmu M, for 10 min) induces a fast proteolytic degradation of FDP\(_{Gi}\). The process is likely to be mediated by the proteasome, as it can be prevented by incubation of \textit{Giardia} trophozoites with the proteasome inhibitor MG132\(^{19}\). Other potential targets of H\(_2\)O\(_2\), including the NADH-oxidase and the yet unknown proteins implicated in the electron transfer to FDP\(_{Gi}\), can be envisaged. Taken together, these observations suggest that following cell exposure to H\(_2\)O\(_2\), among a number of (expected) detrimental effects, the reduction of O\(_2\) to H\(_2\)O is severely impaired, the proteasome is activated and the FDP\(_{Gi}\) is rapidly degraded, so that the reaction of O\(_2\) with ROS-producing enzymes, such as, DT-diaphorase\(^{16}\), is favoured. Consistently, O\(_2\) consumption by H\(_2\)O\(_2\)-treated parasites was shown to lead to H\(_2\)O\(_2\) accumulation\(^{19}\). This appears to be a vicious circle whereby toxicity of O\(_2\) and H\(_2\)O\(_2\) is enhanced, eventually causing cell death.

**Enzymatic Detoxification of NO in \textit{G. intestinalis}**

Among its numerous physiological functions in higher organisms, NO is produced by the immune system to fight microbial pathogens\(^{49,50}\). At present, very little is known regarding \textit{G. intestinalis} and its defense mechanisms against NO. Trophozoites were reported to inhibit the \textit{in vitro} production of NO by human intestinal epithelial cells through the consumption of arginine\(^{10}\), one of the substrate of NO synthase. It is unclear whether arginine is simply taken up by the parasite for metabolic purposes or is extracellularly metabolized by arginine-deiminase (ADI). Interestingly, Ringqvist and co-workers\(^{51}\) in 2008 found that ADI is secreted by \textit{Giardia}, together with other metabolic enzymes\(^{52}\), in response to the interaction with the intestinal epithelial cells. Very recently, the same authors have shown that the ADI-encoding gene is up-regulated in \textit{Giardia} trophozoites during the early phase (1.5 h) of the interaction with Caco-2 cells\(^{51}\). Conversely, in the late phase (18 h), ADI and carbamate kinase are down-regulated, thus resulting into a down-regulation of arginine metabolism\(^{51}\).

Most of the information available on NO and \textit{Giardia} has been acquired in \textit{in vitro} studies; whereas, currently, little is known about the control of \textit{Giardia} infection by NO \textit{in vivo}. There is a single report on NOS-deficient mice showing that the neuronal (NOS-1), rather than the inducible (NOS-2) isoform of NOS, contributes to \textit{Giardia} elimination\(^{53}\). In that study, however, the effect of NOS-1 on parasite clearance was attributed to the increased gastrointestinal motility favoured by NO, eventually resulting in a faster elimination of \textit{Giardia}, rather than to NO toxicity against the parasite. In other studies carried out in minimal media (RPMI medium or PBS), different nitrosative stressors (S-nitroso-acetyl-penicillamine, sodium nitroprusside, Roussin’s black salt or nitrite) were shown to induce cytotoxic effects in \textit{Giardia} trophozoites\(^{54-57}\). Specifically, under these conditions, nitrosatively stressed cells underwent loss of plasma membrane potential accompanied by ultrastructural changes, reduced capacity to consume oxygen, decreased flagellar motility and minor capacity to resist to osmotic stress\(^{55}\). All together, these effects explain the loss of cell viability observed in these studies upon nitrosative stress. In contrast, Eckmann and co-workers\(^{10}\) reported cytostatic, rather than cytotoxic effects in \textit{Giardia} trophozoites, following incubation with nitrosoglutathione (GSNO) or nitrite. NO did not affect cell viability, but it inhibited proliferation and development (encystation and excystation) of the parasite. The cytostatic effect of NO is interesting, as it leads to suggest that \textit{Giardia} is endowed with defense mechanisms against this radical, which enables the parasite to survive nitrosative stress. Interestingly, the protozoan pathogen \textit{Giardia} codes for a flavohemoglobin (FlavoHb\(_{Gi}\))\(^7\), a NO-detoxifying bacterial enzyme.
FlavoHb shown that the interaction of membrane protein (HCMp), cysteine desulfurase and response to oxidative/nitrosative stress, including a in stress response, such as, heat shock proteins, characterised by our group metabolizing enzyme was recently and concurrently possibly acquired via lateral gene transfer. This NO-metabolizing enzyme was recently and concurrently characterized by our group and by Yee and co-workers (Fig. 2).

In the presence of NADH and under aerobic conditions, the purified recombinant protein metabolizes NO with high efficacy ($V_{\text{max}}=116\pm10 \text{ s}^{-1}$ at [O$_2$]=200 µM) with an apparent O$_2$ affinity ($K_m=22\pm7$ µM) compatible with the physiological O$_2$ levels in the small intestine. In response to nitrosative stress, the expression of FlavoHb$_{Gi}$ markedly increases (~8 fold) in the trophozoites, which thereby become able to efficiently metabolize NO under aerobic conditions. Based on these results, it was proposed that physiologically FlavoHb$_{Gi}$ protects Giardia from NO toxicity.

Based on immunoblotting analysis, the protein in unstressed trophozoites is expressed at low levels and, accordingly, these cells aerobically metabolize NO with low efficacy. In response to nitrite-induced nitrosative stress, however, the expression of FlavoHb$_{Gi}$ markedly increases (~8 fold) in the trophozoites, which thereby become able to aerobically metabolize NO with higher efficiency. In line with previous studies on bacterial FlavoHbs and by Yee and co-workers (Fig. 2).

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Based on microarray analysis, it was recently shown that the interaction of Giardia trophozoites with Caco-2 cells stimulates the expression of FlavoHb$_{Gi}$ and other proteins putatively involved in response to oxidative/nitrosative stress, including a thioredoxin-like protein, a Group 1 high cysteine membrane protein (HCMP), cysteine desulfurase and nitroreductase. In this regard, it is interesting that an altered expression of several genes involved in stress response, such as, heat shock proteins, nitroreductase and protein disulphide isomerase PD14, was also found to correlate with resistance of Giardia to the antibiotics, nitazoxanide and metronidazole.

Concluding Remarks
Recent studies provided new insights into the molecular mechanisms underlying the response of human protozoan parasite, Giardia intestinalis to oxidative/nitrosative stress. The disease-causing Giardia trophozoites have been shown to express a flavohemoglobin protein likely involved, as the previously characterized NADH oxidase, in the O$_2$ detoxification, and a flavohemoglobin that efficiently catalyzes the degradation of NO. Noteworthy, FDP$_{Gi}$ and FlavoHb$_{Gi}$ were likely acquired from prokaryotes via lateral gene transfer events possibly to promote Giardia survival into the host. In this respect, these enzymes may be essential for Giardia survival under oxidative/nitrosative stress conditions in vivo, thereby representing potential targets for new anti-parasitic drugs.

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