Hydrogen peroxide induces apoptosis-like death in *Entamoeba histolytica* trophozoites

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Programmed cell death (PCD) is an essential process in the growth and development of multicellular organisms. However, accumulating evidence indicates that unicellular eukaryotes can also undergo PCD with apoptosis-like features. This study demonstrates that after exposure to 0.8 mM H₂O₂ for 9 h *Entamoeba histolytica* presents morphological and biochemical evidence of apoptosis-like death. Morphological characteristics of apoptosis-like death including DNA fragmentation, increased vacuolization, nuclear condensation and cell rounding were observed for H₂O₂-exposed trophozoites with preservation of membrane integrity. Biochemical alteration in ion fluxes is also a key feature in PCD, and H₂O₂-exposed trophozoites showed overproduction of reactive oxygen species, increased cytosolic Ca²⁺ and decreased intracellular pH. Phosphatidylserine was also found to be expressed in the outer leaflet of the plasma membrane of the H₂O₂-treated trophozoites. Pretreatment with the cysteine protease inhibitor E-64d, the extracellular and intracellular Ca²⁺ chelators EGTA and BAPTA/AM, and the Ca²⁺ influx inhibitor verapamil prior to H₂O₂ exposure abolished DNA fragmentation. The oxidatively stressed trophozoites also showed an increased calpain activity, indicating involvement of Ca²⁺-dependent calpain-like cysteine proteases in PCD of *E. histolytica*. A homogeneous caspase assay showed no significant caspase activity, and administration of caspase 1 inhibitor also did not prevent the death phenotype for the oxidatively stressed trophozoites, indicating a caspase-independent apoptosis-like death. Our observations clearly demonstrate that there is a distinct calpain-dependent but caspase-independent pathway for apoptosis-like death in oxidatively stressed *E. histolytica* trophozoites.

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INTRODUCTION

Entamoeba histolytica, an enteric protozoon, is a well-established causative agent of amoebic dysentery and liver abscesses in humans (WHO, 1998). The parasite usually lives and multiplies within the human gut, which is an environment of low oxygen concentration. During tissue invasion *E. histolytica* is exposed to elevated amounts of exogenous reactive oxygen species (ROS) such as the

superoxide radical anion (O₂⁻) and H₂O₂ (Clark *et al.*, 1986; Murray *et al.*, 1981). The protozoa also encounter the ROS produced by endogenous enzymes and the respiratory burst of the host immune system.

Programmed cell death (PCD) is a suicidal pathway that has clear benefits for multicellular organisms, by eliminating cells which are damaged, infected or simply no longer required. Thus, in mammals, PCD is extremely important during development (Jacobson *et al.*, 1997) and in immune system functions (Greil *et al.*, 1998; Evan & Littlewood, 1998). In contrast to necrosis, cells that die by apoptosis are not lysed, but are eliminated by phagocytosis, thus preventing the development of an inflammatory response.

The benefits of PCD in unicellular organisms are less evident. In such organisms, it leads to death of the entire organism and therefore it is hard to understand how 'suicide' might be advantageous. There are, however, an increasing number of reports describing that some unicellular organisms may also

Abbreviations: AMC, 7-amino-4-methylcoumarin; BAPTA/AM, 1,2-bis(2-aminophenoxy)ethane-*N,N,N',N'*-tetraacetic acid tetrakis(acetoxymethyl ester); BCECF, 2,7-bis(2-carboxyethyl)-5-(and 6)-carboxyfluorescein; [Ca²⁺]_i, intracellular calcium; E-64d, (2S,3S)-trans-epoxysuccinyl-L-leucylamido-3-methylbutane ethyl ester; eh29, thiol-dependent peroxidase; H₂DCFDA, 2',7'-dichlorodihydrofluorescein diacetate; MDA, malondialdehyde; MTT, 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazolium bromide; PCD, programmed cell death; PI, propidium iodide; PS, phosphatidylserine; ROS, reactive oxygen species; SOD, superoxide dismutase; Fe-SOD, iron-containing superoxide dismutase; TG, thapsigargin.

undergo PCD under certain conditions (Welburn *et al.*, 1997; Ameisen, 1996; Christensen *et al.*, 1998a, b). One apparent advantage of apoptosis to a protist 'colony' occurs during periods of nutrient depletion: if a proportion of the colony undergoes apoptosis, nutrients can be conserved and recycled, and less fit individuals can be culled from the population (Debrabant & Nakhasi, 2003). Altruistic suicide associated with nutrient conservation has been suggested to occur in a cohort of the insect gut stage of kinetoplastids (Welburn, 1996) and the mosquito midgut stages of *Plasmodium berghei* (Al-Olayan *et al.*, 2002). Evidence for a cell-suicide pathway in unicellular organisms that is analogous to metazoan apoptosis strongly suggests that PCD confers evolutionary advantages upon micro-organisms, including (i) selection of the best-adapted individuals in response to environmental changes (Lee *et al.*, 2002; Verma & Dey, 2004), (ii) regulation of the competition of parasites for limited resources in the gut or within host tissues (Dale *et al.*, 1995), (iii) regulation of the cell cycle and cell differentiation (Hesse *et al.*, 1995), and (iv) selection of specific parasitic forms, as non-infectious forms do not contribute to perpetuation of the parasite and might compete with the infectious parasites for available nutrients (Welburn *et al.*, 1997).

Various forms of PCD have been described in at least 13 unicellular eukaryotic species belonging to six different branches of the phylogenetic tree. These include non-parasitic organisms such as the slime mould *Dictyostelium discoideum* (Cornillon *et al.*, 1994), the dinoflagellate *Peridinium gatunense* (Vardi *et al.*, 1999) and the ciliate *Tetrahymena thermophila* (Christensen *et al.*, 1998a, b). In parasitic organisms, PCD has been described for kinetoplastid parasites of the genera *Trypanosoma* and *Leishmania* (Shaha, 2006; Duzenko *et al.*, 2006), *Giardia lamblia* (Chose *et al.*, 2003), *Blastocystis hominis* (Nasirudeen *et al.*, 2004), *Trichomonas vaginalis* (Chose *et al.*, 2002) and *Plasmodium falciparum* (Picot *et al.*, 1997).

Apoptosis induces cellular and biochemical changes including caspase activation, DNA fragmentation, externalization of phosphatidylserine (PS), increase in intracellular Ca^{2+} and mitochondrial dysfunction, as well as physical changes such as cell shrinkage, alteration in cell volume, cytoplasmic blebbing and vacuolization, chromatin condensation and nucleosomal fragmentation. Apoptosis is energy dependent and requires ATP for signalling from the cytoplasm to the nucleus of the cell. The physiological roles of the apoptosis in protozoa are unknown but this phenomenon is related to altruistic behaviour, with clear benefit for entire population or as a mechanism to avoid host death (Wanderley *et al.*, 2005).

H_2O_2 , an important ROS, is a key mediator for eliciting PCD in mammalian and yeast cells (Li *et al.*, 2000; Vollgraf *et al.*, 1999; Clement & Pervaiz, 1999; Madeo *et al.*, 1999). H_2O_2 also induces apoptosis-like death in *Leishmania donovani* promastigotes (Das *et al.*, 2001), and caspase activation and PCD in *Tritrichomonas foetus* (Mariane

et al., 2003). But it is not established whether exposure to H_2O_2 can bring about apoptosis-like death in *E. histolytica* trophozoites, although it is well established that H_2O_2 can kill them (Ghadirian *et al.*, 1986). Recent reports suggested that apoptosis-like death can be induced by the aminoglycosidic antibiotic G418 (Villalba *et al.*, 2007) and nitric oxide species (Ramos *et al.*, 2007) in *E. histolytica* trophozoites. In the present study we aimed to demonstrate H_2O_2 -induced death of *E. histolytica* trophozoites as a model to study the death-associated phenotype and to identify possible biochemical pathways.

METHODS

Cultivation and oxidative stress of *E. histolytica*. Trophozoites of *E. histolytica* strain HM1:IMSS were cultured axenically in TYI-S-33 medium (Diamond *et al.*, 1978), supplemented with heat-inactivated bovine serum and 3% complete vitamin mixture at 35.5 °C. For stress experiments, trophozoites in the exponential growth phase were washed once with pre-warmed complete TYI-S-33 medium and chilled on ice for 5 min to dislodge the cells from tube surfaces. Briefly, 2×10^6 trophozoites in 20 ml complete TYI-S-33 medium were transferred aseptically to a 90 mm tissue culture Petri dish and kept at 35.5 °C for different time periods with different concentrations of H_2O_2 . After each exposure, the Petri dish was chilled to dislodge the amoebae, which were harvested by centrifugation from the culture supernatant for further experiments.

In vitro cytotoxicity assay. The *in vitro* cytotoxic activity of H_2O_2 on *E. histolytica* trophozoites was investigated by treating 2×10^6 *E. histolytica* cells kept in TYI-S-33 medium with H_2O_2 concentrations varying from 0.08 mM to 8 mM for time periods ranging from 0 h to 9 h, using appropriate controls for each set. The viability of trophozoites was examined by Trypan Blue exclusion (0.5 mg ml^{-1}). In each test there were three replicates and data are expressed as the mean $\pm$ SD of three experiments.

Measurement of cell viability by MTT assay. Viability of untreated and 0.8 mM H_2O_2 -treated *E. histolytica* cells (2×10^6 each set) for time periods ranging from 3 h to 9 h was determined using an MTT-based *in vitro* toxicology assay kit (Sigma). MTT [3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazolium bromide] is converted by active cellular dehydrogenases of living cells to its blue-coloured formazan salt derivative, which was measured spectrophotometrically at 570 nm. The cells from each set were incubated and assayed following the kit protocol. The viability of trophozoites treated with H_2O_2 for 9 h ('9 h stress-induced trophozoites') pretreated with EGTA was also determined using the MTT assay. The 9 h stress-induced trophozoites were reseeded in fresh TYI-S-33 medium (H_2O_2 free) and their viability was evaluated after 24 h. In each test there were three replicates and data are expressed as the mean $\pm$ SD of three experiments.

Cellular morphology using microscopy and flow cytometry for untreated and stress-induced trophozoites. To observe changes in cell morphology for untreated and 0.8 mM H_2O_2 -treated *E. histolytica* cells (2×10^6 each set) incubated for time periods ranging from 3 h to 9 h, the cells were examined under a phase-contrast microscope (Olympus BX41). Cellular morphology was also investigated for trophozoites treated with H_2O_2 for 9 h after pretreatment with EGTA and E-64d.

Changes in size and light-scattering properties for untreated and 9 h stress-induced trophozoites (1×10^6 each set) were determined by flow cytometry using a BD FACSCalibur equipped with Cell Quest software

using a 488 nm argon laser. A specific gate based on the properties of control trophozoites was selected to determine their positions on a forward scatter vs side scatter dot-plot. Light scattered in the forward direction is roughly proportional to cell size whereas light scattered at a 90° angle (side scatter) is proportional to cell density.

Assay for membrane integrity. Measurement of the release of the cytosolic enzyme lactate dehydrogenase serves as an indicator of loss of membrane integrity and thus cell viability. As *E. histolytica* lacks lactate dehydrogenase, the release of parasite cellular dehydrogenases into the culture medium was assayed by incubating samples of culture medium with sodium pyruvate in the presence of NADH. Dehydrogenases present in the culture medium catalyse the reduction of pyruvate and the concomitant oxidation of NADH to NAD⁺. The rate of conversion of NADH to NAD⁺ was measured spectrophotometrically at 340 nm. Untreated and 0.8 mM H₂O₂-treated *E. histolytica* cells (2 × 10⁶ each set) were incubated for time periods ranging from 3 h to 9 h. After incubation, a 100 µl aliquot of medium was collected for each set; 750 µl NADH (0.3 mg ml⁻¹) solution and 150 µl 20 mM sodium pyruvate were added to it, and activity was measured immediately at 340 nm. A mixture of 100 µl cell-free medium, 750 µl NADH (0.3 mg ml⁻¹) solution and 150 µl 20 mM sodium pyruvate was used as blank. A positive control for almost 100 % cell lysis was included in the assay, in which cells were lysed by incubating them with 1 % Triton X-100 for 15 min. In each test there were three replicates and data are expressed as the mean ± SD of three experiments.

Lipid peroxidation assay. Untreated and 0.8 mM H₂O₂-treated *E. histolytica* cells (2 × 10⁶ each set) were incubated for time periods ranging from 3 h to 9 h. A 100 µl aliquot of each sample was used to determine lipid peroxidation, measuring thiobarbituric-acid-reactive substances (TBARS) by a fluorescence method (Fraga *et al.*, 1987). Briefly, 0.05 ml 4 % (w/v) BHT and 1 ml PBS were added to 0.1 ml of each sample. After incubating for 30 min at 35.5 °C, 1.5 ml 20 % (v/v) acetic acid and 1.5 ml 0.8 % (w/v) 2-thiobarbituric acid were added. The mixture was heated for 45 min in boiling water and TBARS were extracted into 5 ml n-butanol. After brief centrifugation, the fluorescence of the butanol layer was measured at 515 nm excitation and 553 nm emission in a Perkin-Elmer LS 50 spectrofluorometer. Values were expressed as pmol TBARS [malondialdehyde (MDA) equivalents] per mg protein. An MDA standard was prepared from 1,1,3,3-tetraethoxypropane (El Hafidi & Baños, 1997). In each test there were three replicates and data are expressed as the mean ± SD of three experiments.

Detection of accumulation of ROS. Intracellular oxidant levels were determined by the use of H₂DCFDA (2',7'-dichlorodihydrofluorescein diacetate), which is oxidized inside the cell to fluorescent dichlorofluorescein. Untreated and 0.8 mM H₂O₂-treated *E. histolytica* trophozoites (2 × 10⁶ each set) incubated for time periods ranging from 3 h to 9 h were incubated with 0.4 mM (final concentration) H₂DCFDA for 15 min in the dark in PBS (pH 7.2). The cells were washed once in PBS (pH 7.2) and were harvested and lysed in lysis buffer (1 % SDS and 1 % Triton X-100 in 10 mM Tris/HCl, pH 8.0) and the fluorescence intensity was measured in the supernatant using a spectrofluorimeter (Perkin Elmer LS 50) with excitation at 504 nm and emission at 529 nm. The fluorescence intensity measured was directly proportional to the accumulation of ROS. In each test there were three replicates and data are expressed as the mean ± SD of three experiments.

Comparison of expression level of stress-combating enzymes between untreated and stress-induced *E. histolytica* by RT-PCR

Isolation of RNA. Total RNA was isolated from untreated and 0.8 mM H₂O₂-treated *E. histolytica* trophozoites incubated for time

periods ranging from 3 h to 9 h using TRIzol reagent (Invitrogen), a monophasic solution of phenol and guanidine isothiocyanate, following the manufacturer's protocol.

Semiquantitative RT-PCR. Reverse transcription was performed using 0.2 µg total RNA from untreated and 0.8 mM H₂O₂-treated *E. histolytica* cells incubated for time periods ranging from 3 h to 9 h using an anchored oligo-dT (GenHunter; H-dT₁₁M, where M represents A, C or G). The synthesized cDNAs were amplified by PCR for specific genes (eh29F 5'-TCAATCAGTCAAATGTCTTGC-3' and eh29R 5'-GAATGTCAACTTTCCTACTCC3'; sodF 5'-AATGCTCTTGAGCCTCA-3' and sodR 5'-CTTCCAGTTGACTACATTCC-3'; oxidoreductase F 5'-TTATAGCAACAAGAAATGGT-3' and oxidoreductase R 5'-AAAGAATGGAAGTTCTTTT-3'; actF 5'-GAGGATATGCTTTCACCACT-3' and actR 5'-ATAGCTGGTCCAGATTCATC-3'). In all PCRs after initial denaturation at 94 °C for 5 min, targeted genes were amplified by 25 amplification cycles (94 °C for 30 s, 55 °C or 50 °C for 30 s, and 72 °C for 1 min) followed by a final extension at 72 °C for 5 min. All PCRs were performed for 25 cycles, which was within the linear range of amplification of the corresponding mRNA species. The products were run on 1 % agarose gel, stained with ethidium bromide and finally documented and quantified using the Bio-Rad Gel Documentation System and associated Quantity One software. All the RT-PCR products were normalized with respect to the actin RT-PCR product.

Assay for stress-responsive enzymes

Untreated and 0.8 mM H₂O₂-treated *E. histolytica* cells (2 × 10⁶ each set) were incubated for time periods ranging from 3 h to 9 h. After incubation, different enzyme assays were performed; all assays were done at least in triplicate.

Peroxioredoxin. The consumption of H₂O₂ by *E. histolytica* peroxiredoxin was determined using a reaction mixture containing 50 mM Tris/HCl (pH 8.0), 0.2 mM dithiothreitol, 50 mM H₂O₂, and different amounts of protein. Before addition of H₂O₂, protein samples were preincubated with 0.2 mM dithiothreitol for 30 min. The reaction was stopped after 30 min by trichloroacetic acid precipitation (10 % final concentration). After removal of the precipitated protein, 0.2 ml 10 mM ferrous ammonium sulfate and 0.1 ml 2.5 M potassium thiocyanate were added to a 1 ml aliquot of the supernatant. H₂O₂ was determined spectrophotometrically (at 480 nm) using known amounts of H₂O₂ (1–50 mM) as a standard (Thurman *et al.*, 1972).

Superoxide dismutase (SOD). The activity of SOD was determined by the reduction of ferricytochrome *c* according to standard methods (McCord & Fridovich, 1969).

NAD(P)H:flavin oxidoreductase. Activity was determined as described by Lo & Reeves (1980), by measuring the initial rate of NAD(P)H oxidation at 340 nm ($\epsilon = 6.22 \text{ M cm}^{-1}$) at 25 °C. One unit of NAD(P)H:flavin oxidoreductase activity was defined as the amount of enzyme which catalyses the oxidation of 1 µmol NAD(P)H min⁻¹.

DNA fragmentation assay of untreated and stress-induced *E. histolytica* trophozoites. Pellets of 5 × 10⁶ trophozoites from untreated and 0.8 mM H₂O₂-treated *E. histolytica* cells incubated for time periods ranging from 3 h to 9 h were resuspended in 500 µl digestion buffer (10 mM EDTA, 50 mM Tris, 0.5 % SDS, 1 % Sarkosine, pH 8.0) containing 0.5 mg Proteinase K ml⁻¹ and incubated at 55 °C for 3 h. The suspensions were extracted twice with phenol/chloroform/isoamyl alcohol and then once with chloroform/isoamyl alcohol. The aqueous layer was combined with 50 µl 3 M sodium acetate and 2.5 vols 100 % ethanol and kept at -20 °C overnight for precipitation of DNA. Precipitated DNA was washed with ice-cold

70% ethanol, air-dried and resuspended in TE buffer (10 mM Tris, 1 mM EDTA, pH 8.0). DNA fragmentation assay was also performed with 9 h stress-induced trophozoites reseeded in fresh TYI-S-33 medium (H_2O_2 free) after 24 h.

Effect of cysteine protease inhibitor E-64d, various Ca^{2+} chelators and Ca^{2+} flux inhibitors and a caspase 1 inhibitor on DNA fragmentation. *E. histolytica* trophozoites (5×10^6) were incubated with 15 μM E-64d (Sigma) for 1 h and then were induced to undergo PCD by co-incubation with 0.8 mM H_2O_2 for 9 h. Similarly, pretreatments with 1 mM EGTA, 50 μM 1,2-bis(2-aminophenoxy)ethane-*N,N,N',N'*-tetraacetic acid tetrakis(acetoxy-methyl ester) (BAPTA/AM), 50 μM verapamil, 50 nM thapsigargin (TG) and a combination of TG and EGTA were performed in separate sets prior to exposure to H_2O_2 for 9 h. A pretreatment with the caspase 1 inhibitor Z-WEHD-FMK (100 μM , Roche) was also performed in a separate experiment. Finally a DNA fragmentation assay as described above was conducted with these samples.

Transmission electron microscopy analysis of untreated and stress-induced cells. Untreated and 0.8 mM H_2O_2 -treated *E. histolytica* cells (2×10^6 each set) for 9 h were harvested, washed twice with PBS and fixed for 1 h with 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer, pH 7.4. Fixed trophozoites were washed twice with 0.1 M sodium cacodylate buffer, post-fixed with 2% osmium tetroxide, dehydrated with ethanol at increasing concentrations and treated with propylene oxide. The trophozoites were then embedded in epoxy resin. Semithin sections were stained with toluidene blue for light microscopic examination. Thin sections were stained with uranyl acetate followed by lead citrate, and examined with an electron microscope.

Cell death assay using an annexin-V-FLUOS staining kit. Annexin V staining distinguishes apoptotic cells from necrotic cells. The expression of PS in the outer membrane for untreated and 0.8 mM H_2O_2 -treated *E. histolytica* cells (2×10^6 each set) incubated for time periods ranging from 3 h to 9 h was monitored by labelling with annexin-V-FITC and evaluated by confocal microscopy (LSM 510, Carl Zeiss) and flow cytometry (BD, FACSCalibur). Untreated and stress-induced cells collected after incubation were washed twice in PBS (pH 7.4) and labelled using the annexin-V-FLUOS Staining kit (Roche), following the manufacturer's protocol with a slight modification in the incubation time of annexin V reaction. The cells were analysed by confocal microscopy (LSM 510, Zeiss). The excitation wavelength was 488 nm for both FITC and propidium iodide (PI), and the emission wavelength was 530 nm for FITC and 585 nm for PI. The cells were also analysed by flow cytometry (FACSCalibur). Fluorescence parameters from single cells were collected using a logarithmic amplifier after gating on the combination of forward scatter and side scatter. Red fluorescence from PI was collected through the FL-2 channel and green fluorescence from annexin-V-FITC through the FL-1 channel. The fluorescence distribution was displayed as a two-colour dot-plot and the percentage of fluorescent cells in each quadrant was determined. Cells scoring annexin V positive and PI negative are apoptotic and those scoring both annexin V and PI positive are necrotic. PS externalization was also investigated for the 9 h stress-induced trophozoites pretreated with EGTA and E-64d, and for 9 h stress-induced trophozoites reseeded in fresh TYI-S-33 medium (H_2O_2 free) after 24 h.

Homogeneous caspase assay. To determine caspase-dependent or -independent cell death of *E. histolytica* following exposure to 0.8 mM H_2O_2 for 9 h, a fluorogenic homogeneous caspase assay was performed using the Homogeneous Caspase Assay kit (Roche). *E. histolytica* trophozoites (1×10^5) untreated or treated with H_2O_2 were examined for caspase activity following the manufacturer's protocol.

Briefly, after treatment, the caspase substrate, prediluted in incubation buffer, was added and incubated for 2 h at 37 °C. The fluorimetric measurement was recorded with an LS 50 instrument (Perkin Elmer), with excitation at 504 nm and emission at 529 nm; the amount of developed fluorochrome is proportional to the concentration of activated caspases. For a positive control, the human cell line HeLa was treated with actinomycin D for 24 h and analysed similarly. In each test there were three replicates and data are expressed as the mean $\pm$ SD of three experiments.

Calpain activity. To determine calpain activity during H_2O_2 -mediated cell death, *E. histolytica* cells were exposed to 0.8 mM H_2O_2 for 9 h and calpain activity was assayed using Suc-Leu-Leu-Val-Tyr-AMC (calpain substrate) as described previously (Kohli *et al.*, 1997). Cytosolic extract was first prepared from untreated and 9 h H_2O_2 -treated cells and the cytosolic protein concentration in each case was measured. A 40 μl sample of cytosol (1 mg protein ml^{-1}) was added to 160 μl 50 μM Suc-Leu-Leu-Val-Tyr-AMC (dissolved in DMSO and buffer containing 100 mM Tris/HCl and 145 mM NaCl, pH 7.3). Calpain activity was assayed in the presence of 10 mM Ca^{2+} . AMC (7-amino-4-methylcoumarin) released was measured by fluorometry using 360 nm excitation and 430 nm emission filters. In each test there were three replicates and data are expressed as the mean $\pm$ SD of three experiments. Standard curves were generated for each experiment using AMC of known concentrations. Calcium-dependent calpain activity was expressed as pmol AMC released per mg cytosolic protein at 10 mM Ca^{2+} .

Cytosolic Ca^{2+} concentrations. Changes in intracellular Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$) were monitored with the fluorescent probe Fura-2/AM. Untreated and 0.8 mM H_2O_2 -treated *E. histolytica* cells (1×10^6 each set) incubated for time periods ranging from 0 h to 9 h were harvested, and washed twice at 400 g for 10 min at 4 °C in buffer I (0.116 M NaCl, 5.4 mM KCl, 0.08 mM MgSO_4 , 5.5 mM D-glucose and 0.05 M HEPES, pH 7.0). The cells were resuspended in loading buffer (1×10^6 trophozoites ml^{-1}) that contained 0.116 M NaCl, 5.4 mM KCl, 0.08 mM MgSO_4 , 5.5 mM D-glucose, 1.5% sucrose, 0.05 M HEPES, pH 7.4, and 6 mM Fura-2/AM. The trophozoite suspension was incubated for 1 h at 37 °C with occasional agitation. The trophozoites were then washed four times with ice-cold buffer I to remove extracellular dye. Fura-2/AM was excited at 340 nm, and emission at 510 nm was registered with a Perkin Elmer LS 50 spectrofluorimeter. The $[\text{Ca}^{2+}]_i$ (nM) was determined at 30 °C using the formula $[\text{Ca}^{2+}]_i = K_d \times (F_2 - F_3) / (F_4 - F_1)$, where F_1 is the fluorescence signal obtained from the entire cell, F_2 the fluorescence signal after addition of 1 mM EGTA, F_3 the fluorescence following cell lysis with 0.04% Triton X-100 in 0.03 M Trizma base, and F_4 the fluorescence after adding 4 mM CaCl_2 . K_d is the dissociation constant, for which a value of 224 nM was used, as reported by Grynkiewicz *et al.* (1985). A pretreatment with 1 mM EGTA, 50 μM BAPTA/AM, 50 μM verapamil, 50 nM TG and a combination of TG and EGTA in separate sets before exposure to H_2O_2 for 9 h was performed and $[\text{Ca}^{2+}]_i$ was measured for each set. In each test there were three replicates and data are expressed as the mean $\pm$ SD of three experiments.

Intracellular pH measurement. Untreated and 0.8 mM H_2O_2 -treated *E. histolytica* cells (1×10^6 each set) incubated for time periods ranging from 0 h to 9 h were resuspended in TYI-S-33 medium and washed twice with buffer A (0.14 M KCl, 4 mM CaCl_2 , 25 mM HEPES/Tris, pH 7.4). Then, trophozoites were loaded with 10 mM BCECF [2,7-bis(2-carboxyethyl)-5-(and 6)-carboxyfluorescein] for 45 min in 1 ml buffer A. Nigericin (1 $\mu\text{g ml}^{-1}$) was added to the loading incubation. After loading, trophozoites were washed twice with buffer and resuspended in fresh buffer. Fluorescence was registered at 535 nm in a spectrofluorimeter (Perkin Elmer LS 50). At the end of each experiment, an *in situ* pH calibration procedure with

nigericin was used to relate the fluorescence intensities at 485 nm to the pH value. When cells are exposed to depolarizing high- K^+ buffers of different pH values (5.7–7.7), nigericin, an H^+-K^+ exchanger ionophore, sets $[K^+]_o = [K^+]_i$ and $pH_o = pH_i$, where $[K^+]_o$ is extracellular potassium concentration, $[K^+]_i$ is intracellular potassium concentration, pH_o is extracellular pH and pH_i is intracellular pH. In each test there were three replicates and data are expressed as the mean $\pm$ SD of three experiments. The two-tailed P -value of the mean of the differences of the intracellular pH was calculated using Graph Pad software.

RESULTS

Dose and time kinetics of H_2O_2 -mediated death of *E. histolytica*

The amoebicidal activity of H_2O_2 against *E. histolytica* strain HM1:IMSS was determined by exposing *E. histolytica* trophozoites to three different concentrations (0.08, 0.8 and 8 mM) of H_2O_2 for time periods ranging from 0 h to 9 h. The percentage of dead *E. histolytica* for a particular dose increased with time of incubation (Fig. 1a). The viability of the trophozoites was also decreased in a dose-dependent manner for a particular time period (Fig. 1a). The maximum percentage of trophozoite death was found after 9 h incubation with 8 mM H_2O_2 solution, whereas 0.8 mM H_2O_2 caused death of ~50% trophozoites after 9 h incubation. To confirm that the killing of amoebae in this system was induced in the presence of H_2O_2 , the amoebae were pretreated with catalase (100 U ml⁻¹) prior to H_2O_2 treatment. The percentage of dead trophozoites decreased significantly on co-incubation with H_2O_2 and catalase (data not shown).

MTT assay of metabolically active cells upon exposure to H_2O_2

The proportion of metabolically active stress-induced trophozoites was determined using an MTT-based *in vitro* toxicology assay kit (Sigma). The MTT assay of the trophozoites showed that 3 h stress-induced trophozoites had very similar metabolic activity to untreated trophozoites, but 6 h and 9 h H_2O_2 -stressed trophozoites were 39% and 48% less metabolically active than the untreated trophozoites (Fig. 1b). The viability of 9 h stress-induced cells pretreated with EGTA was about 80% of that of untreated trophozoites (data not shown). It was also found that the viability of 9 h stress-induced trophozoites reseeded in fresh TYI-S-33 medium (H_2O_2 free) after 24 h decreased by 86% compared to 24-h-old trophozoites which had not been pre-exposed to oxidative stress (data not shown).

H_2O_2 induces morphological changes in a time-dependent manner and precipitates apoptosis-like death

Phase-contrast microscopy and flow cytometry were performed with the untreated and stress-induced tropho-

zoites to determine the morphological changes characteristic of apoptosis-like death. Phase-contrast microscopy of untreated and 3 h stress-induced trophozoites showed typical amoebic forms with irregular shape and abundant small food vacuoles (Fig. 1c, panel A). However, a dramatic change in cell shape was observed after 6 h of incubation with H_2O_2 . After this treatment most of the cells become rounded, and the cytoplasm became condensed and also contained large vacuoles (Fig. 1c, panel A). Despite the rounding of the cells following treatment the trophozoites showed no sign of membrane rupture during the total period of incubation. In the flow cytometric analysis, the cell population in the upper right and upper left quadrants of the dot-plot increased substantially after exposure to H_2O_2 stress for 9 h in comparison to the untreated trophozoites and there was also a marked increase in the Y-mean value in the stress-induced trophozoites compared to the control (from 30.5 to 84.1), but the concomitant change in the X-mean value (from 71.6 to 77.6) was rather slight (Fig. 1c, panel B). This result clearly revealed a marked increase in granularity (approximately threefold) for 9 h stress-induced parasites in comparison to the untreated trophozoites, but there was no significant change in cell size. Cell rounding and increased vacuolization were also observed for 9 h stress-induced trophozoites pretreated with E-64d and EGTA (data not shown).

Release of cellular dehydrogenases and lipid peroxidation as a marker of membrane integrity

Membrane integrity after exposure to H_2O_2 was investigated by the release of cellular dehydrogenases from the untreated and stress-induced trophozoites. Cells lysed with 1% Triton X-100 served as the positive control, where almost 100% cell lysis was expected. The positive control demonstrated a very high value of enzyme activity as the membrane is totally ruptured (Fig. 1d). Although the loss in membrane integrity with respect to the positive control was much less in both untreated and H_2O_2 -treated trophozoites (Fig. 1d), 9 h stress-induced trophozoites showed slightly higher loss of membrane integrity than untreated trophozoites (Fig. 1d). The membrane integrity was also investigated by measuring the lipid peroxidation level in untreated and H_2O_2 -treated trophozoites. The MDA level (an index of lipid peroxidation) was similar for the untreated and H_2O_2 -treated trophozoites, with a slight increase in the later hours of incubation (Fig. 1e). These results show that the membrane remains almost intact during the whole period of incubation, with a slight decrease in membrane integrity at the later times.

Time-dependent accumulation of ROS

The intracellular accumulation of ROS by trophozoites with increasing time of exposure to H_2O_2 was determined by using H_2DCFDA . ROS accumulation increased with time of exposure, with a 1.3-, 4.0- and 6.2-fold increase in ROS levels being observed in the 3 h, 6 h and 9 h

Fig. 1. (a) Time-course assay of cell death for untreated and 0.08, 0.8 and 8 mM H₂O₂-treated *E. histolytica* trophozoites. Cell death was determined at 3 h intervals for 9 h. In each test there were three replicates and the data are means $\pm$ SD of three independent experiments. (b) Measurement of metabolically active cells for untreated and 3, 6 and 9 h 0.8 mM H₂O₂-treated *E. histolytica* trophozoites using the MTT assay. Data are means $\pm$ SD of three independent experiments. (c) Morphology of untreated and stress-induced *E. histolytica* trophozoites analysed by phase-contrast microscopy and flow cytometry. Panel A, phase-contrast micrographs showing the morphologies of untreated (C) and 3, 6 and 9 h 0.8 mM H₂O₂-treated trophozoites. Scale bars, 5 μ m. Panel B, flow cytometry dot-plots indicating cell size (forward scatter) and granularity (side scatter) for untreated and 9 h 0.8 mM H₂O₂-treated trophozoites. Region R1 represents the gate of the trophozoite population selected for the experiment. (d) Spectrophotometric measurement of loss of membrane integrity for untreated and 3, 6 and 9 h 0.8 mM H₂O₂-treated *E. histolytica* trophozoites. Release of cytosolic dehydrogenases served as an indicator of loss of membrane integrity. Data (units mg⁻¹) are means $\pm$ SD of three independent experiments. (e) Spectrofluorimetric measurement of lipid peroxidation, measured as MDA equivalents generated, as an indicator of membrane integrity for untreated (Un) and 3, 6 and 9 h 0.8 mM H₂O₂-treated *E. histolytica* trophozoites.

stress-induced trophozoites, respectively, in comparison to untreated trophozoites (Fig. 2a).

Expression level and enzyme activity of SOD, eh29 and NAD(P)H:flavin oxidoreductase in untreated and stress-induced *E. histolytica*

The expression level and enzyme activity of the important stress-regulatory enzymes superoxide dismutase (SOD), thiol-dependent peroxidase (eh29) and NAD(P)H:flavin oxidoreductase was investigated. The mRNA level of SOD and eh29 decreased gradually with the increase in duration of oxidative stress. The SOD mRNA level decreased by 11 % in 3 h, 43 % in 6 h and 51 % in 9 h, and that of eh29 level decreased by 8 % in 3 h, 29 % in 6 h, and 60 % in 9 h with respect to the untreated trophozoites (Fig. 2b). In contrast, the mRNA level of NAD(P)H:flavin oxidoreductase, which is responsible for the generation of H_2O_2 , was found to increase gradually with increased time of exposure to oxidative stress. The oxidoreductase mRNA

level increased by 24 % in 3 h, 62 % in 6 h and 67 % in 9 h with respect to the untreated trophozoites (Fig. 2b). The assay of enzyme activity for both SOD and eh29 showed a similar gradual decrease with the increase in time of exposure, whereas a similar gradual increase in NAD(P)H:flavin oxidoreductase activity with time was observed for the stress-induced trophozoites (Table 1).

DNA fragmentation in *E. histolytica* trophozoites upon exposure to H_2O_2

In mammalian cells the hallmark of apoptosis is the intranucleosomal degradation of genomic DNA. Therefore, we investigated the DNA fragmentation pattern for untreated and stress-induced trophozoites. The stress-induced trophozoites showed a DNA fragmentation pattern after 9 h exposure to oxidative stress, identified by electrophoresis on a 1.5 % agarose gel (Fig. 3a, panel 1). The ladder pattern was not as clear as a metazoan DNA ladder and showed some degree of smearing with

Fig. 2. (a) Generation of ROS for untreated and 3, 6 and 9 h 0.8 mM H_2O_2 -treated *E. histolytica* trophozoites. Intracellular ROS generation was analysed spectrofluorimetrically using H_2DCFDA . In each test there were three replicates and the data are means $\pm$ SD of three independent experiments. (b) Semiquantitative RT-PCR analysis of SOD, eh29 and NAD(P)H:flavin oxidoreductase (OXR) for untreated and 3, 6 and 9 h 0.8 mM H_2O_2 -treated *E. histolytica* trophozoites. Ethidium-bromide-stained PCR products were photographed and the images were analysed densitometrically. Actin PCR was conducted to show uniform expression of a housekeeping gene in untreated and treated parasites for the whole time interval. Data are means $\pm$ SD of three independent experiments. Panel A shows the gel images; panel B is a graphical representation of the densitometric data.

Table 1. Activities of stress-responsive enzymes for untreated and stress-induced *E. histolytica*

H ₂ O ₂ treatment	SOD (units mg ⁻¹)	eh29 (nmol H ₂ O ₂ min ⁻¹ mg ⁻¹)	NAD(P)H:flavin oxidoreductase (units mg ⁻¹)
None	6.4 ± 0.4	3.2 ± 0.3	2.3 ± 0.1
3 h	5.9 ± 0.2	2.8 ± 0.2	2.9 ± 0.05
6 h	4.3 ± 0.3	1.7 ± 0.05	3.7 ± 0.3
9 h	2.3 ± 0.1	1.4 ± 0.1	3.9 ± 0.2

occasional bands. The untreated, and 3 h and 6 h stress-induced trophozoites did not show any characteristics of DNA fragmentation (Fig. 3a, panel 1). The 9 h stress-induced trophozoites reseeded in fresh TYI-S-33 medium (H₂O₂ free) showed DNA fragmentation after 24 h (data not shown).

Effect of E-64d, Ca²⁺ chelators, Ca²⁺ flux inhibitors and a caspase 1 inhibitor on DNA fragmentation

To investigate the role of cysteine proteases, caspase 1 and Ca²⁺ in DNA fragmentation of the stress-induced trophozoites, prior to exposure to H₂O₂ the trophozoites were preincubated with cysteine protease inhibitor E-64d, caspase 1 inhibitor Z-WEHD-FMK, Ca²⁺ chelators and Ca²⁺ flux inhibitors. Preincubation of trophozoites with E-64d abolished DNA fragmentation in 9 h stress-induced trophozoites, but Z-WEHD-FMK was unable to abolish DNA fragmentation (Fig. 3a, panel 1). E-64d treatment led to a high-molecular-mass DNA band similar to that observed with untreated trophozoites (Fig. 3a, panel 1), whereas Z-WEHD-FMK-treated trophozoites showed DNA ladder having some degree of smearing with occasional bands (Fig. 3a, panel 1). These results clearly indicate that cysteine protease(s) are involved in DNA fragmentation of stress-induced trophozoites, whereas caspase 1 does not play a role in the fragmentation. Preincubation with the extracellular Ca²⁺ chelator EGTA, the [Ca²⁺]_i chelator BAPTA/AM and the Ca²⁺ influx inhibitor verapamil abolished DNA fragmentation and led to a high-molecular-mass DNA band similar to that in untreated trophozoites (Fig. 3a, panel 1). Treatment with thapsigargin (TG), known to activate release of Ca²⁺ from intracellular reservoirs by blocking Ca-ATPases in the endoplasmic reticulum, led to DNA fragmentation after 9 h exposure to oxidative stress in the presence of external Ca²⁺, but the DNA fragmentation was abolished and a high-molecular-mass DNA band was observed when preincubation was performed with TG in the presence of EGTA (Fig. 3a, panel 1).

Transmission electron microscopy of *E. histolytica* trophozoites after exposure to H₂O₂

Transmission electron microscopy of untreated trophozoites revealed a round nucleus having a dense peripheral

chromatin with a central endosome (Fig. 3a, panel 2). The 9 h stress-induced trophozoites showed a different distribution of chromatin within the nucleus: the chromatin was condensed and gathered mainly at one side of the nuclear envelope (Fig. 3a, panel 2).

Externalization of PS upon exposure to H₂O₂

The apoptotic mode of cell death was confirmed by the PS externalization of stress-induced trophozoites. The confocal images clearly demonstrated that the untreated and 3 h stress-induced trophozoites were both annexin V and PI negative, while the 6 h and 9 h stress-induced trophozoites were annexin V positive and PI negative (Fig. 3b, panel 1). Therefore, from 6 h onwards the externalization of PS starts under oxidative stress.

Flow cytometric analysis revealed that the percentage of apoptotic cells following H₂O₂ treatment increased in a time-dependent manner. Untreated trophozoites and 3 h stress-induced trophozoites showed a very small number of annexin-V-positive and PI-negative cells (0.02 % and 9.4 % respectively), whereas 6 h and 9 h stress-induced trophozoites showed a significant increase in the annexin-V-positive and PI-negative population (36.2 % and 53 % respectively) (Fig. 3b, panel 2).

It was also found that the 9 h stress-induced trophozoites reseeded in fresh TYI-S-33 medium (H₂O₂ free) showed PS externalization after 24 h. The 9 h stress-induced trophozoites pretreated with EGTA and E-64d also showed PS externalization (data not shown).

Caspase-independent cell death of *E. histolytica* after H₂O₂ exposure

The fluorogenic homogeneous caspase assay determines the presence of any of the active caspases including 2, 3, 6, 7, 8, 9 and 10. The homogeneous caspase assay was performed for the untreated and stress-induced trophozoites to determine the involvement of caspases in the oxidative-stress mediated death of the trophozoites. Untreated and 9 h stress-induced trophozoites showed no significant caspase activity whereas the positive control actinomycin D-treated HeLa cell line showed significant caspase activity (Fig. 3c). The basal level of activity for H₂O₂-treated and untreated trophozoites can be attributed to some autodegradation of the fluorogenic substrate.

Fig. 3. (a) Time-course of DNA fragmentation in trophozoites after H_2O_2 treatment and effect of cysteine protease inhibitor E-64d, various Ca^{2+} chelators and Ca^{2+} flux inhibitors and a caspase 1 inhibitor on DNA fragmentation as analysed by agarose gel electrophoresis, and transmission electron micrographs of nuclear condensation for untreated and 9 h stress-induced trophozoites. Panel 1, electrophoretic analysis of DNA fragmentation on a 1.5% agarose gel for untreated and 3, 6 and 9 h $0.8 \text{ mM H}_2\text{O}_2$ -treated *E. histolytica* trophozoites, and for trophozoites preincubated with (as indicated above the lanes) cysteine protease inhibitor E-64d, caspase 1 inhibitor Z-WEHD-FMK, extracellular Ca^{2+} chelator EGTA, $[\text{Ca}^{2+}]_i$ chelator BAPTA/AM, Ca^{2+} influx inhibitor verapamil (VP), Ca^{2+} -releasing agent from intracellular reservoirs TG (in presence of extracellular Ca^{2+}), and TG + EGTA (to chelate extracellular Ca^{2+}) before 9 h $0.8 \text{ mM H}_2\text{O}_2$ -treatment. Panel 2, transmission electron micrographs of the nucleus of untreated (Un) and 9 h 0.8 mM stress-induced *E. histolytica* trophozoites. Scale bars, $2 \mu\text{m}$. (b) Assay of cell death after stress induction by confocal microscopy and flow cytometry using an annexin-V-FLUOS staining kit. In panel 1, column 1 shows annexin-V-positive images, column 2 shows PI-positive images, column 3 shows the phase-contrast images and column 4 shows the merged images for untreated (Un) and 3, 6 and 9 h $0.8 \text{ mM H}_2\text{O}_2$ -treated *E. histolytica* trophozoites. Scale bars, $10 \mu\text{m}$. Panel 2 shows the corresponding flow cytometry dot-plots indicating the apoptotic population (annexin V positive but PI negative) in the lower right panel. (c) Spectrofluorimetric analysis of caspase activity for untreated (Un) and 9 h 0.8 mM stress-induced trophozoites. Actinomycin-D-treated HeLa cell line (HeLa + AD) served as a positive control. Data are means $\pm$ SD of three independent experiments. (d) Fluorogenic calpain activity assay of untreated and 9 h $0.8 \text{ mM H}_2\text{O}_2$ -treated trophozoites. Calpain activity is expressed as pmol AMC released $\text{min}^{-1} (\text{mg protein})^{-1}$. Data are means $\pm$ SD of three independent experiments.

Calpain-dependent cell death of *E. histolytica* after H_2O_2 exposure

The assay for the Ca^{2+} -dependent cysteine protease calpain was performed with untreated and stress-induced trophozoites to determine the involvement of this protease in the oxidative stress-mediated death of the trophozoites. The calpain activity was found to be approximately fourfold higher in the 9 h oxidatively stressed trophozoites compared to the untreated trophozoites (Fig. 3d), indicating the involvement of this enzyme in stress-induced death of the trophozoites.

Increased $[\text{Ca}^{2+}]_i$ accumulation after H_2O_2 exposure

In mammalian organisms free radical production in conjunction with changes in Ca^{2+} homeostasis is a common feature of PCD (Lipton & Nicotera, 1998). The increase in $[\text{Ca}^{2+}]_i$ was measured spectrofluorometrically during the total period of exposure to ROS. The intracellular Ca^{2+} level remained stable at around 20 nM over the entire period of incubation for untreated trophozoites (Fig. 4a). However, for stress-induced trophozoites the $[\text{Ca}^{2+}]_i$ level increased from $\sim 20 \text{ nM}$ to $\sim 50 \text{ nM}$ at 6 h of incubation and thereafter the increase in $[\text{Ca}^{2+}]_i$ was relatively slight (Fig. 4a). The extracellular Ca^{2+} chelator EGTA decreased the $[\text{Ca}^{2+}]_i$ in both untreated and stress-induced trophozoites (Fig. 4a). The elevation of the intracellular Ca^{2+} level of stress-induced trophozoites was not significant in presence of the $[\text{Ca}^{2+}]_i$ chelator BAPTA/AM or the Ca^{2+} influx inhibitor verapamil (Fig. 4a). Treatment with TG, which activates release of Ca^{2+} from intracellular reservoirs by blocking Ca-ATPases in the endoplasmic reticulum, led to elevation of the $[\text{Ca}^{2+}]_i$ for stress-induced trophozoites in the presence of external Ca^{2+} , but only a slight increase was observed with TG in the presence of the extracellular Ca^{2+} chelator EGTA, i.e. in the absence of external Ca^{2+} (Fig. 4a).

Fig. 4. (a) Measurement of $[\text{Ca}^{2+}]_i$ for untreated and stress-induced *E. histolytica* trophozoites and effect of various Ca^{2+} chelators and Ca^{2+} flux inhibitors in regulation of the $[\text{Ca}^{2+}]_i$ level: Ca^{2+} concentrations were evaluated fluorimetrically using Fura-2/AM dye in untreated and $0.8 \text{ mM H}_2\text{O}_2$ -treated *E. histolytica* trophozoites at 3 h intervals for 9 h. Pretreatments with 1 mM EGTA, $50 \mu\text{M}$ BAPTA/AM, $50 \mu\text{M}$ verapamil, 50 nM TG and a combination of TG and EGTA in separate sets before exposure to H_2O_2 for 9 h were performed and $[\text{Ca}^{2+}]_i$ was measured for each set. Data are means $\pm$ SD of three independent experiments. (b) Change in intracellular pH for untreated and $0.8 \text{ mM H}_2\text{O}_2$ -treated *E. histolytica* trophozoites, measured at 3 h intervals for 9 h by using the fluorescent dye BCECF. Data are means $\pm$ SD of three independent experiments.

Acidification of intracellular pH after H₂O₂ exposure

In other organisms showing features of PCD, increased endogenous ROS and [Ca²⁺]_i are responsible for the loss of mitochondrial and endoplasmic reticulum membrane potential which results in a concomitant decrease in the intracellular pH (Demaurex *et al.*, 2003). To evaluate the effect of H₂O₂ treatment on intracellular pH, a fluorescence-based method was used. Trophozoites were loaded with an acetomethyl ester derivative of BCECF, a dye whose fluorescence emission is sensitive to intracellular pH variations. The excitation wavelengths used were 440 and 490 nm, and the emission wavelength was 535 nm. The intracellular pH of untreated trophozoites remained almost constant at ~7.6 over the entire incubation period. In contrast, a significant decrease in intracellular pH from ~7.6 to ~6.0 was seen after 6 h of exposure to ROS (Fig. 4b). The two-tailed *P*-value of the mean of the differences of the intracellular pH is 0.0173, which was considered to be statistically significant. These results clearly demonstrated that the intracellular pH of stress-induced trophozoites is more acidic than that of untreated trophozoites.

DISCUSSION

In this investigation an attempt was made to understand the involvement of stress-responsive genes in the viability of the parasite *E. histolytica* upon H₂O₂ treatment and also the mode of cell death occurring while the parasite is exposed to a high degree of oxidative stress. This parasite usually lives and multiplies in the low-oxygen environment of the human gut. When *E. histolytica* invades human tissues, it is challenged by a high-oxygen environment (~0.24 mM) (Ramos-Martínez *et al.*, 2009) and by the ROS produced by endogenous enzymes and activated phagocytes during the respiratory burst (Santi-Rocca *et al.*, 2009). Moreover, the steady-state intracellular concentration of H₂O₂ is regulated at concentrations up to 10⁻⁹–10⁻⁷ M. The physiological or pathological variation of catalase concentration in different organs and tissues will lead to different steady-state levels of H₂O₂ concentration for the same rate of H₂O₂ generation (Chance *et al.*, 1979). If the parasite survives in such a high oxidatively stressed condition it will be able to cause tissue damage. During exposure to oxidative stress most of the damage is caused by OH generated from H₂O₂ via Fenton's reaction. Therefore, H₂O₂ is the major ROS involved in the death of trophozoites under oxidative stress conditions. In this study the oxidatively stressed condition encountered in the liver was probably mimicked by incubating trophozoites with 0.8 mM H₂O₂ *in vitro*.

After changing the trophozoites from a microaerophilic environment (low oxygen) to a highly oxidatively stressed environment generated by exposure to 0.8 mM H₂O₂, survival decreased with increased duration of incubation. The 9 h stress-induced trophozoites showed nearly 50 %

less viability, suggesting that upon exposure to exogenous H₂O₂ and subsequent proportional increase in the endogenous ROS level the physiological activities of the trophozoites had been impaired. These data were supported by the MTT and membrane integrity assay. The MTT assay showed that 3 h stress-induced trophozoites had similar metabolic activity to untreated trophozoites, but 6 h and 9 h oxidatively stressed trophozoites were considerably less metabolically active than the untreated controls (Fig. 1b). Membrane integrity as determined by the release of dehydrogenases from the trophozoites and lipid peroxidation level was slightly less in the 9 h oxidatively stressed trophozoites as compared to 6 h, 3 h and untreated trophozoites (Fig. 1d, 1e). The slight increase in parasite cellular dehydrogenase activity and MDA level (index of lipid peroxidation) for the 9 h stress-induced trophozoites may also be an indicator of the late stage of apoptosis. The almost intact membranes visualized in the phase-contrast micrographs (Fig. 1c, panel A) and insignificant PI staining of the 9 h oxidatively stressed trophozoites (Fig. 3b, panel 2) also indicates a slight loss in the membrane integrity for the stress-induced trophozoites at the late stage of apoptosis.

Stress-induced trophozoites also showed marked alteration in cellular morphology. The oxidatively stressed trophozoites showed cell rounding, cytoplasmic condensation and formation of large vacuolar structures inside the cytoplasm by phase-contrast microscopy after 6 h of incubation while the untreated and 3 h stress-induced trophozoites showed typical amoebic shapes with food vacuoles in the cytoplasm (Fig. 1c, panel A). Flow cytometry analysis showed that after 6 h of incubation with H₂O₂ there was a significant increase in cell granularity with a less significant alteration in size (Fig. 1c, panel B). Increased granularity can be attributed to increased vacuolization for stress-induced trophozoites after 6 h of incubation. Vacuolization has been reported in PCD of *Caenorhabditis elegans* (Robertson & Thomson, 1982), *Dictyostelium discoideum* (Cornillon *et al.*, 1994) and some types of higher eukaryotes (Wyllie *et al.*, 1980; Clarke, 1990), and Villalba *et al.* (2007) also made similar observations with *E. histolytica* trophozoites when treated with an aminoglycosidic antibiotic to induce PCD.

Molecules involved in resistance to ROS in *E. histolytica* have been well characterized. *E. histolytica* produces Fe-SOD, which is induced by O₂⁻ to produce H₂O₂ (Bruchhaus & Tannich, 1994). In addition, direct divalent reduction of O₂ to H₂O₂ is catalysed by an NADPH:flavin oxidoreductase (Bruchhaus *et al.*, 1998). Catalase and glutathione reductase systems are absent in *E. histolytica* (Sen *et al.*, 2007), but the H₂O₂ generated by both pathways can be further transformed to H₂O by the action of peroxiredoxin (eh29) (Bruchhaus *et al.*, 1997). The mRNA level of SOD and eh29 was found to decrease gradually with the increase in duration of exposure to oxidative stress. The SOD and eh29 level showed a more than twofold decrease after 9 h exposure to oxidative stress with respect to the untreated trophozoites (Fig. 2b, panel

B). However, the mRNA level of NAD(P)H:flavin oxidoreductase, which is responsible for the generation of H_2O_2 , increased gradually with increased incubation time, and showed an approximately fourfold increase in expression level in 9 h stress-induced trophozoites (Fig. 2b, panel B). These data suggest that upon prolonged exposure the endogenous H_2O_2 generated is not efficiently detoxified and becomes extremely toxic for the parasite, resulting in decreased viability. The mRNA level analysis of the stress-responsive genes alone does not provide complete evidence of their involvement in regulation of oxidative stress and concomitantly cell death. Therefore, the activity of the enzymes was also assayed; the results supported those of the mRNA expression analysis (Table 1).

In multicellular organisms, apoptosis is characterized by two prominent changes in the nucleus: destruction of the normal structural organization owing to collapse of the chromatin into condensed electron-dense masses, and fragmentation of nuclear DNA into oligonucleosomal fragments. We did not observe typical ladder-like DNA fragmentation after exposure to oxidative stress; rather we saw some smearing in the DNA gels with occasional bands after 9 h exposure (Fig. 3a, panel 1). This finding is not uncommon because DNA smearing in the absence of DNA ladders during apoptosis is known in yeast (Madeo *et al.*, 1999) and some metazoan cell types (Oberhammer *et al.*, 1993). But the untreated and the stress-induced trophozoites up to 6 h did not show any kind of DNA fragmentation pattern (Fig. 3a, panel 1). DNA fragmentation was less significant in 6 h compared to 9 h oxidatively stressed trophozoites. There are some reports indicating that DNA fragmentation cannot always be regarded as a hallmark for apoptosis, as certain cells display morphological and biochemical features of apoptosis without a typical ladder-like DNA fragmentation (Collins *et al.*, 1992; Howell & Martz, 1987; Barbieri *et al.*, 1992; Mesner *et al.*, 1992; Falcieri *et al.*, 1993; Vaux *et al.*, 1994; Hirata *et al.*, 1998). Ramos *et al.* (2007) reported four bands smaller than 500 bp and Villalba *et al.* (2007) found a more heterogeneous digestion pattern with a smear of degraded DNA containing faint ladder bands for *E. histolytica* trophozoites when induced to PCD by nitrosative stress and aminoglycosidic antibiotic G418 respectively. These observations are similar to ours for *E. histolytica* trophozoites exposed to H_2O_2 . Transmission electron micrographs of stress-induced trophozoites confirmed the occurrence of a PCD-like phenomenon, showing characteristic nuclear condensation and chromatin fragmentation (Fig. 3a, panel 2), which correlates with the finding of Villalba *et al.* (2007).

In normal cells, PS is found predominantly in the inner layer of the plasma membrane bilayer. Apoptotic cells lose this phospholipid asymmetry, leading to the exposure of PS on their surface. In the early stages of eukaryotic apoptosis, cells externalize PS while maintaining membrane integrity (Gatti *et al.*, 1998). The FITC-conjugated annexin V was bound to the externalized PS, and PI could not enter the cell and hence did not bind to the nucleus due to the

intactness of the cell membrane of the stress-induced trophozoites from 6 h onwards (Fig. 3b, panels 1 and 2). Normal cell permeability and PS externalization is a positive signal of apoptosis and hence the trophozoites start undergoing apoptosis-like death after 6 h of exposure to ROS. The untreated trophozoites and the stress-induced trophozoites prior to 6 h were viable because they did not bind annexin V or PI, indicating no externalization of PS prior to 6 h incubation (Fig. 3b, panels 1 and 2). This is in contrast to the report of Villalba *et al.* (2007), who could not detect annexin V binding for *E. histolytica* trophozoites; they suggested that this might be due to the low content of PS in the *E. histolytica* membrane. Our positive results could be due to the longer annexin V reaction period used. Our lab had previously shown that after 6 h exposure to oxidative stress, *E. histolytica* shows annexin-positive staining (Sen *et al.*, 2007).

Apoptosis-associated nuclear condensation and segmentation are generally attributed to caspase activity that leads to the cleavage of substrates such as lamins, caspase-activated DNase or acinus (Rao *et al.*, 1996; Liu *et al.*, 1997; Sakahira *et al.*, 1998; Sahara *et al.*, 1999). *In silico* evidence for the presence of a putative caspase-like protein in the *E. histolytica* genome (TIGR 9712) was sought by Villalba *et al.* (2007) and their results did not show any matches that suggested the presence of a caspase-like protein. Our homogeneous caspase assay also showed no significant caspase activity for the H_2O_2 -exposed trophozoites (Fig. 3c). Zhang *et al.* (2000) had earlier demonstrated that one of the *E. histolytica* cysteine proteases has substrate specificity similar to that of caspase 1, and therefore we investigated the effect of the caspase 1 inhibitor Z-WHED-FMK on the death phenotype of oxidatively stressed trophozoites. This caspase 1 inhibitor was unable to prevent the death phenotype, as the DNA fragmentation was not abolished in presence of the inhibitor (Fig. 3a, panel 1). Hence, caspase may have no role in inducing apoptosis-like death for the stress-induced trophozoites. But the parasite contains 50 cysteine protease genes (Bruchhaus *et al.*, 2003; Tillack *et al.*, 2007). Calpain, a Ca^{2+} -dependent cysteine protease, has also been reported to be present in *E. histolytica* (Tillack *et al.*, 2007). The 9 h oxidatively stressed trophozoites showed an approximately fourfold increase of calpain activity in comparison to the untreated trophozoites, and this may indicate calpain-mediated apoptosis of stress-induced trophozoites. Earlier reports demonstrated that E-64d, a cell-permeable variant of E-64, can efficiently block calpain-mediated apoptosis (Ray *et al.*, 2001). We therefore investigated the effect of E-64d on one of the most important features of apoptosis-like death, i.e. DNA alteration. Preincubation of trophozoites with E-64d prior to H_2O_2 exposure abolished DNA degradation (Fig. 3a, panel 1). This finding strongly suggests that calpain participates in stress-induced PCD of *E. histolytica*. This would be in accordance with the work of Villalba *et al.* (2007), where involvement of cysteine protease in apoptotic death of G418-induced trophozoites

was demonstrated. In contrast, a recent study by Ghosh *et al.* (2010) demonstrated H₂O₂-induced cysteine-protease-independent apoptosis-like cell death in *E. histolytica*. They showed that E-64 could not rescue the cells from PCD. E-64 is an extracellular cysteine protease inhibitor and hence in our study we used its cell-permeable variant, E-64d; we found a cysteine-proteinase-dependent calpain-mediated apoptosis-like death of *E. histolytica* upon exposure to H₂O₂.

Earlier studies revealed that exposure to oxidative stress causes an increase in [Ca²⁺]_i levels, which is another common feature of apoptosis (Jiang *et al.*, 1994). Our results showed a significant increase in the [Ca²⁺]_i from ~20 nM to ~50 nM upon exposure to ROS for 9 h, suggesting a pivotal role for Ca²⁺ in apoptosis-like death of *E. histolytica*. The increase of [Ca²⁺]_i induced by oxidative stress could be due either to extracellular Ca²⁺ influx through the plasma membrane or to Ca²⁺ release from intracellular stores. To address this issue, drugs known to function as regulators of [Ca²⁺]_i in several eukaryotic cells were used and the changes in Fura-2/AM-loaded trophozoites were monitored. Pretreatment with Ca²⁺ chelators might have detrimental effects on several Ca²⁺-dependent physiological functions. Therefore, we checked the viability 9 h stress-induced cells pretreated with EGTA. The cells pretreated with EGTA remained about 80% viable with respect to untreated cells. Therefore, pretreatment with Ca²⁺ chelator does not show detrimental effect on Ca²⁺-dependent physiological functions up to 9 h (although an effect may be apparent at later time points). In order to explore the role of extracellular Ca²⁺ in the modulation of [Ca²⁺]_i, the effect of oxidative stress in cells maintained in the absence of external Ca²⁺ using the extracellular Ca²⁺ chelator EGTA was tested. In these cells the increase in [Ca²⁺]_i was not observed (Fig. 4a). Stress-induced cells previously treated with the [Ca²⁺]_i chelator BAPTA/AM also did not show any increase in the [Ca²⁺]_i (Fig. 4a). Pretreatment with the Ca²⁺ influx inhibitor verapamil also abolished the rise in [Ca²⁺]_i level upon exposure to oxidative stress (Fig. 4a). To further study a possible participation of Ca²⁺ release from amoebic intracellular stores, [Ca²⁺]_i was monitored in trophozoites depleted of Ca²⁺ from intracellular pools by treatment with thapsigargin (TG). TG induced a transient increase of [Ca²⁺]_i initially by releasing Ca²⁺ from internal stores, but following exposure to oxidative stress this transient increase in [Ca²⁺]_i secondarily activates plasma-membrane Ca²⁺ channels and induces the influx of external Ca²⁺ further (Fig. 4a). In contrast, only a transient increase [Ca²⁺]_i level was produced by TG in the absence of external Ca²⁺ (Fig. 4a). These data suggest that external Ca²⁺ influx is an important mechanism in the oxidative-stress-induced increase in the [Ca²⁺]_i level but not the Ca²⁺ release from the internal stores, and that cells depleted of [Ca²⁺]_i cannot respond to the oxidative-stress-mediated [Ca²⁺]_i increase.

Ca²⁺ is necessary for the activation of different enzymes, including calpains that participate in PCD (Tagliarino *et al.*,

2001). The extracellular Ca²⁺ chelator EGTA, [Ca²⁺]_i chelator BAPTA/AM and Ca²⁺ influx inhibitor verapamil abolished the increase in [Ca²⁺]_i level for stress-induced trophozoites (Fig. 4a), and as [Ca²⁺]_i is essential for activation of calpain and other enzymes responsible for PCD, this eventually leads to a decrease in apoptotic cell death as evidenced by abolished DNA fragmentation after exposure to ROS for 9 h (Fig. 3a, panel 1). Pretreatment with TG led to elevation of the [Ca²⁺]_i level for stress-induced trophozoites in the presence of external Ca²⁺ (Fig. 4a), and DNA fragmentation was seen (Fig. 3a, panel 1). However, a transient increase was observed with TG in absence of external Ca²⁺ (Fig. 4a) and the death phenotype was also inhibited, which is demonstrated by abolished DNA fragmentation (Fig. 3a, panel 1). Therefore, increase in the [Ca²⁺]_i level by Ca²⁺ influx is responsible for the apoptotic death of the stress-induced trophozoites, but the release of Ca²⁺ from the internal stores in the absence of Ca²⁺ influx is not sufficient to bring about apoptosis-like death. Control incubations showed that pretreatment with EGTA, BAPTA/AM and verapamil did not have detrimental effects on Ca²⁺-dependent physiological functions for 9 h stress-induced trophozoites.

An increased level of [Ca²⁺]_i generates the signal necessary for calpain-dependent DNA fragmentation of apoptotic cells. Therefore, pretreatment with the calpain inhibitor E-64d and the Ca²⁺ chelator EGTA only abolishes the DNA fragmentation (late event of apoptosis), but the trophozoites demonstrated other early features of apoptosis such as PS externalization, cell rounding and increased vacuolization.

Apoptosis can be divided into two stages: the commitment stage and the execution stage. Cells can be rescued from by repair mechanisms if they are not yet committed to apoptosis, but once they are committed to apoptosis they cannot be rescued and will die. Nine-hour stress-induced trophozoites appeared to be committed to apoptosis: when reseeded in fresh TYI-S-33 medium these cells showed an 86% decrease in viability compared to 24-h-old trophozoites not pre-exposed to oxidative stress. They also showed the characteristic events of PCD such as PS externalization and DNA fragmentation.

There is published ultrastructural evidence that smooth endoplasmic reticulum and Golgi-like elements are present in *E. histolytica* (Chávez Munguía *et al.*, 2000). Therefore, it may be suggested that immediately after the loss of the membrane potential of the endoplasmic-reticulum-like elements, protons are released into the cytosol, thus contributing to intracellular acidification (Facompre *et al.*, 2001). pH changes modulate the apoptotic responsiveness of the cells, and also amplify the apoptotic programme by regulating enzymic activities; this is in accordance with the H₂O₂-induced PCD, where a fall in intracellular pH from 7.6 to 5.8 is observed in the stress-induced *E. histolytica* trophozoites (Fig. 4b).

In this study, we have shown, to our knowledge for the first time, apoptosis-like death in *E. histolytica* induced by

H₂O₂. This process is orchestrated by coordinated alterations in intracellular ion fluxes and subsequent morphological and biochemical changes that are analogous to the events observed during PCD in other organisms. Altogether, our results suggest that a caspase-independent but calpain-mediated apoptosis-like death mechanism may exist in this amitochondriate organism. This work will provide the necessary groundwork for further studies on cell death and identification of the molecular components of *E. histolytica* that are involved in this process. This knowledge should provide new insights into the host-parasite relationship and potential molecular targets for rational drug design.

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