Synopsis

In the human body about 1,00,000 cells are produced every second by mitosis and equal number die by apoptosis and necrosis (Vaux and Korsmeyer, 1999). In a year, this amounts to the proliferation and subsequent destruction of a mass of cells equal to an individual's body weight. Any faulty regulation will give rise to diseases like cancer and autoimmune diseases hence the processes of cell death and proliferation are very tightly regulated in the body. Poly (ADP-ribose) polymerase-1 (PARP-1) plays an important role in both these processes. It binds to damaged DNA and gets activated consequent to DNA damage. It is one of the immediate DNA damage sensors playing very important role in DNA repair. Besides, this 116-kDa protein is found to be involved in various processes owing to its wide range of substrates. This enzyme transfers ADP-ribose moiety over to substrate proteins using NAD^+ as the ADP-ribose donor. Under conditions of extensive DNA damage and subsequent PARP over activation, depletion of NAD^+ is seen. ATP levels get depleted in an effort to replenish the depleted cellular NAD^+. ATP depletion leads to glycolysis blockade, energy failure, and ultimately cell death. Thus PARP overactivation has been associated with both apoptosis and necrosis (Virag and Szabo, 2002). Because of this reason, nowadays PARP is an emerging target for the treatment of oxidative stress related diseases.

Cell death can be induced by a number of agents including reactive oxygen species (ROS) (Hasnain *et al.,* 1999). ROS, among other effects cause DNA damage and thus act as potent cell death stimuli. We have recently reported the involvement of PARP in oxidative stress induced cell death in *D. discoideum* (Rajawat *et al.,* 2007; 2011). The mechanism by which PARP-1 activation leads to cell death appears linked to the rapid utilization of NAD^+ during formation of poly(ADP-ribose) (PAR) (Pieper *et al.*, 1999). PAR has a fast turnover rate, with a half-life approaching 1 min, due to rapid degradation by the endo-exoglycosidase poly(ADP-ribose) glycohydrolase (PARG) (D'Amours *et al.*, 1999). In contrast to PARP, little is known about the role of PARG in cell function. There are, however, at least two mechanisms by which PARG could influence PARP-mediated cell death. First, PARG inhibition could slow the turnover of PAR and thereby limit NAD^+ depletion. Second, PARG inhibition could prevent the removal of PAR from PARP-1. Because PARP-1 is inhibited by extensive poly-(ADP-ribosyl)ation, PARG inhibitors could thereby indirectly inhibit PARP-1 activity. Thus PARG inhibition would prevent the removal of PAR from PARP and also

maintain the levels of NAD^+ and ATP in the cell and prevent cell death via preventing mitochondrial membrane potential (MMP) changes and apoptosis inducing factor (AIF) release. Even though inhibition of PARG and PARP have opposing effects on poly(ADP-ribose) formation, both approaches provide remarkable protection to the cells (Kim *et al.*, 2005). Although pharmacological inhibition of PARP during conditions of oxidative stress is found to be beneficial, its inhibition could lead to adverse effects due to its involvement in various physiological processes. However, PARG inhibition with gallotannin could serve as a better option in curing diseases involving PARP overactivation. The role of PARP in apoptotic cell death in several eukaryotic systems is well characterized, but the role of PARP and PARG in paraptotic cell death is yet to be addressed.

Cadmium is an important heavy metal environmental toxicant, which is classified as a human carcinogen. However, the mechanism of carcinogenic activity of cadmium are not clearly defined. In this study, our present work shows the mechanism of cadmium (Cd^{2+}) induced cytotoxicity and involvement of PARP during cadmium induced cell death using *D. discoideum*. We have used molecules namely benzamide (PARP inhibitor), gallotannin (PARG inhibitor), NAD^+ or ALLN (Calpain inhibitor) to explore the potential of PARP, PARG, NAD^+ and calpain as pharmacological targets to rescue oxidative stress induced cell death. Kinetic studies of cell death parameters were done using cadmium and cumene H_2O_2 as cell death inducers.

Families of cysteine proteases called caspases carry out apoptosis in higher eukaryotes. Functional caspases are not present in *D. discoideum* (Uren *et al.*, 2000). Although paracaspase gene is reported in *D. discoideum*, knock out of paracaspase does not affect development, suggesting paracaspase is not required for developmental cell death (Roisin-Bouffay *et al.*, 2004). Thus *D. discoideum* is a good model to study caspase independent cell death or paraptosis. Moreover, it would be very interesting to study the effect of heterologous expression of *Sf*-1 (*Spodoptera frugiperda*-1) caspase on *D. discoideum* cell death and development.

Objectives of the present study

1) Mechanism of cadmium (Cd^{2+}) induced cell death and development in *Dictyostelium discoideum*

2) Involvement of poly (ADP-ribose) polymerase during cadmium (Cd^{2+}) induced cell death and heterologous expression of *Sf*-1 caspase in *Dictyostelium discoideum*

Kinetic studies of cell death parameters were done using Cd^{2+} as the cell death inducer. As monitored by trypan blue exclusion method LD_{50} for Cd^{2+} was found to be 0.5mM and our PS-PI dual staining results suggest that, 0.2mM and 0.5mM Cd^{2+} yielded paraptotic and necrotic cell death, However *D. discoideum* did not show apoptotic cell death due to the absence of caspases (Oli *et al.*, 1998). We have further confirmed that Cd^{2+} induces paraptotic or necrotic cell death via ROS generation. This study demonstrates several important findings that help in understanding the sequence of events happening during Cd^{2+} induced PARP mediated cell death in *D. discoideum.* ROS could be beneficial or exhibit pathophysiological role as cell death inducer and severity of the stress determines whether cells undergo necrosis or apoptosis (Palomba *et al.*, 1996). It has been reported that 0.01 mM Cd^{2+} treatment showed caspase independent cell death while 0.05 mM Cd^{2+} induced necrotic cell death in mesangial cells (Douglas *et al.*, 2010). During our studies Cd^{2+} yielded caspase independent (paraptotic) cell death by 0.2 mM Cd^{2+} (exposure for 1 hour) while 0.5 mM Cd^{2+} (exposure for 1 hour) showed necrosis in *D. discoideum.* This finding suggests that *D. discoideum* cells are atleast 10-20 fold more resistant to Cd^{2+} than mesanglial cells.

D. discoideum showed significant induction of either *cat*A mRNA or CatA enzyme activity under oxidative stress, however, cat A mutant was found to be 160 fold more sensitive to H_2O_2 (Garcia *et al.,* 2003). 2-5 fold more catalase activity was found in *D. discoideum* compared to other organisms and this could also confer resistance to oxidative stress to this organism. Furthermore, we have reported an increase in glutathione peroxidase (GPx) activity under oxidative stress (Katoch and Begum, 2003). Thus presence of high level of catalase throughout its life cycle and significant induction of GPx activity could confer *D. discoideum* cells higher resistance to oxidative stress. Furthermore, *D. discoideum* has a high content of unsaturated fatty acids making up 75-90% of the fatty acids of the organism and of the membrane (Weeks and Herring, 1980). However, the fatty acids are monounsaturated or with two double bonds and hence are less susceptible to lipid peroxidation compared to polyunsaturated fatty acids. Thus non-significant changes in lipid peroxidation (LPO) levels

(Katoch and Begum, 2003) under oxidative stress could also offer an explanation for the high resistance of *D. discoideum* to cadmium induced oxidative stress.

Reduced glutathione and other thiol containing proteins play a key role in cellular protection against Cd^{2+} toxicity. Elevation of GSH levels has been demonstrated to be protective against Cd^{2+}-induced lethality in rats (Singhal *et al.*, 1987). The ionic species, Cd^{2+}, which is regarded as being responsible for Cd^{2+}-induced toxicity, can be scavenged by glutathione to prevent its interaction with critical cellular targets. However, either continuous exposure or exposure to toxic doses of Cd^{2+} may overwhelm the cellular supply of GSH and the related defense system so as to result in toxicity, including carcinogenesis (Liu *and* Templeton, 2007). In general, the expression of antioxidant genes such as those coding for the synthesis of superoxide dismutase and catalase is repressed by Cd^{2+} (Casalino *et al.*, 1997) and thus offers the underlying reason for Cd^{2+}-induced lipid peroxidation, oxidative stress, and the associated toxicity. In contrast, there are some reports suggesting an increase in activity of SOD and catalase in tissues adapted to Cd^{2+} by prolonged exposure (Zikic *et al.*, 1998). These findings reinforce the conclusions from the data on Cd^{2+}-induced ROS generation. Our results suggest that Cd^{2+} induced cell death could be via ROS generation in *D. discoideum* hence we have also studied cumene H_2O_2 induced cell death. Interestingly, GSH confers strong protection against Cd^{2+} induced ROS production, MMP changes, cell death and development.

Cadmium induced changes in *D. discoideum* development

Multicellular organisms have evolved sophisticated systems by which cells coordinate their respective functions. *Dictyostelium discoideum*, as a social amoeba, represents an unusual mode of multicellularity by which previously independent cells group together to form a multicellular organism, but requires a similar degree of communication between different cells as do multicellular plants and animals. The present study provides evidence that *D. discoideum* utilizes superoxide as a signaling intermediate at the onset of its development from unicellular to multicellular existence. But ROS is required only in moderate levels for development (Bloomfield and Pears, 2003).

Cadmium induces subcellular accumulation of $O_2^{\cdot-}$ and H_2O_2 in pea leaves (Sandalio *et al.*, 2001). These ROS mediate cytotoxic effects via observed DNA damage. Cadmium exposed *D. discoideum* cells showed dose dependent delay in development. Glutathione is the first line of defense against cadmium toxicity and glutathione is required for growth and prespore

cell differentiation in *D. discoideum* (Kim *et al.*, 2005) and also glutathione is important to scavenge ROS in the cells. This is also supported by our study as only cadmium treatment is showing developmental block or delay in dose dependent fashion but GSH pretreated cells with same doses of Cd^{2+} are showing developmental restoration. Interestingly, cadmium treated *D. discoideum* cells showed delayed differentiation at slug formation. Effect of Cd^{2+} on development i.e. delay at slug stage, resembles that of CatB mutant as shown by Garcia *et al.*, 2003. This could be due to Cd^{2+} affecting CatB activity/expression during development.

Effect of NAD^+ supplementation, PARP inhibition, PARG inhibition, calpain inhibition and GSH on Cd^{2+} induced cell death

The role of PARP in apoptotic death in several eukaryotic systems is well characterized, but the role of PARP and PARG in paraptotic cell death is yet to be addressed. PARP over activation leading to mitochondrial changes is well reported, however the nuclear-mitochondrial cross talk is yet to be known. Attempt was made to understand the nuclear-mitochondrial cross talk. *D. discoideum* cells were pretreated independently with NAD^+ supplementation, benzamide (PARP inhibitor), gallotannin (PARG inhibitor), ALLN (Calpain inhibitor) or GSH each 2 hrs prior to oxidative stress (via cadmium or cumene H_2O_2 exposure).

10mM NAD^+ supplementation showed reduction in cell death 52% and 40% paraptotic dose and necrotic dose of Cd^{2+} respectively. Cell death with 1mM NAD^+ supplementation was found to be reduced from 25% to 11% and 50% to 30% in paraptotic and necrotic doses of cumene H_2O_2 induced cell death respectively. These results suggest that NAD^+ could rescue oxidative stress induced cell death in a dose dependent manner. It has been reported that extracellular application of NAD^+ was able to restore intracellular NAD^+ levels and counteract cell death induced by FK866, suggesting NAD^+ addition may affect physiological NAD^+ homeostasis (Billington *et al.*, 2008). It has also been shown that NAD^+ when exogenously applied to the culture medium of neurons can be transported across the axonal membrane (Araki *et al.*, 2004).

To study the involvement of PARP during Cd^{2+} induced cell death, *D. discoideum* cells were pretreated with 1 mM benzamide and cell death was monitored. PARP inhibition showed reduction in cell death from 25% to 15% and 50% to 35% at paraptotic and necrotic dose of Cd^{2+} respectively. Similar trend was also observed with H_2O_2 induced cell death also. These results suggest that PARP plays an important role in *D. discoideum* cell death induced by

oxidative stress. Interestingly PARG inhibition by gallotannin also showed ~40 % and ~32 % rescue in Cd^{2+} induced paraptotic and necrotic cell death respectively. These data indicate that PARG also plays important role in *D. discoideum* cell death.

Altogether these results imply that PARP, PARG and NAD^+ depletion contribute significantly to *D. discoideum* cell death induced by oxidative stress (induced by Cd^{2+} or H_2O_2). Ours is the first report on the role of PARP in *D. discoideum* cell death, where we have shown the involvement of PARP during oxidative stress mediated delayed development and also during normal aggregation (Rajawat *et al.*, 2007; 2011).

Evidence suggests that MNNG-induced DNA damage leads to calpain activation through PARP-1. Calpain in turn activates Bax resulting in its translocation from cytosol to mitochondria, where it facilitates the release of AIF from the mitochondrial intermembrane space to the cytosol. Activated calpain also regulates AIF release by cleaving the membrane-anchored AIF to the soluble and cell death-active form, tAIF (Moubarak *et al.*, 2007). Calpain inhibition also rescues cell death induced by H_2O_2.

Effect of NAD^+ supplementation, PARP inhibition and PARG inhibition, calpain inhibition or GSH pre treatment on Cd^{2+} induced ROS production

The amount of ROS produced under oxidative stress in *D. discoideum* cells was measured fluorimetrically as well as with fluorescence microscope using DCFDA dye. Both the doses of Cd^{2+} produced ROS in a dose and time dependent manner. ROS estimations were done with various pre-treatments to find out if NAD^+ supplementation, benzamide and gallatonin rescued H_2O_2 induced paraptotic and necrotic cell death in *D. discoideum* by scavenging the ROS. These results suggest ROS production to be an event upstream to PARP activation. Similar results were obtained with H_2O_2 stress. However GSH pre treatment after Cd^{2+} stress prevented ROS production significantly. These results suggest GSH could block cell death at an up stream stage.

Effect of NAD^+ supplementation, Benzamide, Gallotannin and ALLN pre treatment on Cd^{2+} induced PARP activation

Effect of NAD^+ supplementation, benzamide, gallotannin and ALLN pretreatments on PARP activity was measured by indirect immunofluroscence method immediately after 10 min of oxidative stress, as peak PARP activity is found at this time point after oxidative stress induction. There was a dose dependent effect of oxidative stress on PARP activation at 10

min. Not surprisingly, NAD^+ being a substrate for PARP, its supplementation showed increased PARP activation in a dose dependent manner, whereas pretreatment with benzamide and gallotannin showed decreased PARP activity as benzamide and gallotannin are inhibitors of PARP and PARG respectively. Nevertheless, all these agents i.e. benzamide, gallaotannin and NAD^+ had opposing effect on PARylation. This implies that PARP activation may not be the actual culprit leading to cell death. Significant rescue in cell death with exogenous NAD^+ despite of increasing PARP activity further project PARP as an **"Innocent killer protein"** its role is to recruit DNA repair machinery upon DNA damage. Interestingly inhibition of calpain, did not affect the oxidative stress induced PARP activation, suggesting calpain activation is a downstream event in cell death cascade.

NAD^+ depletion leads to MMP changes in *D. discoideum*

Paraptotic cells exhibit a change in mitochondrial membrane potential compared to control cells. This change in MMP was measured fluorimetrically and by fluorescence microscopy using $DiOC_6$ dye. Our results showed a dose dependent effect of oxidative stress on MMP changes. Change in MMP is taken as an indicator of changes in the mitochondrial physiology which could be associated with the release of various pro apoptotic proteins from mitochondria into the cytosol. With Cd^{2+} stress change in MMP was observed at 3 hours.

Changes in the mitochondrial membrane potential is associated with release of apoptosis-related killer proteins into the cytoplasm that are usually present in the inter-membrane space (IMS) of the healthy cell (Newmeyer and Ferguson-Miller, 2003). AIF, one such protein, plays an important role in inducing nuclear chromatin condensation as well as large-scale DNA fragmentation. *D. discoideum* exhibits caspase independent cell death and also AIF mediated cell death is proposed to be predominating (Arnoult *et al.*, 2001).

However PARP activation and mitochondrial changes did occur but the link between them is yet to be addressed. To find out the signal which is involved during Nuclear-Mitochondrial cross talk we monitored the NAD^+ levels. Our results showed 62 and 78% reduction in NAD^+ level in paraptotic and necrotic doses of H_2O_2. NAD^+ depletion caused by PARP overactivation triggers changes in MMP following AIF release from mitochondria to cytosol and then nucleus. Additionally, NAD^+ supplementation showed maximum rescue in cell death. As can be seen NAD^+, benzamide, and gallotannin pretreated *D. discoideum* cells could prevent NAD^+ depletion followed by MMP changes induced by oxidative stress. In other words, NAD^+ depletion is a turning point during entire cell death cascade, suggesting

NAD^+ to be the **"Currency coin"** during nuclear-mitochondrial cross talk; thus preventing NAD^+ depletion could block the downstream events leading to cell death.

Also as stated earlier PARP is important for various physiological processes, hence its inhibition would not be an appropriate approach for treatment of PARP associated diseases. Our results strongly support that replenishment of NAD^+, despite it being the central metabolite in a number of biological pathways, would serve the best pharmacological agent against diseases related to oxidative stress induced PARP mediated cell death. Thus this study sheds light on the current use of NAD^+, benzamide, gallotannin ALLN and GSH as pharmacological agents for treatment of diseases associated with oxidative stress induced PARP mediated cell death.

Heterologous expression of *Sf*-1 caspase using pA15-cas (constitutive promoter) in *Dictyostelium discoideum*

Heterologous expression of caspase-3/CED-3 in yeast has shown that substrates for caspase-3 are present in *S. cerevesiae* that may participate in the normal cell growth and division processes. Evidence suggest that overproduction of the single metacaspase YCA1 resulted in autocatalytic processing and rendered cells more sensitive to exogenous or aging-related oxidative stress, as determined by reduced clonogenicity (Madeo *et al.*, 2002). A yeast strain with a disrupted YCA1 gene (Δyca1) was also shown to be three-fold less sensitive to H_2O_2, and 5% of the cells escaped from aging-related cell death (Madeo *et al.*, 2002). Interestingly, extracts of H_2O_2-treated YCA1-overproducing yeast were highly active toward the synthetic caspase substrates Val-Glu-Ile-Asp-AMC and Ile-Glu-Thr-Asp-AMC. Thus, YCA1 metacaspase behaved as a bona fide caspase; in other words metacaspases constitute a new group of cysteine proteases homologous to caspases. (Madeo *et al.*, 2002). Heterologous expression of *Trypanosoma brucei* metacaspase TbMCA4 in the budding yeast *Saccharomyces cerevisiae* resulted in growth inhibition, mitochondrial dysfunction and clonal death (Alexander *et al.*, 2002). As mentioned above *D. discoideum* lacks caspases while it interestingly possesses PARP and paracaspase providing a tempting model organism for dissecting the evolutionary conserved cell death pathway. However paracaspase is not required for *D. discoideum* developmental cell death (Uren *et al.*, 2000).

We made an attempt for heterologous expression of *Sf*-1 caspase in *D. discoideum* using constitutive expression vectors. The aim was to study the effect of caspase on *D. discoideum* growth and development using constitutive hetelogous expression (pA15 cas). pA15-cas

transfected *D. discoideum* cells did not survive however, pA15-GFP (vector control) transfected *D. discoideum* cells grew normally and also showed development. Hence stage specific expression of caspase may be attempted in future. The "induced proximity model" is predicted on the empirical observation that the zymogen forms of unprocessed caspases are not entirely inactive but rather possess weak protease activity. When brought into close contact through protein interactions, the zymogens can trans-process each other, producing the fully active proteases (Wang *et al.,* 2005). In support with this report, our caspase transfectants *D. discoideum* cells might have induced cell death via mechanism similar to induced proximity model of caspase activity.

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Effect of oxidative stress and involvement of poly(ADP-ribose) polymerase (PARP) in *Dictyostelium discoideum* development

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Dictyostelium discoideum, a unicellular eukaryote, exhibits multicellularity upon nutrient starvation and is a good model system for developmental studies, and for the study of various signal transduction pathways. Reactive oxygen species at low doses act as signaling molecules; however, at high doses they are known to cause DNA damage that results in the activation of poly(ADP-ribose) polymerase (PARP). We have earlier reported the high resistance of the unicellular stage of *D. discoideum* to oxidative stress, and we now show the response of this organism to oxidative stress and the role of PARP during development. We used hydroxylamine (HA) to induce *in situ* generation of H_2O_2 and monitored the effect of benzamide, a PARP inhibitor, on oxidative stress-induced changes in *D. discoideum* development. Interestingly, oxidative stress resulted in PARP activation within 5 min that was inhibited by benzamide. Oxidative stress-induced delay in developmental pattern was also partially restored by benzamide. We studied the long-term effects of PARP inhibition under oxidative stress, and our results demonstrated that spores formed under HA stress exhibited significant delay in germination in comparison to benzamide-pretreated HA-stressed cells. However, second-generation cells showed normal development, signifying that PARP inhibition has no deleterious effect on *D. discoideum* development under oxidative stress.

Dictyostelium discoideum, a unicellular eukaryote, exhibits multicellularity upon nutrient starvation and thus provides a simple but excellent model system for the study of various signal transduction pathways [1], the findings of which can later be confirmed with complex eukaryotic systems. *D. discoideum* in the unicellular stage is known to be highly resistant to DNA-damaging agents and oxidative stress [2,3]. However, the response of *D. discoideum* development to oxidative stress is not clearly understood. Recent studies showed that superoxide plays a vital role in the aggregation process of *D. discoideum* cells [4], as inhibition of superoxide-dependent signaling events affects the transition from the unicellular to the multicellular phase. During development, *D. discoideum* cells produce nitric oxide, which is also postulated to act as a signaling molecule [5].

Reactive oxygen species (ROS) nevertheless also have deleterious effects and are known to cause DNA damage [6], which in turn results in the activation of poly(ADP-ribose) polymerase (PARP). This catalyzes the transfer of ADP-ribose moieties to acceptor proteins by utilizing NAD^+ as the substrate, and helps in DNA repair [7,8]. PARP also monitors the status of DNA before entry into mitosis [9,10], and hence has been implicated in checkpoint control. Cells are

Abbreviations
FITC, fluorescein isothiocyanate; HA, hydroxylamine; LD, lethal dose; PAR, poly(ADP-ribose); PARP, poly(ADP-ribose) polymerase; PBA, phosphate-buffered agar; ROS, reactive oxygen species; SB, Sorenson's buffer.

arrested at different phases of the cell cycle, depending upon the extent of PARP activation [11] under stress conditions. Thus, in higher eukaryotic cells, PARP contributes to cell homeostasis under mild stress conditions, and conversely, during conditions of moderate/severe cellular stress, PARP overactivation leads to cell death, which results in several disease conditions [12]. Pharmacological inhibition of PARP during moderate/severe cellular stress is beneficial [13,14]; however, the consequences of such inhibition for genomic integrity are not yet understood. *D. discoideum* is reported to have nine potential PARP genes [15], unlike another unicellular eukaryote, *Saccharomyces cerevisiae* [16]. Hence, we selected *D. discoideum* as a model system to study the role of PARP in its development under oxidative stress conditions.

We have studied the dose-dependent effect of hydroxylamine (HA) (for *in situ* H_2O_2 generation) on *D. discoideum* development and also the role of PARP in oxidative stress-induced effects on development. Our present study is the first report on the activation of PARP under oxidative stress in *D. discoideum*, and our results suggest that *D. discoideum* is an excellent model system with which to investigate the long-term effects of PARP inhibitors for two successive generations.

Results

Dose-dependent effect of oxidative stress on *D. discoideum* cell death

Cell death was induced by treating *D. discoideum* cells for 1 h with different concentrations (1.0, 2.5 and 4.0 mM) of HA, a known catalase inhibitor [17], in order to promote *in situ* generation of H_2O_2. HA-induced cell death was measured after 24 h by the Trypan blue exclusion method. The percentage of cells undergoing cell death was found to increase from 15% to 90% as the concentration of HA was increased from 1.0 mM to 4.0 mM, and 50% cell death was seen at 2.5 mM HA (Fig. 1).

D. discoideum growth under oxidative stress

To monitor the effect of HA on the *D. discoideum* cell cycle, a growth curve was obtained. The growth curve showed a dose-dependent increase in the lag phase from 36 h to 60 h, 72 h and 96 h at lethal dose (LD)15 (1 mM), LD50 (2.5 mM) and LD90 (4 mM), respectively. Furthermore, the log phase was shortened to 48 h, 48 h and 36 h at LD15, LD50 and LD90, followed by faster attainment of stationary phase

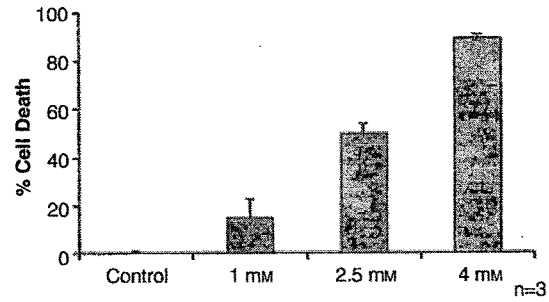

Fig. 1. Dose-dependent effect of HA on *D. discoideum* cell death determined by the Trypan blue exclusion method. Cells were treated with different doses of HA, and cell death was assessed by the Trypan blue method after 24 h. HA at 1 mM caused 15% cell death, and hence this dose was considered to be LD15; a 2.5 mM dose was found to be LD50, as 50% of cells were dead; 4 mM HA was found to be LD90, as this dose caused 90% cell death.

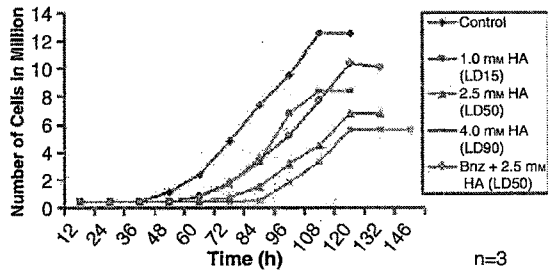

Fig. 2. Effect of PARP inhibition during oxidative stress-induced growth changes in *D. discoideum*. Under oxidative stress, the growth curve showed a dose-dependent increase in the lag phase. The log phase was shortened, and this was followed by faster attainment of stationary phase. Benzamide-pretreated cells showed a reduction in the lag phase from 72 to 60 h at LD50, followed by a longer log phase. Results are means of three independent experiments performed in duplicate.

(Fig. 2), suggesting that HA caused cell cycle arrest leading to an increased lag phase.

D. discoideum development under oxidative stress

To study the effect of oxidative stress on differentiation, developmental studies were performed. The dose-dependent effect of HA on *D. discoideum* development was studied by exposing the cells to different concentrations of HA (1.0, 2.5 and 4.0 mM) for 1 h and then allowing them to develop. As can be seen from Table 1 and Fig. 3A, development was delayed in a dose-dependent manner at the loose aggregation stage by 2 h and 12 h at LD15 and LD50 of HA,

Table 1. Developmental stages of *D. discoideum* at different time intervals. Cells (2.5×10^6) were treated with 2.5 mM and 4 mM HA for 1 h, plated on non-nutrient agar, and observed at different time points. Also shown is the effect of PARP inhibition by benzamide during oxidative stress on *D. discoideum* development. LA, loose aggregate; TA, tight aggregate; SF, slug formation; FBF, fruiting body formation; CD, cell death; FB, fuiting body; –, no development until after 1 week.

	LA (h)	TA (h)	SF (h)	FBF (h)	% CD	% FB
HA (mM)						
0.0	6	12	18	24	1	100
1.0	8	14	20	26	15	100
2.5	18	24	30	36	50	30
4.0	–	–	–	–	90	–
HA (mM) + 1 mM benzamide						
0.0	6	12	18	24	1	100
1.0	6	12	18	24	5	100
2.5	12	17	23	29	20	70
4.0	18	24	30	36	40	20

respectively, as compared to control cells. At 18 h of development, 40% loose aggregates were seen in 2.5 mM HA as compared to controls. The percentage involvement of cells was slightly increased with time. Nevertheless, cells treated with LD90 of HA showed no development until after 1 week, suggesting that development was arrested. HA-treated *D. discoideum* cells exhibited dose-dependent decreases in the number and size of fruiting bodies as compared to control cells (Fig. 3B).

Oxidative stress induces PARP activation

PARP activity in *D. discoideum* was assayed at various time points (5, 10, 20 and 60 min and 4 h) after HA stress. PARP activity was increased initially, and significant peak PARP activity was seen at 5 min after exposure of the cells to 2.5 mM HA (Fig. 4A,B). No difference in fluorescence intensities was observed at time points after 10 min.

PARP inhibition by benzamide

To address the role of PARP under oxidative stress, PARP inhibition studies were performed. Peak PARP activity, which was observed after 5 min of 2.5 mM HA exposure, was significantly inhibited by 1 mM benzamide (Fig. 4A,B), confirming PARP activation in *D. discoideum* under oxidative stress.

PARP inhibition during oxidative stress-induced growth changes in *D. discoideum*

PARP inhibition conferred protection against 2.5 mM HA-induced delay in growth. The lag phase in benzamide-pretreated cells was reduced from 60 h to 50 h, and was followed by a longer log phase (Fig. 2).

Role of PARP during *D. discoideum* development

The role of PARP in *D. discoideum* development was investigated by its inhibition with benzamide.

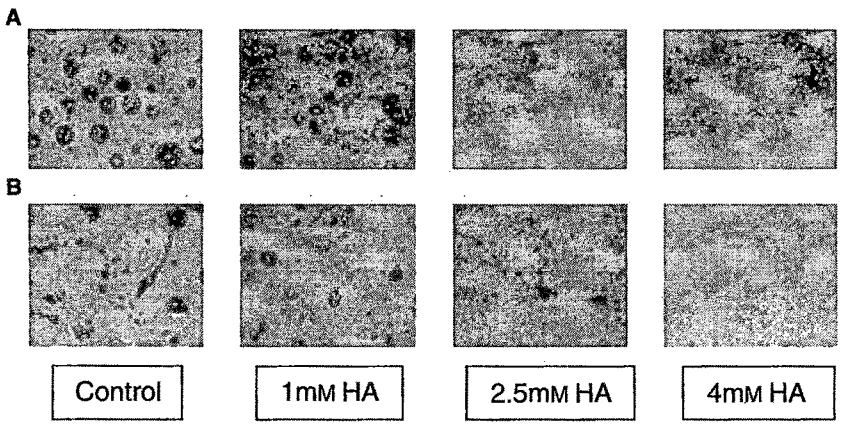

Fig. 3. Development of *D. discoideum* cells at 12 and 24 h under oxidative stress. (A) Developmental phenotypes of control and 1 mM HA-treated *D. discoideum* cells at 12 h. Cells after HA treatment were starved on nutrient-free agar medium and photographed at 4x magnification. (B) Developmental stages of control cells, and 2.5 mM and 4 mM HA-treated cells, at 24 h. Scale bar, 10 μm. Results are means of three independent experiments performed in duplicate.

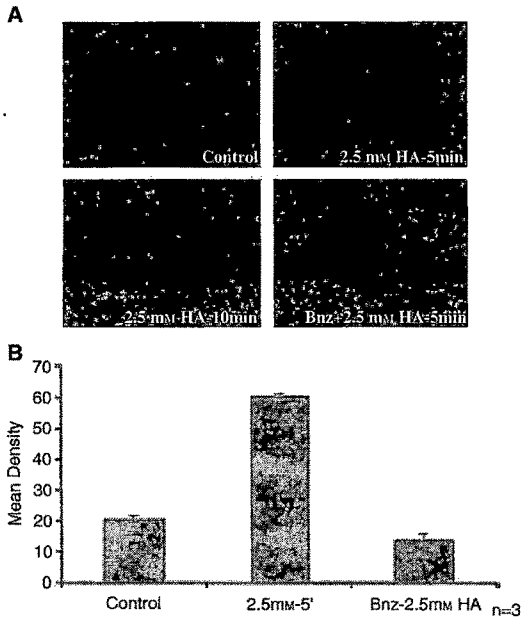

Fig. 4. Fluorescence images for PARP assay under 2.5 mM HA stress at varying time intervals. (A) Cells after treatment with HA were fixed and incubated with antibody to PAR, and were then treated with FITC-conjugated secondary antibody to assess PARP activity. PAR immunoreactivity was barely detectable in controls, whereas peak activity was seen at 5 min after 2.5 mM HA stress, and was reduced to basal level by 10 min. Benzamide significantly inhibited peak PARP activation. (B) Representation of the results for PARP activation in the form of a histogram; a significant increase in PARP activity was seen at 5 min. $P < 0.001$.

Benzamide (1.0, 2.0 and 3.0 mM) did not show any effect on development. However, benzamide at 4 mM caused a 3–4 h delay in the tight aggregate-to-slug transition (Table 2). Interestingly, *D. discoideum* cells treated with 3.0 and 4.0 mM benzamide showed abnormal fruiting bodies with larger fruits.

PARP involvement during oxidative stress-induced developmental changes in *D. discoideum*

To determine the role of PARP in oxidative stress-induced developmental changes, *D. discoideum* cells were exposed to benzamide (1 mM for 24 h) prior to HA (LD15, LD50 and LD90) treatment, and allowed to develop; the results are shown in Table 1. Benzamide-pretreated cells, upon exposure to a high dose of HA (2.5 mM), exhibited development, and the delay at the loose aggregation stage was reduced from 18 h to 12 h (Table 1). The percentage of loose aggregates formed was also increased, whereas in the case of

Table 2. Effect of the PARP inhibitor benzamide on *D. discoideum* development. LA, loose aggregate; TA, tight aggregate; SF, slug formation; FBF, fruiting body formation; CD, cell death; FB, fruiting body.

Benzamide (mM)	LA (h)	TA (h)	SF (h)	FBF (h)	% CD	% FB
0.0	6	12	18	24	1	100
1.0	6	12	18	24	2	100
2.0	6	12	18	24	2	100
3.0	6	12	18	24	4	95
4.0	6	12	22	28	10	95

LD90, delayed development could be observed in the presence of benzamide, as compared to developmental arrest of 4 mM HA-treated cells. The fruiting bodies formed were very small, with poor stalks and small fruits, and the fruits were few in number (Fig. 5).

PARP inhibition restored spore germination that was delayed due to oxidative stress

To investigate the germination efficiency of spores and the fate of the germinated amoebae, spore revival was attempted. Control and benzamide-treated spores germinated within 108–120 h, whereas the spores formed under 2.5 mM HA stress showed a significant delay, i.e. ~56 h ($P < 0.001$) in germination. There was a partial rescue of the developmental delay, i.e. ~32 h ($P < 0.012$) in the presence of benzamide. Spores formed from benzamide-pretreated and 4 mM HA-treated cells germinated after 60 h as compared to controls (Fig. 6). To avoid ambiguity in the number of fruiting bodies added to each flask, fruiting bodies were picked up from at least four different areas and it was ensured that a single fruiting body was inoculated per milliliter of medium. Our results were also confirmed by microscopically counting the number of cells germinated from each spore, and this was found to be the same for each dose.

For spore revival when log phase had been reached (2.5×10^6 cells/mL), the cells were plated on phosphate-buffered agar (PBA) plates for development, and cells treated with 2.5 mM and 4 mM HA exhibited normal development (data not shown).

Discussion

Among the eukaryotic organisms, the cellular slime mold *D. discoideum* is an excellent model system for studying cell death and developmental aspects [18]. The ability of living cells to cope with various stresses is very crucial for maintaining their correct development. ROS at lower concentrations have physiological

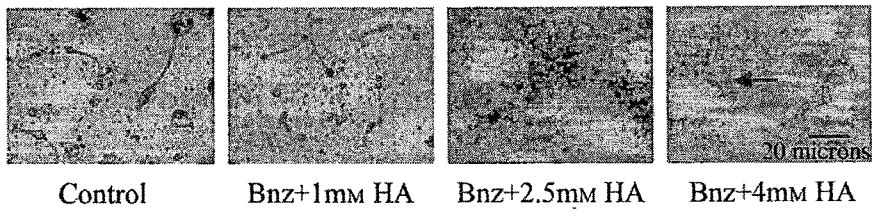

Fig. 5. Effect of PARP inhibition during oxidative stress-induced developmental changes in *D. discoideum*. Cells were preincubated with 1 mM benzamide for 24 h, treated with HA, washed, and plated at a density of 2×10^5 cells·cm^{-2}. Benzamide pretreatment restored the development that was delayed by 2.5 mM HA, and rescued the developmental arrest of 4 mM HA-treated cells. The arrow indicates the fruiting body. Fruiting body formation at different time intervals in the development of HA-treated cells pre-exposed to benzamide is shown. The fruiting body was small in comparison to that of controls. Scale bar, 20 μm. Data are means of three independent experiments performed in duplicate.

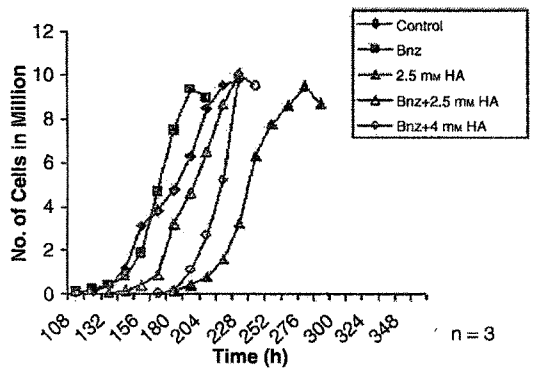

Fig. 6. Effect of PARP inhibition on the fate of spores that were developed under oxidative stress. Spores of control cells germinated within 108 h, whereas spores formed under oxidative (2.5 mM HA) stress exhibited a 56 h delay in germination, which was partially rescued by benzamide pretreatment. Spores formed from cells that were pre-exposed to benzamide and HA-stressed (2.5 and 4 mM HA) germinated earlier than cells treated only with 2.5 mM HA; 4 mM HA-treated cells showed no development and hence no spores. Data are means of three independent experiments performed in duplicate.

functions and serve as second messengers in different signal transduction pathways [19]; however, ROS at higher concentrations cause DNA damage [20] among other cytotoxic effects. PARP is known to play an important role under oxidative stress [21]; however, there is no report on the role of PARP in *D. discoideum* development. We have investigated the role of PARP in *D. discoideum* development by inhibiting its activity with the known PARP inhibitor benzamide, and studied its effects on development and oxidative stress-induced development. Our results suggest that 2.5 mM HA delayed development due to cell cycle arrest, whereas 4 mM HA caused 90% cell death, meaning that cell density was not sufficient for aggregation, leading to complete developmental arrest. Our results show that *D. discoideum* exhibits basal PARP activity (Fig. 4A), and its inhibition by benzamide (1–3 mM) did not affect development. However, benzamide (4 mM)-treated *D. discoideum* cells were unable to differentiate properly (Table 2) and exhibited delayed development, especially at the differentiation stage of prestalk and prespore formation. These results suggest that lower doses of benzamide have no deleterious effects on *D. discoideum* development.

HA-induced oxidative stress activates PARP within 5 min (Fig. 4A,B), and its role during oxidative stress is further confirmed by the use of low concentrations of benzamide. Preincubation of cells with benzamide prevented the peak activity observed during oxidative stress (Fig. 4A,B). Under oxidative stress, partial inhibition of PARP activity led to altered growth, suggesting that oxidative stress could be leading to cell cycle arrest [22] and that PARP inhibition possibly overcomes this arrest. PARP inhibition also rescued the oxidative stress-induced delay in development (Table 1), although the fruiting body was smaller than in controls (Fig. 5). Thus, our results suggest not only the presence of PARP in *D. discoideum*, but also its overactivation under moderate to severe oxidative stress. Our present study is the first report on the role of PARP in *D. discoideum* development.

PARP inhibitors are powerful cell-protective agents that block cell death in response to oxidative stress and hence are used as therapeutic molecules to control oxidative stress-related diseases [12]. However, the consequences of the blockade of cell death by PARP inhibitors for long-term cell survival are not entirely clear. In this context, we have studied the effect of PARP inhibition under oxidative stress on two generations by reviving the spores and monitoring growth and doubling time. It was found that in normal cells, PARP inhibition (1 mM benzamide) has no effect on spore germination. However, when cells were exposed to oxidative stress (2.5 mM HA) and allowed to develop, the spores remained dormant for longer time

as compared to control spores, as the spores took more time (56 h) to germinate as compared to control spores. Conversely, when cells were exposed to oxidative stress (2.5 mM and 4 mM HA) with PARP inhibition and allowed to develop, the spores showed faster germination (32 h and 60 h) as compared to cells exposed to oxidative stress alone (2.5 mM HA), as seen in Fig. 6. Interestingly, the amoebae thus formed due to spore germination (2.5 and 4 mM HA with and without PARP inhibition) exhibited normal development (data not shown), suggesting that second-generation cells had overcome the effect of oxidative stress. Thus, our results demonstrate that partial PARP inhibition under mild or severe oxidative stress did not affect repair of the damage incurred due to oxidative stress, as the amoebae formed upon spore germination exhibited normal growth and development for two successive generations. Our data support the idea that PARP inhibition is beneficial under oxidative stress and that PARP inhibitors are potential therapeutic molecules for the control of oxidative stress-related diseases. This study also opens the possibility for identifying the genes involved in *D. discoideum* spore dormancy under stress conditions.

Experimental procedures

Materials

Hydroxylamine, benzamide and anti-mouse IgG (whole molecule) fluorescein isothiocyanate (FITC) conjugate developed in rabbit were obtained from Sigma Aldrich (St Louis, MO), and mouse mAb (10H) to poly(ADP-ribose) (PAR) (Ab-1) was obtained from Calbiochem (San Diego, CA, USA).

Cell culturing

D. discoideum cells (Ax-2 strain) were grown in suspension in HL5 medium with shaking at 150 r.p.m. and 22 °C. Developmental studies were carried out on non-nutrient agar plates. All the experiments were carried out with *D. discoideum* cells at mid-log phase with a cell density of 2.5×10^6 cells·mL^{-1}. Amoebae were washed with 1 × Sorenson's buffer (SB) (17 mM potassium phosphate, pH 6.4) by centrifugation at 300 *g* for 5 min, and spread on phosphate-buffered agar (PBA) plates at a density of 2.5×10^5 cells·cm^{-2}. The plates were allowed to develop at 22 °C.

Dose-dependent effect of HA on *D. discoideum* cell death

Cells (2.5×10^6) were harvested by centrifugation at 300 *g* for 5 min at 4 °C, resuspended in HL5 medium, exposed to different doses (1.0, 2.5 and 4.0 mM) of HA, and shaken at 150 r.p.m. at 22 °C for growth [23]. Cell death was checked by a Trypan blue exclusion method after 24 h.

Effect of HA on *D. discoideum* growth

Cells (0.5×10^6) were harvested by centrifugation at 300 *g* for 5 min at 4 °C, resuspended in 4 mL of HL5 medium so that the cells entered lag phase, and then exposed to different concentrations (1.0, 2.5 and 4.0 mM) of HA for 1 h. The cells were washed with 1 × SB two or three times, and finally suspended in HL5 medium (pH 6.5) and shaken at 150 r.p.m. and 22 °C for growth. The cells were counted using a hemocytometer every 12 h up to 132 h (6 days) [23].

Effect of HA on *D. discoideum* development

Cells (2.5×10^6) were harvested and processed as described above for HA treatment (1.0, 2.5 and 4.0 mM), and the cells were then resuspended in 100 μL of 1 × SB and spread on non-nutrient agar plate (PBA plates). The plates were kept at 22 °C, and different stages of development were observed. Grids 1 mm square were made on a 35 mm plate, and then fruiting bodies in five such squares of different regions were counted under a microscope. Approximately 40 fruiting bodies were counted in the experiment.

PARP activation under HA stress

Cells treated with different doses of HA were centrifuged and washed once with NaCl/Pi, fixed in 70% chilled methanol for 10 min at −20 °C, washed with blocking solution (1.5% BSA with 0.05% Tween-20 in NaCl/P_i), and then incubated for 1 h with antibody to PAR raised in mouse at a concentration of 0.5 μg·mL^{-1} [24]. Cells were washed two or three times with blocking solution, and incubated for 1 h with FITC-conjugated anti-mouse IgG as secondary antibody, used at a dilution of 1 : 200. Cells were washed two or three times with NaCl/P_i, and fluorescence was observed at 60× magnification using a Nikon (Tokyo, Japan) fluorescence microscope with a charge-coupled device camera; results are shown for 2.5 mM HA only. Data were analyzed by IMAGE PROPLUS software to calculate the mean density of fluorescence from different fields, and ~50 cells were examined for each dose.

PARP inhibition by benzamide

A culture in log phase with a cell count of 1.0×10^6 cells was incubated with 1 mM benzamide, a PARP inhibitor [25], for 24 h. Cells were then treated with 2.5 mM HA and observed for PARP activation as for the PARP assay.

Effect of benzamide on HA-induced changes to *D. discoideum* growth

Cells (0.5×10^6) were treated with the 1 mM benzamide for 24 h, and then the cells were exposed to HA (2.5 mM) for 1 h. Cells were washed and resuspended in 4 mL of sterile HL5, and growth was monitored for 6 days.

Dose-dependent effect of benzamide on *D. discoideum* development

Cells (1.0×10^6) were harvested, resuspended in HL5 medium, and exposed to different concentrations (1.0, 2.0, 3.0 and 4.0 mM) of benzamide for 24 h at 22 °C. After 24 h of incubation, the cells were washed three times with 1 × SB and processed for development.

Effect of benzamide on oxidative stress-induced changes to *D. discoideum* development

Cells (1.0×10^6) were harvested, resuspended in HL5 medium, and exposed to 1 mM benzamide for 24 h at 22 °C. After 24 h of incubation, cells were treated with different concentrations of HA (2.5 and 4.0 mM) for 1 h. The cells were then centrifuged at 300 *g*, washed two or three times with 1 × SB, plated on PBA plates, and monitored for development.

Effect of benzamide on the fate of spores formed under HA stress

Spores formed after treatment with 2.5 and 4 mM HA in the presence and absence of benzamide were picked from different areas with the help of a sterilized nichrome loop, and added to 5 mL of HL5 medium. Flasks were continuously shaken at 150 r.p.m. and 22 °C. After germination, the cells were counted every 12 h using a hemocytometer.

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