INTRODUCTION

Cells contain a specific death-governing network associated with different effector and/or dismantling mechanisms. Apoptosis consists of events occurring via a cascade of caspase activation leading to ordered dismantling of critical cell components and pathways. At the end of apoptosis, the cell is fragmented into apoptotic bodies that undergo phagocytosis by neighbouring cells (Katoch et al., 2002). Thus, these morphological changes are the manifestation of the cell undergoing systematic dismantling and further packaging itself into membrane-bound vesicles to be taken up by neighbouring cells. Because cellular contents are not released, apoptosis occurs without inflammation. However, little is known about the dismantling process in non-apoptotic cell death where caspases are not involved. A major task, for the less well-known non-apoptotic types of cell death, will be the identification and study of cell death types, which reflect dismantling events of effector mechanisms. Dictyostelium discoideum is a good model system to study dismantling events where it shows poly(ADP-ribose) polymerase (PARP)-mediated caspase-independent cell death (Rajawat et al., 2014). The evolutionary aspects of programmed cell death would be illuminated by studying cell death mechanisms in D. discoideum (Mir et al., 2007; Kawal et al., 2011).

Oxidative stress has been shown to be associated with cell death in most systems (Hasnain et al., 1999; Sah et al., 1999; Mohan et al., 2003). We have reported the high resistance of the unicellular stage of D. discoideum to oxidative stress (Katoch & Begum, 2003). D. discoideum devoid of caspases exhibits non-apoptotic cell death mediated by several proteins or factors, PARP being one of them (Rajawat et al., 2014). We have established the role of PARP during development (Rajawat et al., 2007, 2011) and staurosporine- (Mir et al., 2012) and oxidative stress-induced cell death (Rajawat et al., 2014) along with the downstream effects. Our results suggest that PARP and apoptosis inducing factor (AIF) may be the key players in regulating caspase-independent cell death. In the current study we have explored the biochemical events occurring during oxidative stress-induced cell death in D. discoideum.
In mammalian systems, probable candidates involved in cell dismantling could be lysosomal proteases or cytosolic protease, namely calpain. Partial destabilization of lysosomal membrane leads to release of cathepsin D in the cytosol. Cathepsin D triggers Bax activation, which induces the release of AIF into the cytosol (Bidère et al., 2003). Cathepsin D also induces generalized proteolysis leading to caspase-independent cell death. Calpains are believed to participate in intracellular signal processing via limited proteolysis of their targets. Calpains have been shown to act downstream of caspase activation and contribute to the degradation phase of camptothecin-induced apoptosis in HL-60 cells (Wood et al., 1998; Wood & Newcomb, 1999). Sanvicencs et al. (2004) have shown that both caspases and calpains contribute to oxidative stress-induced apoptosis in retinal photoreceptor cells. Thus, to explore the role of proteases in D. discoideum dismantling, we initiated our study with protease inhibitor cocktail (PIC) to inhibit most of the proteases and then used specific inhibitors for calpain and cathepsin D.

**METHODS**

**D. discoideum culture conditions.** D. discoideum strain Ax-2, which is an axenic derivative of Raper’s wild-type NC-4, was used. D. discoideum was grown under different culture conditions. Growing cells (unicellular) were maintained in a liquid suspension (HL5 medium). D. discoideum cells were grown in HL5 medium, pH 6.5, with shaking (150 r.p.m.) at 22 °C (Watts & Ashworth, 1970). Exponential phase cells at a density of ~2.5 × 10^6 cells ml^{-1} were used for experiments.

**Induction of oxidative stress.** Oxidative stress was induced in D. discoideum cells by in situ generation of H\textsubscript{2}O\textsubscript{2} upon addition of hydroxylamine (HA; Sigma), a catalase inhibitor (Kono & Fridovich, 1983), or by exogenous addition of cumene hydroperoxide (Sigma). Exponential phase cells at a density of ~2.5 × 10^6 cells ml^{-1} were exposed to different doses of HA (0, 1, 2.5 mM) and paraptotic (0.03 mM) and necrotic (0.05 mM) doses of cumene H\textsubscript{2}O\textsubscript{2}, as described by Rajawat et al. (2014) in HL-5 medium at 22 °C in a sterile flask.

**Assessment of cell death by Annexin V-FITC/propidium iodide (PI) dual staining.** To differentiate between apoptotic and necrotic cell death, dual staining with Annexin V-FITC/PI (Miller, 2004) was performed using an apoptosis detection kit (Molecular Probes). Then, ~2.0 × 10^6 cells were pelleted and washed twice with 1 × Sorenson’s buffer (SB). D. discoideum cells were then suspended in binding buffer provided in the kit and incubated with Annexin V for 10 min and then with PI for 5 min in the dark at 22 °C. Fluorescence was monitored at ~63 using a Zeiss confocal laser scanning fluorescence-inverted microscope (LSM 710; Carl Zeiss) and quantified by flow cytometry using a FACSCalibur cytometer (BD Biosciences). Data were analysed with FACSDiva software. A dose- and time-dependent study was done to standardize the paraptotic and necrotic doses of HA for further experiments.

**Evaluation of mitochondrial membrane potential (MMP).** The potential sensitive dye 3,3'-dihexyloxacarbocyanine iodide (DiOC\textsubscript{6}; Sigma) was used to evaluate changes in MMP (Koning et al., 1993). To observe the change in MMP, a time-dependent study was done using paraptotic and necrotic doses of HA as standardized by Annexin V–PI dual staining. Approximately 2.0 × 10^6 cells were pelleted and washed twice with 1 × SB. Cells were stained with DiOC\textsubscript{6} (400 nM) for 15 min in the dark and then washed once with 1 × SB and monitored for fluorescence using a Nikon Eclipse TE2000S fluorescence microscope.

MMP was also measured by flow cytometry, by incubating D. discoideum cells (1 × 10^6 ml^{-1}) with 5,5',6,6'-tetramethyl-1,3,3'-tetraethylbenzimidazol carboxyanine iodide (JC-1; Molecular Probes) (Cossarizza & Salvioni, 2001), and quantified by flow cytometry using a FACS ARIA cytometer. Data were analysed with CellQuest software.

**Monitoring AIF release by immunofluorescence.** Release of AIF from mitochondria to cytosol and its translocation to nucleus were monitored by detecting immunofluorescence (Bidère et al., 2003) at different time intervals. D. discoideum cells were pelleted and washed once with PBS (pH 7.4), fixed in 70% chilled methanol for 10 min at –20 °C and then washed with blocking solution (1.5% BSA with 0.05% Tween 20 in PBS) followed by incubation for 1 h in primary antibody using rabbit anti-AIF polyclonal antibodies raised against amino acids 151–180 of human AIF (Cayman Chemical) at 1:1000 dilution and then anti-rabbit IgG (whole molecule) TRITC conjugate (Sigma) at 1:400 dilution. Nuclear counterstaining with DAPI (1 µg ml^{-1}) for 5 min was performed after the removal of excess secondary antibody and observed for fluorescence.

**Detection of caspase activity.** The substrate DEVD-AMC is an oligopeptide that is covalently linked with the fluorophore 7-amino-4-methylcoumarin (AMC). DEVD is the cleavage site for caspases (3 and 7). Cleavage takes place at the C terminus of the last aspartate residue, thus liberating the fluorophore AMC, which can be estimated and/or visualized under the fluorescence microscope (AMC λ\textsubscript{ex}=380 nm; λ\textsubscript{em}=420 nm).

Approximately 2.0 × 10^6 cells were harvested and washed with PBS. Cells were resuspended in 1 ml PBS and an aliquot of 100 µl was taken from it and 10 µl of DEVD-AMC substrate (1 mg ml^{-1}) was added. After incubation for 1 h, cells were observed under the Nikon Eclipse TE2000S fluorescence microscope. AMC liberated from the fluorogenic substrate was measured at 380 nm using a UV filter (Olie et al., 1998). Caspase activity as a function of AMC fluorescence was monitored at different time intervals. The entire procedure was carried out using a caspase-3 assay kit (Sigma) as per the manufacturer’s instructions. The effect of caspases on cell death was also studied by using a broad caspase inhibitor (zVAD-fmk, Sigma).

**Characterization of vesicles formed during paraptotic cell death.** Isolation of vesicles was performed from D. discoideum culture after 16 h of 1 mM HA stress as mentioned by Gauhar & Sharma (2002a). The culture was centrifuged at 1000 g for 4 min at 4 °C; the supernatant was collected and centrifuged at 21 000 g for 45 min at 4 °C. The pellet obtained was washed once with 1 × SB and used for further analysis. Formation of these vesicles was monitored at different time intervals. Isolated vesicles were stained separately with a fluorescent membrane probe, 1,6-diphenyl-1,3,5-hexatriene (DPH), at a concentration of 1 µM, DAPI and Annexin V–PI and then observed using a confocal laser scanning fluorescence-inverted microscope (LSM 710; Carl Zeiss) and fluorimeter (F7000; Hitachi).

**Vacuolization in paraptotic cell death.** Cultured exponential-phase cells exposed to 0.03 mM H\textsubscript{2}O\textsubscript{2} were rinsed in 0.1 M phosphate buffer, and fixed in 2.5 % glutaraldehyde and 2.0 % paraformaldehyde in 1.0 M phosphate buffer, pH 7.4, at 4 °C for 6–8 h. The pellet was then stored in paraformaldehyde and 0.1 M phosphate buffer (1 : 1) and processed for microtomy. Sections were obtained with a Reichert Utracut E ultramicrotome, stained and examined with a Morgagni 268D transmission electron microscope.

**Investigation of proteases involved in cell death.** Cells were pre-incubated with PIC (Sigma) followed by oxidative stress, and MMP change was monitored. Similarly, MMP change, phosphatidylserine
(PS) exposure and vesicle formation were followed in oxidative stress-treated cells pre-incubated for 12 h with 7.5 μM pepstatin A, a cathepsin D inhibitor, and 10 μM N-acetyl-L-leucyl-L-leucyl-L-norleucinal (ALLN), a calpain inhibitor.

**Calpain activity.** Calpain activity present in total cell lysates of 2.0 x 10⁶ cells of control and oxidative stressed *D. discoideum* was determined by cleavage of the fluorescent substrate N-succinyl-LLVY-AMC (Calbiochem). Calcium-dependent fluorescence was measured after 30 min incubation at 37 °C in buffer containing 63 mM imidazole-HCl, pH 7.3, 10 mM β-mercaptoethanol and 5 mM CaCl₂ (Moubarak et al., 2007). Fluorescence was recorded in a fluorimeter. Data were normalized per milligram of protein, as estimated by the Lowry method (Lowry et al., 1951).

**RESULTS**

**Induction of cell death in *D. discoideum* cells by oxidative stress**

Experiments were designed to establish the ability of *D. discoideum* to undertake cell death as a function of oxidative stress. This was achieved by intracellular build up of H₂O₂ by HA treatment. Cells were treated with different concentrations of HA (0, 1, 2.5, 3, 4 mM), a known inhibitor of catalase, leading to intracellular accumulation of reactive oxygen species, and cell death was measured by the trypan blue exclusion method (Fig. 1a). The LD₅₀, i.e. the concentration of HA inducing 50% cell death at 12 h post-treatment, was 2.5 mM.

Staining with Annexin V-FITC in conjunction with vital dyes such as PI allows us to distinguish apoptotic cells (Annexin V-positive, PI-negative) from necrotic cells (Annexin V-positive, PI-positive). Thus, based on Annexin V–PI dual staining results, 2.5 mM HA was necrotic as cells exhibited both Annexin V staining due to PS exposure and PI staining at 3 h. In contrast, 1 mM HA was paraptotic as Annexin V staining due to PS exposure was exhibited by cells after 5 h while PI was observed at 12 h of oxidant treatment (Fig. 1b, c). Paraptotic and necrotic doses of cumene H₂O₂ were also used for further experiments, as described previously (Rajawat et al., 2014).

**Oxidative stress-induced cell death in *D. discoideum* is caspase-independent**

Oxidative stress was generated with HA or cumene H₂O₂. Caspase-3 activity was monitored during oxidative stress-induced cell death. No significant change in caspase activity was seen in basal fluorescence in control and 1 mM HA-treated cells at 1 and 6 h post-stress. A caspase-3-specific inhibitor (DEVD-CHO) could not inhibit the observed caspase activity (Fig. 2a), suggesting absence of caspase activation during oxidative stress-induced cell death in *D. discoideum*. HA at 2.5 mM also exhibited non-significant caspase activity (Fig. 2a).

To demonstrate that the cytotoxicity effect is not due to a caspase-dependent pathway, cell death study was carried out with a broad caspase inhibitor (zVAD-fmk). Our previous reports show that MMP changes following post-oxidative stress. zVAD-fmk had no effect on MMP changes (Fig. 2b) induced by oxidative stress and also on plasma membrane integrity as monitored by PI staining (Fig. 2c). In the presence of 10 μM zVAD-fmk, the cytotoxicity induced by oxidative stress was not inhibited, indicating that *D. discoideum* cells take up a caspase-independent pathway. Further increasing the concentration of zVAD-fmk up to 25 μM did not change the results.

**Characterization of vesicles formed during non-apoptotic cell death**

To examine the fate of dying paraptotic cells, paraptotic vesicle formation was assessed. We could observe the formation of vesicles at a later stage after loss of plasma membrane integrity. Our results suggest that the vesicles formed by paraptotic doses of HA and cumene H₂O₂ were membranous in nature (Fig. 3a, b) and contain DNA (Fig. 3c). PS staining could not be seen, suggesting that vesicles did not exhibit PS exposure (data not shown). Interestingly such vesicles were not observed with 2.5 mM HA/0.05 mM cumene H₂O₂ stress, which is the necrotic dose.

**Vacuolization in paraptotic cell death**

Under the electron microscope, vegetatively growing *D. discoideum* cells showed a highly developed cytoplasmic system of small vacuoles. After 16–22 h of oxidative stress-induced paraptotic cell death (1 mM HA/0.03 mM cumene H₂O₂), vacuoles were more abundant in stressed cells than in control cells. After 12 h in the presence of 1 mM HA/0.03 mM cumene H₂O₂, the cell borders were altered. The most prominent difference was the presence of large cytoplasmic vacuoles, either appearing empty or containing residual material (Fig. 3d).

**Proteases involved in *D. discoideum* caspase-independent cell death**

**Effect of PIC on caspase-independent cell death.** PIC had no effect at 3 h while partial rescue in MMP changes was observed at 5 h (Fig. 4a). Protease inhibition results showed that MMP changes were unaffected, suggesting that lysosomal proteases are not involved in the early phase of paraptotic cell death. However, during the late phase of paraptotic cell death lysosomes seem to be involved in dismantling events, as caspases are absent in *D. discoideum*.

**Calpain activity during caspase-independent cell death**

To explore a possible role of calpain in oxidative stress-induced cell death we examined the effect of HA and H₂O₂ on calpain activity. The kinetics of calpain activity was studied using the fluorescent calpain peptide substrate N-succinyl-leu-leu-val-tyr-7-amino-4-methylcoumarin. Our results suggest an increase in calpain activity by 3 h during 1 mM HA and 0.03 mM cumene H₂O₂ treatments (Fig. 4b).
Effect of calpain and cathepsin D inhibition on MMP changes during oxidative stress-induced cell death

To further identify the specific protease(s) involved during oxidative stress-induced cell death we used ALLN, a calpain inhibitor, and pepstatin A, a cathepsin D inhibitor, and monitored their effects on MMP changes and PS–PI staining. To study the effect of calpain and cathepsin D inhibition during oxidative stress-induced cell death, cells were pre-incubated with 10 μM calpain inhibitor (ALLN)
and 7.5 μM pepstatin A before inducing oxidative stress. No significant effect was observed on MMP with pepstatin alone (Fig. 5a). Nevertheless, calpain inhibitor (ALLN) alone could partially retrieve the changes in MMP induced during stress with 1 mM HA and 0.03 mM H₂O₂. Further combinatorial effects of both inhibitors on cell death were also monitored. The resultant MMP changes as monitored using DiOC₆ are shown in Fig. 5(b, c). As can be seen, calpain inhibitor and pepstatin A pretreatment together gave significant rescue in MMP changes as compared with pepstatin A or ALLN alone on 1 mM HA- and 0.03 mM H₂O₂-induced MMP changes. To confirm the change in MMP, JC-1 staining was performed. Mitochondria containing red JC-1 aggregates in healthy cells are detectable in the FL2 channel, and green JC-1 monomers in apoptotic cells are detectable in the FITC channel (FL1). JC-1 staining also showed a reduction in red fluorescence in ~64% of cells, indicating mitochondrial membrane depolarization at 3 h post-1 mM HA treatment, which showed marked rescue by the calpain inhibitor ALLN and pepstatin A pretreatment: an ~37% increase in red fluorescence as opposed to 5.6% with pepstatin A or 12.56% with ALLN (Fig. 5d). As pepstatin alone could not rescue MMP changes, cathepsin D probably does not have a major role in inducing cell death. As shown in Fig. 6, cells pre-incubated with calpain inhibitor (ALLN) and cathepsin D inhibitor (pepstatin A) subjected to 1 mM HA stress exhibited only PS exposure at 12 h but no PI staining, indicating that plasma membrane integrity remains intact upon treatment with calpain and cathepsin inhibitors during oxidative stress.

### Fig. 2. Caspase-independent cell death in D. discoideum.

(a) Caspase activity during paraptotic cell death. D. discoideum cells were treated with HA stress and caspase activity was assayed at 6 h in the form of DEVD-AMC cleavage and also in the presence of a caspase-3 inhibitor. Caspase activity was non-significant (n.s.). (b) MMP changes in the presence of a broad caspase inhibitor during paraptosis. Data (mean ± se) are from three independent experiments. No significant (n.s.) effect was observed. (c) Propidium iodide staining in the presence of a broad caspase inhibitor during paraptosis. Data (mean ± se) are from three independent experiments.

### Effect of calpain inhibition on AIF translocation

AIF translocation to nucleus was observed at 6 h of 1 mM HA treatment (cells exhibited pink fluorescence). Pretreatment of ALLN and pepstatin A partially prevented the translocation of AIF to nucleus in 1 mM HA-stressed D. discoideum cells (Fig. 7).

### Vesicle formation in the presence of calpain and cathepsin D inhibitors

The vesicles were formed by 16 h with a paraptotic dose of oxidative stress. As can be seen from the fluorescence images, the vesicles are membranous in nature and contain DNA as indicated by staining with DPH and DAPI, respectively. Cells pretreated with cathepsin D and calpain inhibitors, then exposed to 1 mM HA and 0.03 mM H₂O₂ stress showed no vesicle formation while pre-incubation with calpain inhibitor alone followed by treatment with 1 mM HA and 0.03 mM H₂O₂ showed vesicle formation (Fig. 8) although less than under oxidative stress alone.

### Proteases involved during oxidative stress-induced necrotic cell death

#### Calpain activity during necrosis

A significant increase in calpain activity was observed in cells subjected to 2.5 mM HA and 0.05 mM H₂O₂ treatments as compared with control at 3 h (Fig. 9a).

### Effect of PIC on necrosis

Partial rescue in MMP was seen at 2 h with PIC during necrotic cell death, confirming that lysosomal proteases are involved in MMP changes (Fig. 9b).
**Fig. 3.** Vesicle formation and vacuolization during oxidative stress-induced paraptosis. (a) Characterization of paraptotic vesicles formed during oxidative stress using the membrane probe DPH by confocal microscopy. Data are representative of at least three independent experiments. Photographs were taken using a ×63 objective. (b) Fluorimetric analysis of paraptotic vesicles using DPH staining. \( ***P<0.001 \) compared with control. Error bars, ± SEM. (c) Characterization of paraptotic vesicles formed during oxidative stress using the DNA binding dye DAPI. Data are representative of at least three independent experiments. \( ***P<0.001 \) compared with control. Error bars, ± SEM. (d) Electron microscopy of 0.03 mM H\(_2\)O\(_2\)-stressed *D. discoideum* Ax-2 cells at 16 and 22 h. Cells exhibit an irregular shape with a highly developed vacuolar system (V) under oxidative stress.

**Fig. 4.** Effect of PIC on MMP changes and calpain activity during parapoptotic cell death. (a) PIC exhibited partial restoration of MMP changes at 5 h. Results are the mean of three independent experiments ± SEM. \( ***P<0.001, \) \( **P<0.01 \) compared with control; \( aa, bbP<0.01 \) compared with the respective treatments. (b) Calpain activity measured by using its substrate succinyl-AMC during paraptosis at 3 h post-oxidative stress. \( **P<0.001, \) \( *P<0.01 \) compared with control.
**Fig. 5.** MMP changes during paraptosis in *D. discoideum*. (a) Effect of cathepsin D inhibition on MMP changes during oxidative stress. No significant changes were observed with cathepsin D inhibitor, pepstatin A. Results are the mean of three independent experiments ± SEM. (b) Fluorescence images of MMP changes monitored using DiOC₆ dye. Data are representative of three independent experiments. Photographs were taken using a ×60 objective. (c) Densitometric analysis of fluorescence data. Calpain inhibition partially intercepts the MMP changes at both 1 mM HA and 0.03 mM H₂O₂. ***P < 0.001 compared with untreated control; aa, bb P < 0.01 compared with the respective treated controls; b P < 0.05 compared with H₂O₂ treatment. Error bars, ± SEM. (d) Dot plot of red fluorescence (FL2) versus green fluorescence (FL1) resolved in live cells with intact MMP and from 1 mM HA-treated cells (in the presence of ALLN, pepstatin and ALLN + pepstatin) with loss of MMP. Mitochondria containing red JC-1 aggregates in healthy cells are detectable in the FL2 channel, and green JC-1 monomers in apoptotic cells are detectable in the FITC channel (FL1).
Effect of calpain and cathepsin D inhibition on MMP changes during oxidative stress-induced necrotic cell death

No effect was observed on 2.5 mM HA- and 0.05 mM H₂O₂-induced MMP changes by ALLN, a calpain inhibitor, alone. Calpain and cathepsin D inhibition collectively prevented the MMP changes during necrosis (Fig. 9c).

DISCUSSION

Caspase activation is the hallmark feature of apoptotic cell death seen in all multicellular eukaryotes (Saraste & Pulkki, 2000). Interestingly, caspase activation has also been reported during prokaryotic cell death (Gautam & Sharma, 2002a, b) and also during oxidative stress-induced cell death in yeast (Madeo et al., 2002). We have characterized the oxidative stress-mediated cell death in *D. discoideum*, which was found to be PARP- and AIF-mediated but caspase-independent. Reports suggest that developmental cell death is caspase-independent (Roisin-Bouffay et al., 2004). However, Olie et al. (1998) reported that extracts from vegetative Ax-2 cells showed labelling with z-EK(bio)D-aomk, which labels activated caspases and caspase inhibitor, blocked morphogenesis and not cell death. Nevertheless, caspase activation could not be seen in *D. discoideum* under oxidative stress (Fig. 2a), strengthening caspase independence during oxidative stress-induced cell death in *D. discoideum* unlike the yeast system which showed caspase activation during oxidative stress-induced cell death (Madeo et al., 2002). The absence of caspases led us to characterize caspase-independent cell dismantling mechanisms. The sequence of events between oxidative insult and cell dismantling remain unclear. Along with caspase independence, vacuolization continues to be a morphological change in non-apoptotic PARP-mediated death called ‘paraptosis’ (Sperandio et al., 2000). Vacuolization observed under oxidative stress indicates paraptotic cell death (Fig. 3).

To explore the possibility of lysosomal involvement during oxidative stress-induced cell death in *D. discoideum*, PIC was used to monitor MMP changes, which exhibited partial rescue, suggesting that proteases could be acting upstream of MMP changes (Fig. 4a). Cathepsin D and calpain inhibition studies were performed to further elucidate the involvement of specific proteases in dismantling during paraptosis. Cathepsin D, being a lysosomal protease known to be active at cytosolic pH (Zong & Thompson, 2006), could serve as the protease in dismantling the cell (Turk & Stoka, 2007) during oxidative stress-induced cell death. In addition, calpain, a cytosolic protease known to affect MMP (Polster & Fiskum, 2004), and further downstream events during caspase-independent cell death could be involved. Hence

**Fig. 6.** Effect of calpain and cathepsin D inhibition on plasma membrane integrity as monitored by PS–PI dual staining. Both ALLN and pepstatin A delayed the PS exposure during paraptosis. Photographs were taken using a ×60 objective.

**Fig. 7.** Effect of calpain inhibition on AIF translocation. Intensity of green fluorescence was monitored after 6 h of oxidative stress-induced paraptotic cell death. Red colour: AIF; fluorescent green: DAPI (pseudo colour); green: AIF translocated to nucleus (indicated by arrow). Photographs were taken using a ×60 objective.

**Fig. 8.** Paraptotic vesicles stained with membrane probe DPH. Vesicles collected after 12 h of HA and H₂O₂ treatments were incubated with DPH and fluorescence was observed by fluorimetry. ***P<0.001 compared with control; aa, bb P<0.01 compared with the respective treatments; bbb P<0.001 compared with H₂O₂ treatment. Error bars, ± SEM.
experiments were done using a cathepsin D inhibitor (pepstatin A) and calpain inhibitor (ALLN).

Calpain inhibition studies showed a partial rescue in caspase-independent cell death while cathepsin D inhibition alone could not delay death of the cell. When pre-incubated with the calpain inhibitor ALLN, cells showed rescue in MMP changes at 3 h (Fig. 5b, c), while pepstatin A did not show any effect on MMP (Fig. 5a). During necrosis MMP change was partially rescued by PIC (Fig. 9b) and by both calpain and cathepsin D inhibition at 3 h (Fig. 9c).

Inhibition of these two proteases also prevented the loss of plasma membrane integrity (Fig. 6). It has been reported that calpain inhibitor is able to block translocation of AIF and further cell death both in vivo and in vitro (Sanges et al., 2006). AIF translocation to the nucleus was also partially rescued upon calpain inhibition (Fig. 7). Thus, our results imply that calpains function upstream while lysosomal proteases function downstream of mitochondrial changes during oxidative stress-induced cell death.

Cells pre-incubated with both cathepsin D and calpain inhibitors showed complete inhibition of vesicle formation (Fig. 8), suggesting that cathepsin D and calpains facilitate cell dismantling during oxidative stress-induced cell death. Calpain activation as well as its blockade during PARP-mediated cell death was confirmed by using a fluorescent substrate for calpain in the total cell lysate (Figs 4b and 9a), and our results suggest that calpain regulates AIF release during oxidative stress-induced PARP-mediated cell death in D. discoideum. Hence, protection observed with protease inhibitors could be attributed mainly to calpains as caspases are absent in D. discoideum (Olie et al., 1998).

Our data suggest that oxidative stress promotes calpain activity in D. discoideum. We have also shown that proteases are involved in causing MMP changes and downstream events including cell dismantling in the absence of caspases. Our studies suggest that proteases, particularly calpains and cathepsin D, could be the main players involved in the downstream events during oxidative stress-induced cell death as their inhibition prevented dismantling of the cell and thus delayed cell death in D. discoideum.

Mitochondrial uncoupling leads to plasma membrane rupture in necrotic cell death induced by DIF in D. discoideum, whereas exogenous glucose delays it non-glycolytically (Laporte et al., 2007). Our study suggests that necrosis occurs in a programmed fashion with MMP changes manifested by proteases followed by plasma membrane rupture. Proteolysis during oxidative stress-induced necrotic cell death involves calpains and lysosomal proteases. While the pathways for apoptosis and necrosis are distinct, they nonetheless overlap and cross talk in vivo (Hasnain et al., 2003). It would be of interest to explore if such cross talk exists.

Thus, from the above results we suggest an alternative cell death programme that may be regulated by PARP, AIF, calpains and cathepsin D as key players, where PARP may activate calpains by bringing a change in calcium homeostasis, as shown by Moubarak et al. (2007). Activated calpains would cause the release of AIF from mitochondria and cathepsin D from lysosomes. In this context we have revealed two proteases that could be orchestrating cell dismantling. This pathway opens the possibility to further
characterize the mechanism involved in cell dismantling during caspase-independent cell death. Moreover, D. discoideum can thus be used as a model to study the molecular mechanisms involved in non-apoptotic and programmed necrosis, which can then be substantiated in mammalian cells.

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