Proteases involved in cell dismantling during oxidative stress induced paraptosis and necrosis in *D. discoideum*

5.1. INTRODUCTION

Every cell contains 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 the 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. Thus these morphological changes are the manifestations of the cell systematically dismantling and packaging itself in membrane bound vesicles to be taken up by neighboring cells. Because cellular contents are not released, this occurs without inflammation. However, little is known about the dismantling process in nonapoptotic cell death where caspases are not involved. A major task ahead, for the less well known nonapoptotic types of cell death, will be the identification and study of cell death types, which reflect dismantling events of effector mechanisms. *D. discoideum* is a good model system to study dismantling events where experimental accessibility of nonapoptotic cell death is easier and molecular redundancy might be less compared to the other animal models.

Probable candidates involved in dismantling could be lysosomal proteases or cytosolic protease namely calpain. Partial destabilization of lysosomal membrane leads to release of cathepsin D in cytosol. Cathepsin D triggers Bax activation which induces the release of AIF into cytosol (Bidere *et al.*, 2003). It 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 campthotecin induced apoptosis in HL-60 cells (Wood and Newcomb, 1999; Wood *et al.*, 1998). Sanvicens *et al.*, have shown that both caspases and calpains contribute to oxidative stress induced apoptosis in retinal photoreceptor cells (Sanvicens *et al.*, 2004). Thus to explore the role of proteases in 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.

5.2. RESULTS

5.2.1. Proteases involved in *D. discoideum* paraptotic cell death

5.2.1.1. Effect of Protease Inhibitor Cocktail (PIC) on paraptosis

PIC has no effect at 3 hours while partial rescue in MMP changes was observed at 5 hours (Fig. 5.1). 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*.

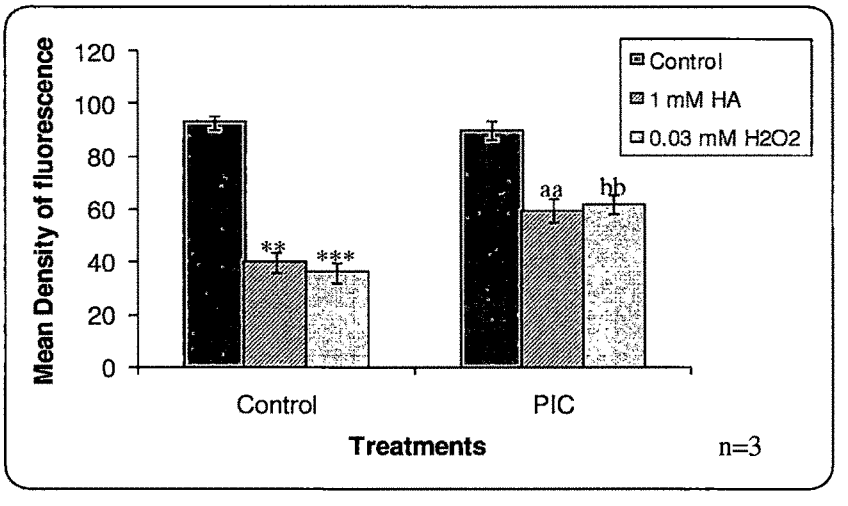

Figure 5.1: Effect of Protease Inhibitor Cocktail (PIC) on mitochondrial membrane potential changes during paraptotic cell death. PIC exhibited partial restoration in MMP changes at 5 hours. Results are the mean of three independent experiments ± SE. *** p value <0.001, ** p value <0.01 compared to control; [aa], [bb] p value <0.01 compared to the respective treatments.

5.2.1.2. Effect of calpain and cathepsin D inhibition on MMP changes during oxidative stress induced paraptotic cell death

To further identify the specific protease/s involved during paraptotic 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 1 and 2.5 mM HA induced cell death, cells were preincubated 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. 5.2).

Nevertheless calpain inhibitor (ALLN) alone could partially retrieve the changes in MMP induced during paraptosis. Further combinatorial effect of both the inhibitors was also monitored on cell death. The resultant MMP changes as monitored using $DiOC_6$ are shown in figs. 5.3 & 5.4. As can be seen from figs. 5.3 & 5.4, calpain inhibitor and pepstatin A pretreatment together gave significant rescue in MMP changes as compared to pepstatin A or ALLN alone on 1 mM HA and 0.03 mM H_2O_2 induced MMP changes. Since pepstatin alone could not rescue MMP changes, probably cathepsin D is not playing a major role in inducing paraptotic cell death.

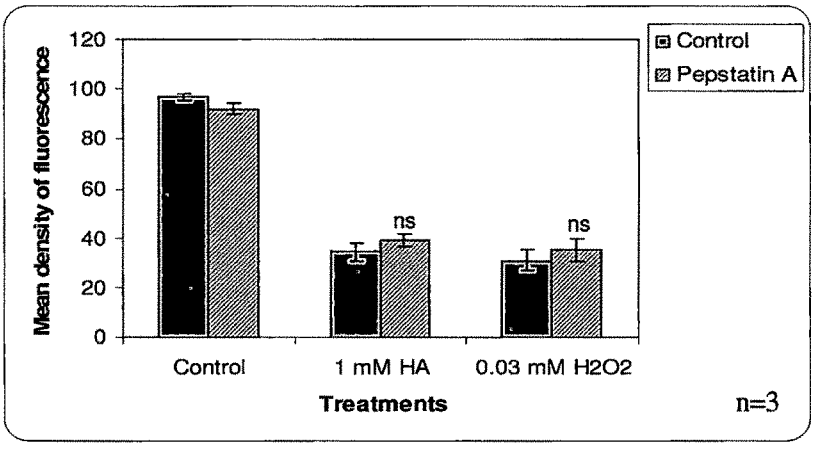

Figure 5.2: Effect of cathepsin D inhibition on MMP changes during oxidative stress. No significant changes were observed with cathepsin D inhibitor, pepstatin A. Results are the mean of three independent experiments ± SE.

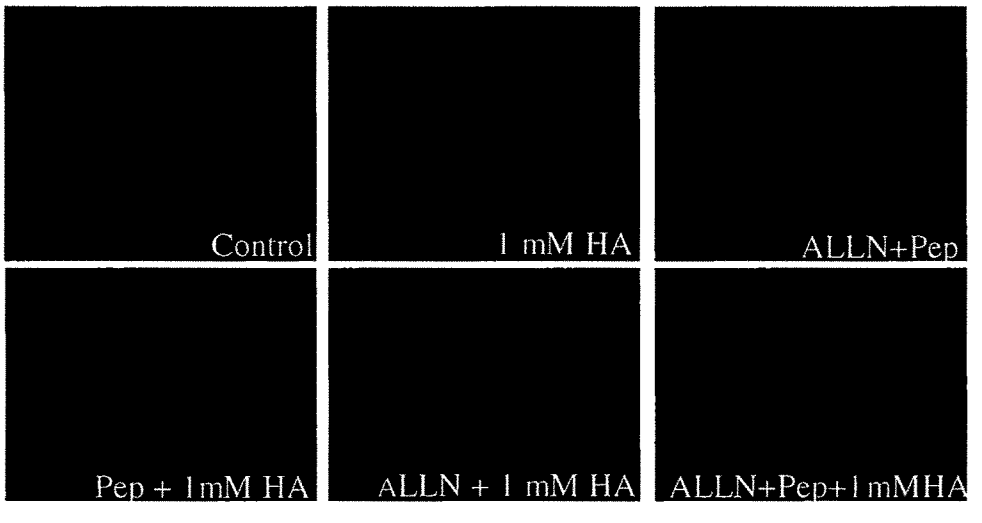

Figure 5.3: Effect of calpain and cathepsin D inhibition on MMP changes during 1 mM HA stress. Data is representative of three independent experiments. Photographs were taken with 60X objective.

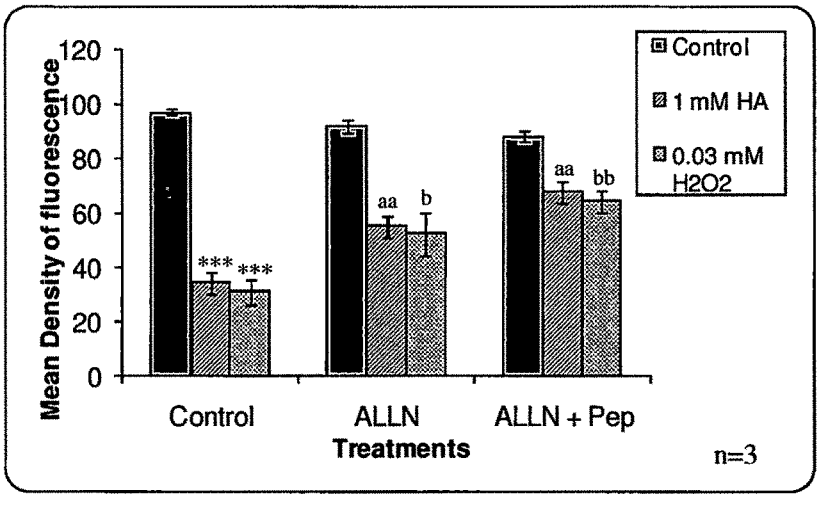

Figure 5.4: Effect of calpain and cathepsin D inhibition on oxidative stress induced mitochondrial membrane potential changes during paraptosis. Calpain inhibition partially intercepts the MMP changes at both 1 mM HA and 0.03 mM H_2O_2. *** p value <0.001 compared to untreated control; [aa, bb] p value <0.01 compared to the respective treated controls; [b] p value <0.05 compared to H_2O_2 treatment.

5.2.1.3. Effect of calpain and cathepsin D inhibition on plasma membrane integrity as monitored by PS-PI dual staining

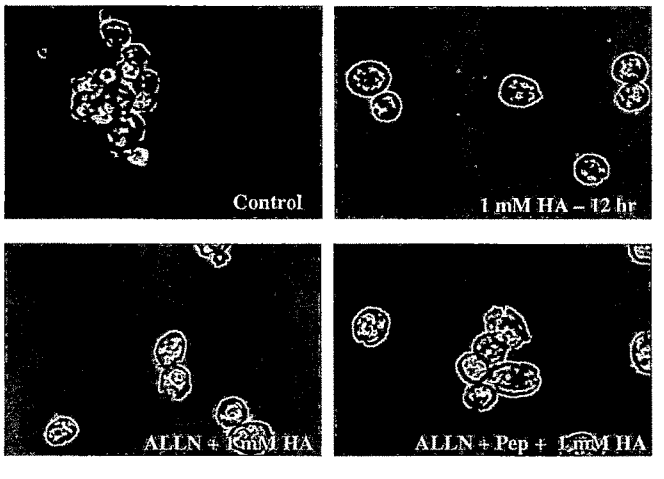

Figure 5.5: Effect of calpain and cathepsin D inhibition on Plasma membrane integrity as monitored by PS-PI dual staining. Both ALLN and pepstatin A delayed the phosphatidylserine exposure during paraptosis. Photographs were taken with 60X objective.

As shown in Fig. 5.5, cells preincubated with calpain inhibitor i.e. (ALLN) and cathepsin D inhibitor i.e. pepstatin A during 1 mM HA stress exhibited only PS exposure at 12 hours but no PI staining which indicates that plasma membrane integrity remains intact upon treatment with calpain and cathepsin inhibitors during oxidative stress.

5.2.1.4. Effect of calpain inhibition on AIF translocation

AIF translocation to nucleus was observed at 6 hours of 1 mM HA treatment (cells exhibited pink fluorescence). Pretreatment of ALLN (calpain inhibitor) and pepstatin A partially prevented the translocation of AIF to nucleus in 1 mM HA stressed *D. discoideum* cells (Fig. 5.6).

Figure 5.6: Effect of calpain inhibition on AIF translocation. Intensity of pink fluorescence was monitored after 6 hours oxidative stress induced paraptotic cell death. Photographs were taken with 60X objective.

5.2.1.5. Paraptotic Vesicle formation in presence of calpain and cathepsin D inhibitor

As discussed in previous chapter 3, paraptotic vesicles were formed by 16 hours with oxidative stress. The dyes used to check paraptotic vesicle formation were DPH and DAPI. As can be seen from the fluorescence images the vesicles are membranous in nature and contain DNA indicated by staining with DPH and DAPI respectively. The cells pretreated with cathepsin D and calpain inhibitors, then exposed to 1 mM HA and 0.03 mM H_2O_2 stress showed no vesicle formation while preincubation with calpain inhibitor only followed by treatment with 1 mM HA and 0.03 mM H_2O_2 showed vesicle formation (Figs. 5.7, 5.8, 5.9) although lesser than oxidative stress alone.

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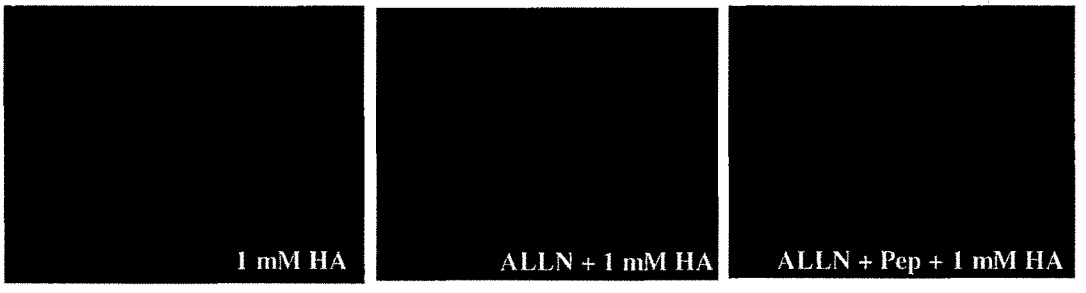

Figure 5.7: Paraptotic vesicle formation as seen by DAPI staining in presence of calpain and cathepsin D inhibitor. Calpain and cathepsin D inhibition (pretreatment with ALLN or ALLN and pepstatin A both) averted the formation of paraptotic vesicles during oxidative stress induced paraptotic cell death. Photographs were taken with 60X objective.

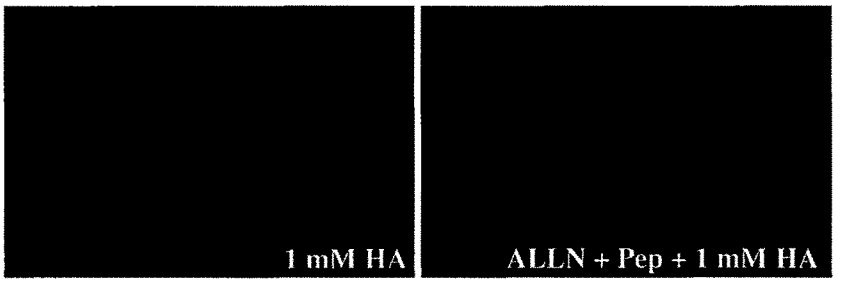

Figure 5.8: Paraptotic vesicles observed by membrane probe DPH in presence of calpain and cathepsin D inhibitor.

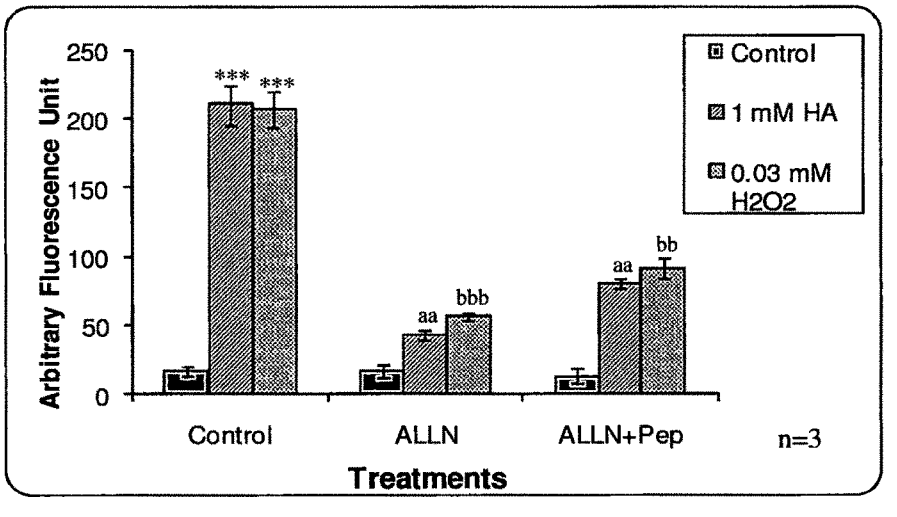

Figure 5.9: Paraptotic vesicles stained with membrane probe DPH. Vesicles collected after 12 hours of HA and H_2O_2 treatments were incubated with DPH and fluorescence was observed by fluorimetry. *** p value <0.001 compared to control; [aa, bb] p value <0.01 compared to the respective treatments; [bbb] p value <0.001 compared to H_2O_2 treatment.

5.2.1.6. Calpain activity during paraptotic cell death

To confirm our hypothesis that calpains are downstream to PARP activation, kinetics of calpain activity was studied. Our results suggest an increase in calpain activity by 3 hours during 1 mM HA and 0.03 mM treatment (Fig. 5.10).

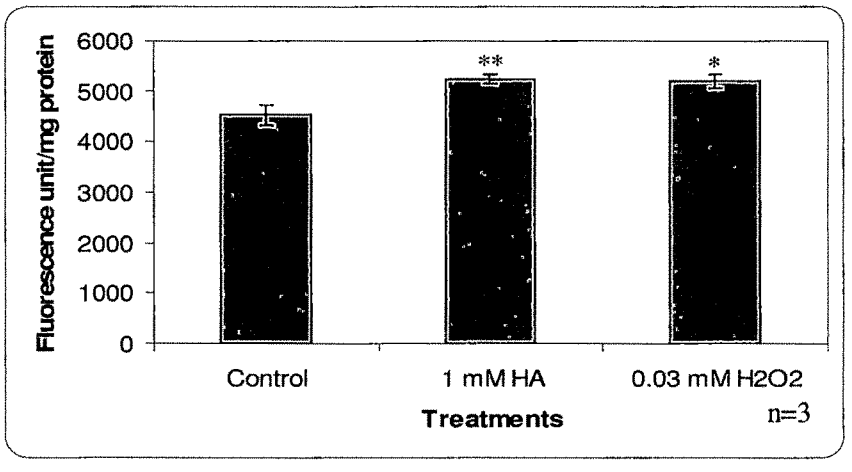

Figure 5.10: Calpain activity measured by using its substrate Succinyl-AMC during paraptosis at 3 hours post oxidative stress. ** p value <0.001 and * p value <0.01 compared to control.

5.2.2. Proteases involved during oxidative stress induced necrotic cell death

5.2.2.1. Effect of Protease Inhibitor Cocktail (PIC) on necrosis

Partial rescue in MMP was seen at 2 hours with protease inhibitor cocktail in necrotic cell death confirming that lysosomal proteases are involved in MMP changes (Fig. 5.11).

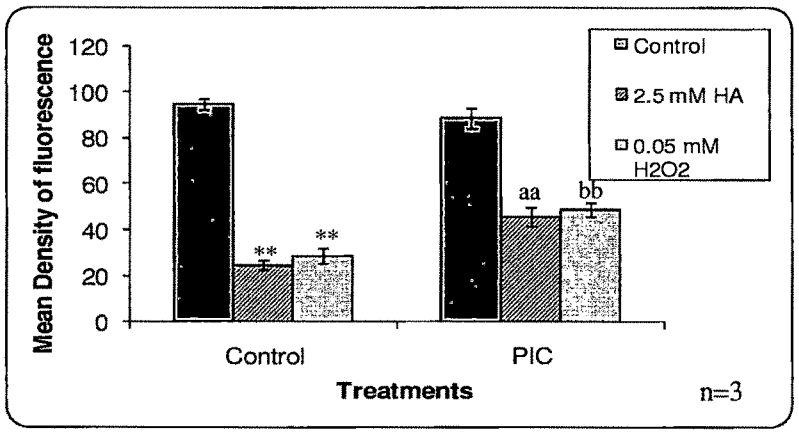

Figure 5.11: Effect of Protease Inhibitor Cocktail (PIC) on mitochondrial membrane potential changes during necrosis. Results are mean of three independent experiments. ** p value <0.01 compared to control; $^{aa, bb}$ p value <0.01 compared to the respective treatments.

5.2.2.2. Effect of calpain and cathepsin D inhibition on MMP changes during oxidative stress induced necrotic cell death

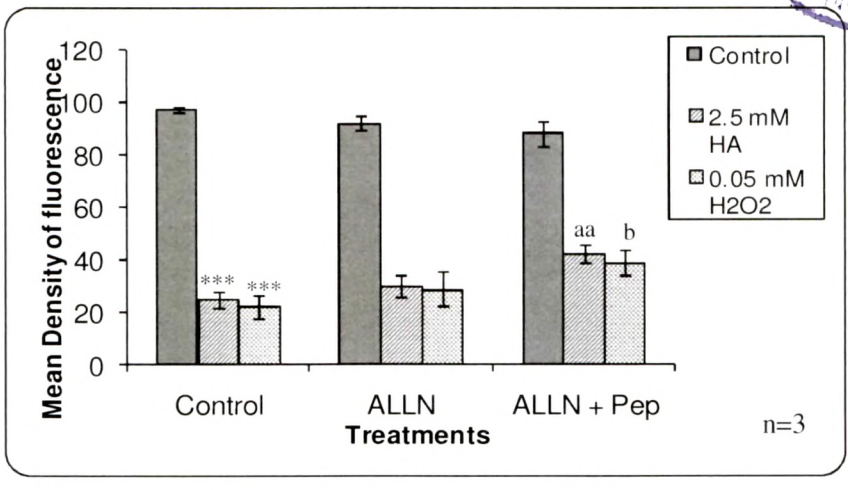

Figure 5.12: Effect of calpain and cathepsin D inhibition on mitochondrial membrane potential changes during necrosis. MMP changes during necrosis are partially rescued by both calpain and cathepsin D inhibition. *** p value <0.001 compared to control; [aa] p value <0.01 compared to the respective treatments; [b] p value <0.05 compared to H_2O_2 treatment.

No effect was observed on 2.5 mM HA and 0.05 mM H_2O_2 induced MMP changes by ALLN, a calpain inhibitor alone. calpain and cathepsin D inhibition collectively prevented the MMP changes during necrosis (Fig. 5.12).

5.2.2.3. Calpain activity during necrosis

Significant increase in calpain activity was observed in cells subjected to 2.5 mM HA and 0.05 mM H_2O_2 treatments as compared to control at 3 hours.

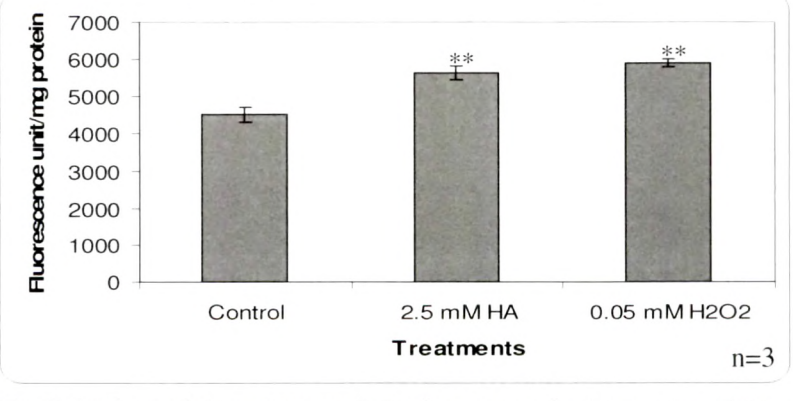

Figure 5.13: Calpain activity measured during necrosis. ** p value <0.01 compared to control.

5.3. DISCUSSION

Caspase activation is the hallmark feature of apoptotic cell death seen in all multicellular eukaryotes (Saraste and Pulkki, 2000). Interestingly caspase activation is also reported during prokaryotic cell death (Gautam and Sharma, 2002a, b). Also, caspase activation was reported during oxidative stress induced cell death in yeast system (Madeo *et al.*, 2002). Nevertheless, caspase activation could not be seen in *D. discoideum* under oxidative stress (as observed in Chapter 1) suggesting that oxidative stress induced cell death in *D. discoideum* is caspase independent and paraptotic type. Absence of caspases led us to characterize caspase independent cell dismantling mechanisms.

To explore the possibility of lysosomal involvement during oxidative stress induced cell death in *D. discoideum,* Protease Inhibitor Cocktail was used to monitor MMP changes and it exhibited partial rescue suggesting that lysosomal proteases could be acting upstream to MMP changes (Figs. 5.1 & 5.2). Cathepsin D and calpain inhibition studies were performed to further elucidate the involvement of proteases in dismantling during paraptosis. Cathepsin D being a lysosomal protease known to be active at the cytosolic pH (Zong and Thompson, 2006) could serve as the protease in dismantling the cell (Turk and Stoka, 2007) during oxidative stress induced cell death. Also calpain, a cytosolic protease known to affect mitochondrial membrane potential (Polster, 2004) and further downstream events during paraptotic cell death could be involved. Hence experiments were done using cathepsin D inhibitor- pepstatin A and calpain inhibitor- ALLN.

Calpain inhibition studies showed a partial rescue in paraptotic cell death while cathepsin D inhibition alone could not delay death of the cell. The cells when preincubated with the calpain inhibitor (ALLN) showed rescue in MMP changes at 3 hours (Figs. 5.3 & 5.4), while pepstatin A did not show any effect on MMP (Figs. 5.3 & 5.4). During necrosis MMP change was partially rescued by both calpain and cathepsin D inhibition at 3 hours (Fig. 5.12). It has been reported that calpain inhibitor is able to block translocation of AIF and further paraptosis both *in vivo* and *in vitro* (Sanges *et al.*, 2006). AIF translocation to nucleus was also partially rescued upon calpain inhibition (Fig. 5.6). Thus our results implicate that calpains function upstream while lysosomal

proteases function downstream to mitochondrial changes during oxidative stress induced paraptosis.

Cells preincubated with both cathepsin D and calpain inhibitors showed complete inhibition of the vesicle formation (Figs. 5.7, 5.8, 5.9) suggesting that cathepsin D and calpains facilitate cell dismantling during oxidative stress induced paraptotic cell death. Calpain activation as well as its blockade during PARP mediated cell death was confirmed by using fluorescent substrate for calpain in total cell lysate (Figs. 5.10 & 5.13), and our results suggests that calpain regulates AIF release during oxidative stress induced PARP mediated cell death in *D. discoideum*. Hence, protection observed with protease inhibitors could be mainly attributed to calpains as caspases are absent in *D. discoideum* (Olie *et al.*, 1998). We have also shown that proteases are involved in causing MMP changes and downstream events including cell dismantling in 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 the paraptotic mode of 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 nonglycolytically (Laporte *et al.*, 2006). Our study suggests that necrosis occurs in a programmed fashion where MMP changes were manifested by proteases (Fig. 5.11) followed by plasma membrane rupture. Proteolysis during oxidative stress induced necrotic cell death involves calpains and lysosomal proteases.

Thus from the above results we put forward that paraptosis, an alternate cell death program may be regulated by the key players - PARP, AIF, calpains and cathepsin D, 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. Thereof we have demonstrated the involvement of calpain in release of AIF in PARP mediated cell death. Moreover, *D. discoideum* can thus be used as a model to study the molecular mechanisms involved in programmed necrosis that can later be substantiated in mammalian cells.

5.4. REFERENCES

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