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 H$_2$O$_2$ 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 H$_2$O$_2$-exposed trophozoites with preservation of membrane integrity. Biochemical alteration in ion fluxes is also a key feature in PCD, and H$_2$O$_2$-exposed trophozoites showed overproduction of reactive oxygen species, increased cytosolic Ca$^{2+}$ and decreased intracellular pH. Phosphatidylserine was also found to be expressed in the outer leaflet of the plasma membrane of the H$_2$O$_2$-treated trophozoites. Pretreatment with the cysteine protease inhibitor E-64d, the extracellular and intracellular Ca$^{2+}$ chelators EGTA and BAPTA/AM, and the Ca$^{2+}$ influx inhibitor verapamil prior to H$_2$O$_2$ exposure abolished DNA fragmentation. The oxidatively stressed trophozoites also showed an increased calpain activity, indicating involvement of Ca$^{2+}$-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.

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 (O$_2^-$) and H$_2$O$_2$ (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...
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; Duszenko 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 Ca²⁺ 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).

H₂O₂, an important ROS, is a key mediator for eliciting PCD in mammalian and yeast cells (Li et al., 2000; Vollgraf et al., 1999; Clement & Pervaz, 1999; Madeo et al., 1999). H₂O₂ also induces apoptosis-like death in Leishmania donovani promastigotes (Das et al., 2001), and caspase activation and PCD in Tritrichomonas foetus (Mariante et al., 2003). But it is not established whether exposure to H₂O₂ can bring about apoptosis-like death in E. histolytica trophozoites, although it is well established that H₂O₂ 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 H₂O₂-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 × 10⁶ 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 H₂O₂. 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 H₂O₂ on E. histolytica trophozoites was investigated by treating 2 × 10⁶ E. histolytica cells kept in TYI-S-33 medium with H₂O₂ 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 ± SD of three experiments.

**Measurement of cell viability by MTT assay.** Viability of untreated and 0.8 mM H₂O₂-treated E. histolytica cells (2 × 10⁶ each set) for time periods ranging from 3 h to 9 h was determined using an MTT-based in vitro toxicity assay kit (Sigma). MTT [3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyldiazotrolium 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 H₂O₂ 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 resuspended in fresh TYI-S-33 medium (H₂O₂ free) and their viability was evaluated after 24 h. In each test there were three replicates and data are expressed as the mean ± 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 H₂O₂-treated E. histolytica cells (2 × 10⁶ 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 H₂O₂ 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 × 10⁶ 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 NAD⁰. The rate of conversion of NADH to NAD⁰ was measured spectrophotometrically at 340 nm. Untreated and 0.8 mM H₂O₂-treated *E. histolytica* cells (2 x 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 x 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% (v/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% (v/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 & Banoš, 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 x 10⁶ each set) incubated for time periods ranging from 3 h to 9 h were resuspended in 500 μl Sarcosine, pH 8.0) containing 0.5 mM Proteinase K ml⁻¹ and incubated at 35 °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 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-DT1, M, where M represents A, C or G). The synthesized cDNAs were amplified by PCR for specific genes (eh29F 5’-TCAATCACTGAAATTGTCG-3’ and eh29R 5’-GAATTGACATCTTCTACCTC-3’; sodF 5’-AATGCTTGTAGGCTCTA-3’ and sodR 5’-GTTCCAGTTGACTACATTCC-3’; oxidoreductase F 5’-TTATAGCAACAAAGAATGTT-3’ and oxidoreductase R 5’-AAAGAAGTGAAGTTCTTITTT-3’. The rate of conversion of NADH to NAD⁰ was measured spectrophotometrically at 340 nm. Untreated and 0.8 mM H₂O₂-treated *E. histolytica* trophozoites (2 x 10⁶ each set) were 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.

**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.
Effect of cysteine protease inhibitor E-64d, various Ca²⁺ chelators and Ca²⁺ flux inhibitors and a caspase 1 inhibitor on DNA fragmentation. E. histolytica trophozoites (5 × 10⁴) were incubated with 15 μM E-64d (Sigma) for 1 h and then were induced to undergo PCV by co-incubation with 0.8 mM H₂O₂ for 9 h. Similarly, pretreatments with 1 mM EGTA, 50 μM 1,2-bis(2-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid tetraakis(acetoxy-methyl ester) (BAPTA/AM), 50 μM verapamil, 50 mM thapsigargin (TG) and a combination of TG and EGTA were performed in separate sets prior to exposure to H₂O₂ 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 H₂O₂-treated E. histolytica cells (2 × 10⁵ 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 H₂O₂-treated E. histolytica cells (2 × 10⁵ 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. Red fluorescence from PI was determined at 30°C using the formula [Ca²⁺]i = Kd × (Fg − Ff)/Ff, where Fg is the fluorescence signal obtained from the entire cell, Ff the fluorescence signal after addition of 1 mM EGTA, Ff the fluorescence following cell lysis with 0.04% Triton X-100 in 0.03 M Trizma base, and Ff the fluorescence after adding 4 mM CaCl₂. Kd is the dissociation constant, for which a value of 224 nM was used, as reported by Gryniewicz 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 H₂O₂ for 9 h was performed and [Ca²⁺]i was measured for each set. In each test there were three replicates and data are expressed as the mean ± SD of three experiments.

Cytosolic Ca²⁺ concentrations. Changes in intracellular Ca²⁺ concentration ([Ca²⁺]i) were monitored with the fluorescent probe Fura-2/AM. Untreated and 0.8 mM H₂O₂-treated E. histolytica cells (1 × 10⁶ 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 1 (0.116 M NaCl, 5.4 mM KCl, 0.08 mM MgSO₄, 5.5 mM d-glucose and 0.05 M HEPES, pH 7.0). The cells were resuspended in loading buffer (1 × 10⁶ trophozoites ml⁻¹) that contained 0.116 M NaCl, 5.4 mM KCl, 0.08 mM MgSO₄, 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 1 to remove extracellular dye. Fura-2/AM was excited at 340 nm, and emission at 510 nm was registered by a Perkin Elmer LS 50 fluorospectrophotometer. The [Ca²⁺]i (nm) was determined at 30°C using the formula [Ca²⁺]i = Kd × (Fg − Ff)/Ff, where Ff is the fluorescence signal obtained from the entire cell, Fg the fluorescence signal after addition of 1 mM EGTA, Ff the fluorescence following cell lysis with 0.04% Triton X-100 in 0.03 M Trizma base, and Ff the fluorescence after adding 4 mM CaCl₂. Kd is the dissociation constant, for which a value of 224 nM was used, as reported by Gryniewicz 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 H₂O₂ for 9 h was performed and [Ca²⁺]i was measured for each set. In each test there were three replicates and data are expressed as the mean ± SD of three experiments.

Intracellular pH measurement. Untreated and 0.8 mM H₂O₂-treated E. histolytica cells (1 × 10⁶ 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 CaCl₂, 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 μg 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

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Phase-contrast microscopy and flow cytometry were used 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 H2O2. 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 H2O2 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 pre-treated 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 H2O2 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 H2O2-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 H2O2-treated trophozoites. The MDA level (an index of lipid peroxidation) was similar for the untreated and H2O2-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 H2O2 was determined by using H2DCFDA. 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 ± 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 ± 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 μm. 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 ± 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$_2$O$_2$, 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$_2$O$_2$**

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...
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 TY1-S-33 medium (H$_2$O$_2$ free) showed DNA fragmentation after 24 h (data not shown).

**Effect of E-64d, Ca$^{2+}$ chelators, Ca$^{2+}$ flux inhibitors and a caspase 1 inhibitor on DNA fragmentation**

To investigate the role of cysteine proteases, caspase 1 and Ca$^{2+}$ in DNA fragmentation of the stress-induced trophozoites, prior to exposure to H$_2$O$_2$ the trophozoites were preincubated with cysteine protease inhibitor E-64d, caspase 1 inhibitor Z-WEHD-FMK, Ca$^{2+}$ chelators and Ca$^{2+}$ 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 Ca$^{2+}$ chelator EGTA, the [Ca$^{2+}$]$_i$ chelator BAPTA/AM and the Ca$^{2+}$ 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 Ca$^{2+}$ 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 Ca$^{2+}$, 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 H$_2$O$_2$**

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 H$_2$O$_2$**

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 H$_2$O$_2$ 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 TY1-S-33 medium (H$_2$O$_2$ 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 H$_2$O$_2$ exposure**

The fluorogenic homogeneous caspase assay determines the presence of any of the active caspses 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 H$_2$O$_2$-treated and untreated trophozoites can be attributed to some autodegradation of the fluorogenic substrate.

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

<table>
<thead>
<tr>
<th>H$_2$O$_2$ treatment</th>
<th>SOD (units mg$^{-1}$)</th>
<th>ch29 (nmol H$_2$O$_2$ min$^{-1}$ mg$^{-1}$)</th>
<th>NAD(P)H:flavin oxidoreductase (units mg$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>6.4 ± 0.4</td>
<td>3.2 ± 0.3</td>
<td>2.3 ± 0.1</td>
</tr>
<tr>
<td>3 h</td>
<td>5.9 ± 0.2</td>
<td>2.8 ± 0.2</td>
<td>2.9 ± 0.05</td>
</tr>
<tr>
<td>6 h</td>
<td>4.3 ± 0.3</td>
<td>1.7 ± 0.05</td>
<td>3.7 ± 0.3</td>
</tr>
<tr>
<td>9 h</td>
<td>2.3 ± 0.1</td>
<td>1.4 ± 0.1</td>
<td>3.9 ± 0.2</td>
</tr>
</tbody>
</table>

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Ca\textsuperscript{2+} TG in the presence of the extracellular Ca\textsuperscript{2+} in the endoplasmic reticulums, led to elevation of the

**Calpain-dependent cell death of E. histolytica after H\textsubscript{2}O\textsubscript{2} exposure**

The assay for the Ca\textsuperscript{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. 4a). The extracellular Ca\textsuperscript{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 [Ca\textsuperscript{2+}]\textsubscript{i} level increased from ~20 nM to ~50 nM at 6 h of incubation and thereafter the increase in [Ca\textsuperscript{2+}]\textsubscript{i} was relatively slight (Fig. 4a). The extracellular Ca\textsuperscript{2+} chelator EGTA decreased the [Ca\textsuperscript{2+}]\textsubscript{i} in both untreated and stress-induced trophozoites (Fig. 4a). The elevation of the intracellular Ca\textsuperscript{2+} level of stress-induced trophozoites was not significant in presence of the [Ca\textsuperscript{2+}]\textsubscript{i} chelator BAPTA/AM or the Ca\textsuperscript{2+} influx inhibitor verapamil (Fig. 4a). Treatment with TG, which activates release of Ca\textsuperscript{2+} from intracellular reservoirs by blocking Ca-ATPases in the endoplasmic reticulums, led to elevation of the [Ca\textsuperscript{2+}]\textsubscript{i} for stress-induced trophozoites in the presence of external Ca\textsuperscript{2+}, but only a slight increase was observed with TG in the presence of the extracellular Ca\textsuperscript{2+} chelator EGTA, i.e. in the absence of external Ca\textsuperscript{2+} (Fig. 4a).

**Increased [Ca\textsuperscript{2+}]\textsubscript{i} accumulation after H\textsubscript{2}O\textsubscript{2} exposure**

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

**Fig. 3.** (a) Time-course of DNA fragmentation in trophozoites after H\textsubscript{2}O\textsubscript{2} treatment and effect of cysteine protease inhibitor E-64d, various Ca\textsuperscript{2+} chelators and Ca\textsuperscript{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 mM H\textsubscript{2}O\textsubscript{2}-treated E. histolytica trophozoites, and for trophozoites preincubated with (as indicated above the lanes) cysteine protease inhibitor E-64d, extracellular Ca\textsuperscript{2+} chelator EGTA, [Ca\textsuperscript{2+}]\textsubscript{i} chelator BAPTA/AM, Ca\textsuperscript{2+} influx inhibitor verapamil (VP), Ca\textsuperscript{2+}-releasing agent from intracellular reservoirs TG (in presence of extracellular Ca\textsuperscript{2+}), and TG+EGTA (to chelate extracellular Ca\textsuperscript{2+}) before 9 h 0.8 mM H\textsubscript{2}O\textsubscript{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 μm. (b) Assay of cell death after stress induction by confocal microscopy and flow cytometry using an annexin-V-FLUO 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 mM H\textsubscript{2}O\textsubscript{2}-treated 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 mM H\textsubscript{2}O\textsubscript{2}-treated E. histolytica trophozoites. Scale bars, 10 μm. 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±SD of three independent experiments. (d) Fluorogenic calpain activity assay of untreated and 9 h 0.8 mM H\textsubscript{2}O\textsubscript{2}-treated trophozoites. Calpain activity is expressed as pmol AMC released min\textsuperscript{-1} (mg protein)\textsuperscript{-1}. Data are means±SD of three independent experiments.

**Fig. 4.** (a) Measurement of [Ca\textsuperscript{2+}]\textsubscript{i}, for untreated and stress-induced E. histolytica trophozoites and effect of various Ca\textsuperscript{2+} chelators and Ca\textsuperscript{2+} flux inhibitors in regulation of the [Ca\textsuperscript{2+}]\textsubscript{i} level: Ca\textsuperscript{2+} concentrations were evaluated fluorimetrically using Fura-2/AM dye in untreated and 0.8 mM H\textsubscript{2}O\textsubscript{2}-treated E. histolytica trophozoites at 3 h intervals for 9 h. Pretreatments 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\textsubscript{2}O\textsubscript{2} for 9 h were performed and [Ca\textsuperscript{2+}]\textsubscript{i} was measured for each set. Data are means±SD of three independent experiments. (b) Change in intracellular pH for untreated and 0.8 mM H\textsubscript{2}O\textsubscript{2}-treated E. histolytica trophozoites, measured at 3 h intervals for 9 h by using the fluorescent dye BCECF. Data are means±SD of three independent experiments.
Acidification of intracellular pH after H$_2$O$_2$ exposure

In other organisms showing features of PCD, increased endogenous ROS and [Ca$^{2+}$], 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 H$_2$O$_2$ 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 H$_2$O$_2$ 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-Martinez 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 H$_2$O$_2$ 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 H$_2$O$_2$ concentration for the same rate of H$_2$O$_2$ 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 H$_2$O$_2$ via Fenton’s reaction. Therefore, H$_2$O$_2$ 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 H$_2$O$_2$ 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 H$_2$O$_2$, survival decreased with increased duration of incubation. The 9 h stress-induced trophozoites showed nearly 50% less viability, suggesting that upon exposure to exogenous H$_2$O$_2$ 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 H$_2$O$_2$ 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 O$_2^-$ to produce H$_2$O$_2$ (Bruchhaus & Tannich, 1994). In addition, direct divalent reduction of O$_2$ to H$_2$O$_2$ is catalysed by a NADPH flavin oxidoreductase (Bruchhaus et al., 1998). Catalase and glutathione reductase systems are absent in *E. histolytica* (Sen et al., 2007), but the H$_2$O$_2$ generated by both pathways can be further transformed to H$_2$O by the action of peroxyreductin (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$_2$O$_2$, 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$_2$O$_2$ 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. These observations are similar to ours for E. histolytica trophozoites exposed to H$_2$O$_2$. 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 segregation 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$_2$O$_2$-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 apoptotic-like death, i.e. DNA alteration. Preincubation of trophozoites with E-64d prior to H$_2$O$_2$ 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.

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was demonstrated. In contrast, a recent study by Ghosh et al. (2010) demonstrated \( \text{H}_2\text{O}_2 \)-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 \( \text{H}_2\text{O}_2 \).

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

\([\text{Ca}^{2+}]_i\) is necessary for the activation of different enzymes, including calpains that participate in PCD (Tagliarino *et al.*, 2001). The extracellular \([\text{Ca}^{2+}]_i\)-chelator EGTA, \([\text{Ca}^{2+}]_i\)-chelator BAPTA/AM and \([\text{Ca}^{2+}]_i\) influx inhibitor verapamil abolished the increase in \([\text{Ca}^{2+}]_i\) level for stress-induced trophozoites (Fig. 4a), and as \([\text{Ca}^{2+}]_i\) 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 \([\text{Ca}^{2+}]_i\) level for stress-induced trophozoites in the presence of external \([\text{Ca}^{2+}]_i\) (Fig. 4a), and DNA fragmentation was seen (Fig. 3a, panel 1). However, a transient increase was observed with TG in absence of external \([\text{Ca}^{2+}]_i\) (Fig. 4a) and the death phenotype was also inhibited, which is demonstrated by abolished DNA fragmentation (Fig. 3a, panel 1). Therefore, increase in the \([\text{Ca}^{2+}]_i\) level by \([\text{Ca}^{2+}]_i\) influx is responsible for the apoptotic death of the stress-induced trophozoites, but the release of \([\text{Ca}^{2+}]_i\) from the internal stores in the absence of \([\text{Ca}^{2+}]_i\) 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 \([\text{Ca}^{2+}]_i\)-dependent physiological functions for 9 h stress-induced trophozoites.

An increased level of \([\text{Ca}^{2+}]_i\) generates the signal necessary for calpain-dependent DNA fragmentation of apoptotic cells. Therefore, pretreatment with the calpain inhibitor E-64d and the \([\text{Ca}^{2+}]_i\) 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 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, cell rounding and increased vacuolization.

There is published ultrastructural evidence that smooth endoplasmic reticulum and Golgi-like elements are present in *E. histolytica* (Chávez Munguiá *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 (Facompré *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 \( \text{H}_2\text{O}_2 \)-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...
H$_2$O$_2$. 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 amitochondrial organism. This work will provide the necessary groundwork for further studies on cell death and identification of the molecular components of Entamoeba 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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