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REVIEW

The oxygen reduction pathway and heat shock stress response are both required for *Entamoeba histolytica* pathogenicity

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Abstract Several species belonging to the genus *Entamoeba* can colonize the mouth or the human gut; however, only *Entamoeba histolytica* is pathogenic to the host, causing the disease amoebiasis. This illness is responsible for one hundred thousand human deaths per year worldwide, affecting mainly underdeveloped countries. Throughout its entire life cycle and invasion of human tissues, the parasite is constantly subjected to stress conditions. Under in vitro culture, this microaerophilic parasite can tolerate up to 5 % oxygen concentrations; however, during tissue invasion the parasite has to cope with the higher oxygen content found in well-perfused tissues (4–14 %) and with reactive oxygen and nitrogen species derived from both host and parasite. In this work, the role of the amoebic oxygen reduction pathway (ORP) and heat shock response (HSP) are analyzed in relation to *E. histolytica* pathogenicity. The data suggest that in contrast with non-pathogenic *E. dispar*, the higher level of ORP and HSPs displayed by *E. histolytica* enables its survival in tissues by diminishing and detoxifying intracellular oxidants and repairing damaged proteins to allow metabolic fluxes, replication and immune evasion.

Keywords *Entamoeba histolytica* · Pathogenicity · Oxidative stress · Heat shock

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Introduction

Entamoeba histolytica is the parasite responsible for amoebiasis in humans; it annually cause one hundred thousand deaths worldwide (World Health Organization 1997). In contrast with other true tissue-destroying microorganisms, like *Clostridium histolyticum* (Oakley and Warrack 1950), the histolytic designation of *Entamoeba* was not supported by a histolytic activity assay of the parasite; instead it was indirectly derived from the presence of tissue necrosis that characterizes the illness. For many years amoebic virulence was mainly attributed to the histolytic activity of the parasite and several cytotoxic molecules like phospholipases, amoebapores and adhesins, among others, were described. Furthermore, an interesting amoebic killing phenomenon named trogocytosis has been recently described (Ralston et al. 2014). In addition, this parasite produces and releases many cysteine proteinases which are able to digest several extracellular matrix components (Keene et al. 1986) and activate host collagenases (Thibeaux et al. 2014). However, although this impressive amoebic repertoire of potentially aggressive molecules occurring in in vitro conditions would be enough to account for tissue destruction in human amoebiasis, up to date there is not a convincing in vivo demonstration to justify the histolytic designation of *Entamoeba*. Instead, using the model of amoebic liver abscess formation in hamsters (ALAH), Tsutsumi et al. (1984) suggested that the immune host response was the principal responsible for tissue destruction in amoebiasis. Later, using leukopenic and immunosuppressed hamsters, this proposal was demonstrated and was also shown that inflammation is required for amoebic survival (Olivos-García et al. 2004, 2007).

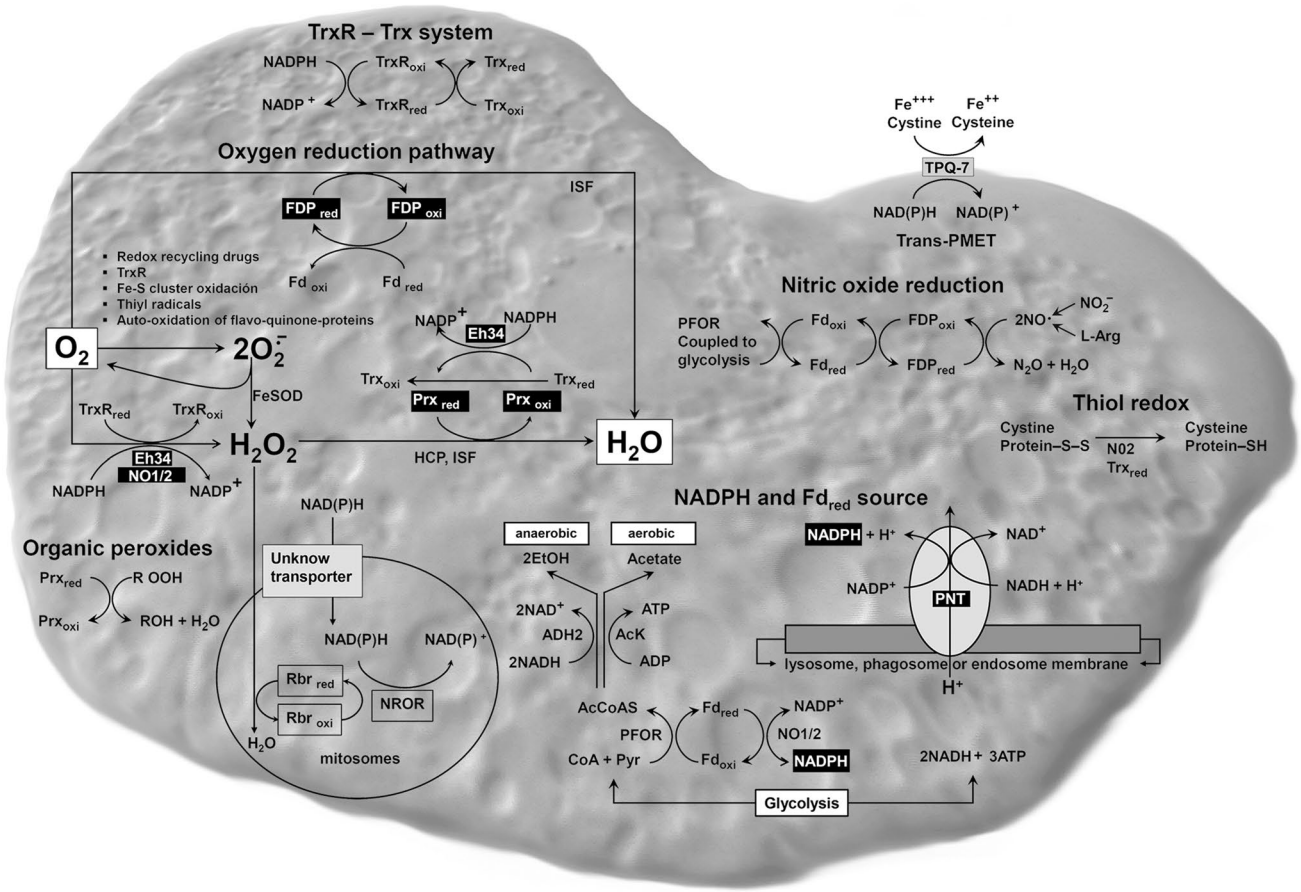

Fig. 1 Antioxidant pathways in *E. histolytica*. The parasite protects itself from O_2 and ROS mainly through the oxygen reduction pathway (ORP) assisted by other contributing systems. Part of the NADH and electrons from reduced Fd, generated by glycolysis, can be transhydrogenated by NADP-dependent oxidoreductases or transhydrogenase to produce NADPH which is further used as the reductive equivalent donor to reduce O_2 and ROS by the antioxidant machinery. Eh34, TrxR and EhNO1/2 generate H_2O_2 through divalent O_2 reduction. H_2O_2 is transformed to H_2O by Prx which is in turn reduced by Eh34 or Trx. Alternatively, using Fd as electron source, the FDP system reduces O_2 to H_2O without ROS generation. On the other hand, the FDP enzyme can detoxify the NO· generated by the parasite or that diffused from the microenvironment. Oxidized Trx is reduced by TrxR at expense of NADPH oxidation. The intracellular $O_2^{\cdot-}$ produced adventitiously is incorporated to ORP by a FeSOD. Rbr and its redox partner NROR, that uses NADPH, protect mitosomes from oxidation by H_2O_2. Although other enzymes as ISF and HCP with antioxidant capacity are present in the genome of *E. histolytica*, their specific function awaits to be revealed. Other functions attributed to Prx are detoxification of organic peroxides, and together with NO2 and Trx they are proposed to maintain the intracellular redox state (cysteine/cystine). Also amoebae may diminish the redox potential of the surroundings through the trans-PMET. Finally, the ORP protects the glycolytic ADH2 and PFOR (among others) from oxidation by O_2 and ROS. Abbreviations: Iron sulfur flavoprotein (ISF); flavodiiron protein (FDP); NAD(P)H-dependent rubredoxin reductase (NROR); rubrerythrin (Rbr); hybrid cluster protein (HCP); NADPH: flavin oxidoreductase (Eh34); peroxiredoxin (Prx); NADPH-dependent oxidoreductases (NO1/2); Fe-superoxide dismutase (FeSOD); transplasma membrane electron transport (Trans-PMET); thermoplasmaquinone-7 (TPQ-7); thioredoxin (Trx); thioredoxin reductase (TrxR); ferredoxin (Fd); pyruvate:ferredoxin oxidoreductase (PFOR); pyridine nucleotide transhydrogenase (PNT); bifunctional aldehyde–alcohol dehydrogenase (ADH2); Coenzyme A (CoA); Pyruvate (Pyr); Acetate kinase (AcK); ADP-forming acetyl-CoA synthethase (AcCoAS); reduced (red); oxidized (oxi)

In microaerobic environments, such as those present in axenic culture or in the lumen of the human colon (O_2 concentration 0.1–2.3 %) (Ladas et al. 2007), detoxification of O_2 by its reduction to H_2O may favor parasite growth (Gillin and Diamond 1981) probably by consumption of electrons generated by glycolysis in the form of NADH and reduced ferredoxin (Fd_{red}) (Fig. 1) (Olivos-García et al. 2012; Cabeza et al. 2015). This active O_2 consumption may justify the designation of *E. histolytica* as a microaerophilic organism.

In culture conditions *E. histolytica* can tolerate up to 5 % PO_2 (Band and Cirrito 1979); however, during the initial stages of ALAH, amoebae are exposed to higher O_2 concentrations (4–14 % PO_2) (Nauck et al. 1981) and host's complement molecules that could be responsible for the massive amoebic death that occurs during the first 12 h

of infection (Rigothier et al. 2002); the increased amoebic sensitivity to complement after O_2 exposure (unpublished observation) supports this proposal. The ischemia generated by inflammation may decrease the parasite contact with dangerous molecules like complement, antibodies and O_2. In addition, since an immune response generates reactive oxidants, the microenvironment in the latest stages of ALAH is expected to be oxidant. Both the amoebic antioxidant system and the heat shock response required to contend with the oxidative stress, are revealed by the study of the loss of *E. histolytica* virulence by prolonged culture: intracellular hypoxia and heat shock response are necessary conditions for amoebic survival in tissues and therefore for pathogenicity (Santos et al. 2015).

This review is based on the ALAH model. However, other important amoebic pathogenicity mechanisms related to intestinal amoebiasis can be found in the recent review by Marie and Petri (2014).

Why amoebae need to maintain intracellular hypoxia in the oxygenated tissues?

Oxygen, $O_2^{\cdot-}$ and H_2O_2 oxidize at different rates the Fe–S centers of some enzymes present in aerobic and microaerophilic cells (Imlay 2003). The most common $[4Fe–4S]^{2+}$ clusters in proteins are oxidized to the form $[3Fe–4S]^{1+}$ and then to $[2Fe–2S]^{2+}$ clusters. If oxidant conditions persist, the $[2Fe–2S]^{2+}$ can be further degraded yielding the apoenzyme forms (Crack et al. 2008). Since most of the Fe–S containing enzymes are involved in energy metabolism or antioxidant activity, their inactivation by oxidation can lead to cellular arrest (by reversible oxidation of the proteins), low ATP and NADH, intracellular acidification, loss of membrane integrity, and death. In contrast with mammalian cells, in anaerobic microorganisms Fe–S clusters of some proteins are exposed to the solvent to work efficiently at low redox potential (~−300 mV) but at the same time this property makes them susceptible to oxidation in aerobic environments (Outten and Theil 2009). As many microaerophilic parasites, *E. histolytica* also obtains energy by fermentative glycolysis (Reeves 1984; Saavedra et al. 2005). Pyruvate:ferredoxin oxidoreductase (PFOR) is involved in oxidative decarboxylation of pyruvate to produce acetyl-CoA, which is further metabolized to ethanol by a bifunctional aldehyde-alcohol dehydrogenase (ADH2). PFOR contains three 4Fe–4S clusters and ADH2 contains a Fe^{2+} atom in the alcohol dehydrogenase domain, essential to preserve its aldehyde dehydrogenase activity. It has been demonstrated that PFOR and ADH2 are inactivated in vitro by O_2 and H_2O_2 and both enzymes are highly inhibited when the parasites are subjected to extreme oxidant conditions affecting energy metabolism parameters (Ramos-Martínez et al. 2009; Pineda et al. 2013). Due to the abundance and extreme sensitivity to reversible/irreversible inactivation by oxidants, amoebic PFOR activity has been used as a marker of oxidative and nitrosative stresses and to evaluate the oxidative status of the amoebic Fe–S clusters (Pineda et al. 2013). Hence, an adequate system for O_2 detoxification is required for proper energy metabolism in the parasite.

The low activity of the O_2 reduction pathway (ORP) of the non-pathogenic *E. dispar* allows it to resist the PO_2 of 0.2–2 % present in the human colon (Ladas et al. 2007); however, it is insufficient to endure the 4–14 % PO_2 present in well-perfused tissues like the liver (Nauck et al. 1981). In contrast, *E. histolytica* contains a proficient ORP that maintains O_2 and its reactive species at permissive levels for cell survival (Santos et al. 2015). The principal enzymes of the ORP are: (a) a flavodiiron protein (FDP) that transforms O_2 into H_2O (Vicente et al. 2012) by the use of Fd as its reductant (Cabeza et al. 2015), (b) a NADPH:flavin oxidoreductase (Eh34) able to reduce O_2 into H_2O_2 at the expense of NADPH oxidation (Bruchhaus et al. 1998), and (c) peroxiredoxin (Prx) that detoxifies H_2O_2 to H_2O using either Trx or Eh34 as redox partners (Bruchhaus et al. 1997). A role of Fe-superoxide dismutase (FeSOD) as part of the main amoebic ORP (Fig. 1) may be disadvantageous because O_2 is a byproduct of the reaction; furthermore, none of the enzymes characterized in *E. histolytica* that detoxify O_2 are able to generate $O_2^{\cdot-}$ as a deliberate stoichiometric product. In any case when FeSOD products O_2 and H_2O_2 emerge, they are detoxified by FDP or the Eh34-Prx couple system. Other enzymes could also aid to detoxify O_2 and reactive oxygen species (ROS) (Fig. 1); the ORP proteins, however, are the best established for that purpose. Their expression is both constitutive and induced in oxidative environments, either in vitro or in vivo conditions (Olivos-García, unpublished results) and the protein overexpression may be regulated at posttranscriptional level since transcriptomic analyses under O_2 and H_2O_2 stresses have not showed significant upregulation of the ORP genes (Vicente et al. 2009; Santos et al. 2015).

ATP and reducing equivalents generated by fermentative glycolysis are required by the enzymatic and non-enzymatic antioxidant pathways to protect against O_2 and ROS (among others) (Fig. 1). A proper glycolytic flux is a necessary requirement for survival in oxygenated environments. The similar cellular distribution of ORP proteins (enrichment of the inner of plasmatic membrane; personal observation) with glycolytic enzymes such as PFOR (Rodríguez et al. 1998) suggests that in oxidative environments, ORP may prevent Fe^{2+} and Fe–S oxidation in proteins. Other reasons that justify the intracellular location of ORP enzymes are their dependence on the cytosolic components NADPH, Fd and Trx-TrxR system, among others (Fig. 1).

In fact, downregulation of Prx protein has shown that in well-oxygenated tissues like the liver, detoxification of intracellular H_2O_2 generated from O_2 reduction is essential for amoebic survival and therefore for pathogenicity (Sen et al. 2007). Thus the main role of FDP and Eh34, in a concerted action with Prx, could be to maintain an intracellular hypoxic environment at an acceptable level for metabolic work. That is why ORP represents a potential therapeutic target.

Why amoebae need to overexpress HSPs in an oxygenated environment?

All cell proteins must fold into precise three-dimensional conformations to fulfill their biological functions. This fundamental process is procured by molecular chaperones to assist in folding, assembly, intracellular localization, secretion, regulation and degradation of different proteins by the ubiquitin–proteasome system. Proteins are major targets for oxidants as a result of their abundance in biological systems and their high rate constants for reaction. During several types of stresses, chaperons increase folding and prevent protein misfolding/aggregation and at the same time promote degradation of oxidized proteins by the lysosome/vacuolar system. Heat shock proteins (Hsp) are ATP-dependent enzymes usually classified according to their molecular weights (Hsp40, Hsp60, Hsp70, Hsp90, Hsp100 and the so-called small Hsps). With some exceptions, members of all these classes are present throughout the three life kingdoms (Vabulas et al. 2010). Several HSPs have been reported in *E. histolytica*: Hsp10, Hsp40, Hsp60, Hsp70, Hsp90, Hsp100 and Hsp101. With the exception of Hsp10 and Hsp60, most of them (transcripts or proteins) are overexpressed by heat (Field et al. 2000; Bernes et al. 2005; Weber et al. 2006), O_2 (Santos et al. 2015), H_2O_2 and NO· (Vicente et al. 2009). The specific function of HSPs is based on the way they interact with substrates, i.e., holders (HSP40), foldases (HSP70 and HSP90) and disaggregases (HSP100, HSP101 and ClpB) (Mokry et al. 2015). In addition, moderated upregulation of ubiquitin genes after such stresses suggests generation of unfolded proteins.

Other important function of HSP70 could be to participate in reparation of Fe–S clusters in proteins. In *E. histolytica* this function is normally performed by the Nitrogen Fixation (NIF) and Cytosolic Iron–sulfur protein Assembly (CIA) systems. However, because the NIF system is sensitive to oxidative stress there must be alternative mechanism(s) to protect Fe–S clusters and NIF system from hyperoxia (Ali and Nozaki 2013). In *E. coli* hscA, a cognate of HSP70, is implicated in de novo synthesis and probably in reparation of Fe–S clusters in proteins (Vickery and Cupp-Vickery 2007). Similarly, when *E. histolytica* is exposed to oxidative environments, such functions may hold on HSP70 since its specific inhibition promotes irreversible PFOR inactivation and amoebic death (Santos et al. 2015). Also, such inhibitions diminished the ability of *E. histolytica* to cause ALAH (Santos et al. 2015). Additionally to its chaperonin activity, during amoebic exposure to tissue hyperoxia the inducible HSP70 protein could play an important role in parasite survival by maintaining the glycolytic fluxes through repairing/de novo synthesis of the Fe–S clusters or iron atoms present in proteins. A similar relationship between resistance to oxidative stress, HSP response and virulence has been observed in *Salmonella typhimurium* (Kaufmann 1991).

How are the antioxidant and HSP responses linked to *E. histolytica* pathogenicity?

During ALAH, amoebic contact with inflammatory cells is more extensive than with liver cells (Tsutsumi et al. 1984). Immune cells are potentially able to produce amoebicidal molecules like ROS, HOCl, NO·, porins and granulysins, among others. In addition, the complement system and antibodies could be dangerous for the parasite. A condition for amoebic pathogenicity is therefore its high ability to evade the immune system, through: (1) neutralizing oxidants, (2) deactivating leucocytes, (3) killing the contacted cells by apoptosis–phagocytosis or trogocytosis, and (4) capping of surface receptors and complement activation in fluid phase. In order to fulfill such functions, the parasite requires at least a well preserved cytoskeleton, plasmatic membrane integrity and both high ATP and reductive equivalents produced by the glycolytic pathway. Since some glycolytic enzymes are susceptible to the oxidants present during tissue invasion (O_2, ROS and NOS), the high activities of antioxidant pathways, including ORP (Fig. 1), as well as the HSP response are necessary to preserve plasmatic membrane integrity and to allow glycolytic fluxes, all related with parasite pathogenicity.

The complete picture of amoebic pathogenic mechanisms will reveal new therapeutic strategies to reduce the morbidity and mortality of human amoebiasis that affects mainly underdeveloped countries.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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