Biochemical basis of the high resistance to oxidative stress in *Dictyostelium discoideum*

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Aerobic organisms experience oxidative stress due to generation of reactive oxygen species during normal aerobic metabolism. In addition, several chemicals also generate reactive oxygen species which induce oxidative stress. Thus oxidative stress constitutes a major threat to organisms living in aerobic environments. Programmed cell death or apoptosis is a physiological mechanism of cell death, that probably evolved with multicellularity, and is indispensable for normal growth and development. *Dictyostelium discoideum*, an eukaryotic developmental model, shows both unicellular and multicellular forms in its life cycle and exhibits apparent caspase-independent programmed cell death, and also shows high resistance to oxidative stress. An attempt has been made to investigate the biochemical basis for high resistance of *D. discoideum* cell death induced by different oxidants. Dose-dependent induction of cell death by exogenous addition of hydrogen peroxide (H_2O_2), *in situ* generation of H_2O_2 by hydroxylamine, and nitric oxide (NO) generation by sodium nitroprusside treatment in *D. discoideum* were studied. The AD_{50} doses (concentration of the oxidants cusing 50% of the cells to die) after 24 h of treatment were found to be 0·45 mM, 4 mM and 1 mM, respectively. Studies on enzymatic antioxidant status of *D. discoideum* when subjected to oxidative stress, NO and nutrient stress reveal that superoxide dismutase and catalase were unchanged; a significant induction of glutathione peroxidase was observed. Interestingly, oxidative stress-induced lipid membrane peroxidative damage could not be detected. The results shed light on the biochemical basis for the observed high resistance to oxidative stress in *D. discoideum*.

1. Introduction

One of the paradoxes of life is that the very molecule which sustains aerobic life i.e. oxygen, is essential for energy metabolism and respiration, but has been implicated in many disease conditions as well (Marx 1985; Fridovich 1983; Halliwell 1997). Thus oxidative stress builds up in a cell when balance between production of reactive oxygen species (ROS) and antioxidant defense system is lost. The evolutionary survival process has provided aerobic organisms well-balanced mechanisms to neutralize the oxidative effects of oxygen and its reactive metabolites. Among other defense mechanisms, damage control is provided by radical scavengers, which prevent initiation and/or propagation of chain reaction and in turn generate new radicals, which are not sufficiently

Abbreviations used: GSH-Px, Glutathione peroxidase; HA, hydroxylamine; MTT, 3-[4,5-Dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide; NO, nitric oxide; PCD, Programmed cell death; ROS, reactive oxygen species; SNP, sodium nitroprusside; SOD, Superoxide dismutase.

reactive to propagate the chain process. Superoxide dismutase (SOD), catalase and peroxidases constitute a mutually supportive team of defense against ROS. While SOD lowers the steady state level of O_2^-, catalase and peroxidases do the same for H_2O_2. The cellular low molecular weight antioxidants – a-tocopherol, ascorbate and reduced glutathione also act in combination. Any imbalance with respect to either excessive free radical generation or lowered efficiency of antioxidant defense system would lead to the accumulation of free radicals which leads to oxidation of various macromolecules including membrane lipids, proteins and DNA. This oxidation of macromolecules disrupts the normal physiology of the cell, and eventually leads to cellular pathogenesis (Halliwell and Gutteridge 1990; Hasnain *et al* 2003). Reactive oxygen species have also been implicated as mediators of cell death: i.e. high degree of oxidative stress can cause necrosis, lower levels will trigger apoptosis (Lennon *et al* 1991; Dypbukt *et al* 1994). The mitochondrial electron transport chain is the most prolific source of cellular ROS producing the superoxide anion, which is subsequently converted to hydrogen peroxide. Alternatively, ROS can result from exposure to exogenous agents such as UV light and a wide variety of environmental and chemical oxidants (Halliwell and Gutteridge 1990).

Programmed cell death (PCD), that is central to the development and homeostasis of multicellular organisms, is a genetically regulated form of cell disassembly in which dying cells and their nuclei exhibit a set of stereotypic morphological and biochemical features including shrinkage, fragmentation and cleavage of chromosomal DNA into internucleosomal repeats (Kerr *et al* 1972; Jacobson *et al* 1997). PCD probably evolved with multicellularity (Ameisen 1996; but see Koonin and Aravind 2002) and is induced by various external or internal signals and ROS is amongst the most potent inducers of PCD (Jacobson 1996; Suzuki *et al* 1997).

D. discoideum offers a good model to study the evolution of PCD as it shows both unicellular and multicellular forms. During induced *in vitro* stalk differentiation, *D. discoideum* exhibits a form of cell death which is characterized by loss of viability, massive vacuolization, prominent cytoplasmic condensation and focal chromatin condensation, apparently, without caspase activation and oligonucleosomal DNA fragmentation (Cornillion *et al* 1994; Olie *et al* 1998). In the course of normal development, cell death during stalk cell differentiation in *D. discoideum* exhibits loss of mitochondrial transmembrane potential, phosphatidylserine surface exposure and caspase activation without oligonucleosomal DNA fragmentation (Kawli *et al* 2002).

Dictyostelium is also known to be unusually resistant to ultraviolet light, gamma radiation and oxidative stress (Yu *et al* 1998). Since, the molecular mechanisms underlying *D. discoideum* cell death and its high resistance to oxidative stress are yet to be understood, an attempt has been made to analyse the biochemical events associated with oxidative stress, nitric oxide (NO) stress and nutrient stress in *D. discoideum*. Chemical agents like hydroxylamine, cumene hydroperoxide and sodium nitroprusside (SNP) were used to study oxidative stress-mediated cell death in *D. discoideum*.

2. Materials and methods

2.1 *Cells and culturing conditions*

Dictyostelium discoideum cells (cloned Ax-2 strain) were grown in suspension in HL5 medium and also maintained on nutrient agar with *Klebsiella aerogenes* and harvested using standard procedures (Sussman 1987; Levraud *et al* 2001). All the experiments were carried out with the *D. discoideum* cells at mid-log phase at a cell density of 2×10^6 cells/ml with > 95% viability (tested with Trypan blue exclusion).

2.2 *Induction of apoptosis in* D. discoideum

Different concentrations of oxidants were used to induce apoptosis in *D. discoideum* cells. The dose showing around 50% cell death was selected for further studies. Two ml of mid-log phase fresh culture (2×10^6 cells/ml) was treated with different concentrations of hydroxylamine (0–8 mM), or cumene hydroperoxide (0–0·90 mM) for 1 h, and incubated at 22°C, 120 rpm shaking for 24 h. For SNP treatment, 2 ml of exponentially growing cells were harvested by centrifugation at 700 g for 5 min at 4°C, washed thrice with KK_2 buffer (17 mM potassium phosphate, pH 6·8) and resuspended in 2 ml of HL5 medium, pH 7·5. The cells were also treated with different concentrations of SNP (0–2 mM) and incubated at 22°C for 1 h, the HL5 medium was changed from pH 7·5 to pH 6·5 and cells were incubated at 22°C with shaking at 120 rpm for 24 h. For studying nutrient stress, 2 ml of exponentially growing cells were harvested by centrifugation at 700 g for 5 min at 4°C, washed thrice with KK2 buffer and resuspended in KK2 buffer and incubated at 22°C, 120 rpm shaking for 24 h. Viability of the cells was monitored with 3-[4,5-Dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) reduction assay and Trypan blue exclusion method at 24 h post treatment.

2.3 *Assay for apoptosis in* D. discoideum *cells*

MTT reduction assay (Sladowski *et al* 1993) was carried out by the addition of MTT stock solution (5 mg/ml) to

each culture being assayed and incubated for 3–4 h. At the end of the incubation period, isopropanol was added directly and the dissolution was accomplished by tituration. Absorbance of the reduced dye was measured at 570 nm with background subtraction at 630–690 nm. Microscopic assay for cell death was carried out by assessing the ability of live cells to exclude Trypan blue. A 45 µl of aliquot of the cells was stained with 0·04% Trypan blue and cells were monitored under a light microscope (40 × magnification). To detect the necrotic cell death, protein concentration (Lowry *et al* 1951) and acid phosphatase activity (Salomon *et al* 1964) in the cell free supernatant were determined.

2.4 *Estimation of the enzymatic antioxidant status and lipid peroxidation in* D. discoideum *cells*

Enzymatic antioxidant status and lipid peroxidation levels in *D. discoideum* cells were monitored at 4 h after treatment with AD_{50} doses of different oxidants as well as after nutrient stress condition. Two ml of mid-log phase fresh culture (2×10^6 cells/ml) was treated with 4 mM hydroxylamine (AD_{50} dose) or 0·45 mM cumene hydroperoxide (AD_{50} dose) or 1 mM sodium nitroprusside (AD_{50} dose) or suspended in KK2 buffer after three washings (nutrient stress) and incubated at 22°C, 120 rpm shaking for 4 h. The cells were lysed by sonication and the activity of catalase (Aebi 1984), GSH-Px (Hafeman *et al* 1974), SOD (Nishikimi *et al* 1972), protein estimation (Lowry *et al* 1951) and lipid peroxidation (Slater 1984) in the cell lysate were assayed according to the standard methods.

3. Results

Experiments were designed to establish the ability of *D. discoideum* to enter cell death as a function of oxidative stress, NO stress and nutrient stress. This was achieved by intracellular build up of H_2O_2 or directly treating *D. discoideum* cells with cumene hydroperoxide; or *in situ* NO generation by sodium nitroprusside treatment as well as nutrient stress by starvation.

3.1 *Intracellular build-up of oxidative stress in* D. discoideum *cells*

Cell death was induced by treating the cells for 1 h with different concentrations (0, 2, 4, 6, 8 mM) of hydroxylamine (HA), a known inhibitor of catalase (Kono and Fridovich 1983), in order to promote intracellular accumulation of ROS. HA-induced cell death in *D. discoideum* cells was measured by the Trypan blue exclusion method

(figure 1a). The number of viable cells was quantitated by the reduction of MTT, a vital dye (Sladowski *et al* 1993) (figure 1b). The percentage of cells undergoing cell death increased from 20% to 98% as the concentration of HA increased from 2 mM to 8 mM (24 h treatment regime) and the AD_{50} was 4 mM. No vesicles resembling the apoptotic bodies were released at 24 h of treatment. No significant change in specific activity of acid phosphatase was observed in the cell free supernatant of cells subjected to HA stress (table 1).

3.2 *Induction of cell death in* D. discoideum *by exogenous addition of* H_2O_2

Dose-dependent induction of cell death in *D. discoideum* was studied by exposing cells to cumene hydroperoxide (cumene H_2O_2) at levels ranging from 0·25 mM to 0·9 mM. In response to increasing doses of cumene H_2O_2, the percentage of cells undergoing cell death increased from 25% to 95% (24 h treatment regime) as shown by
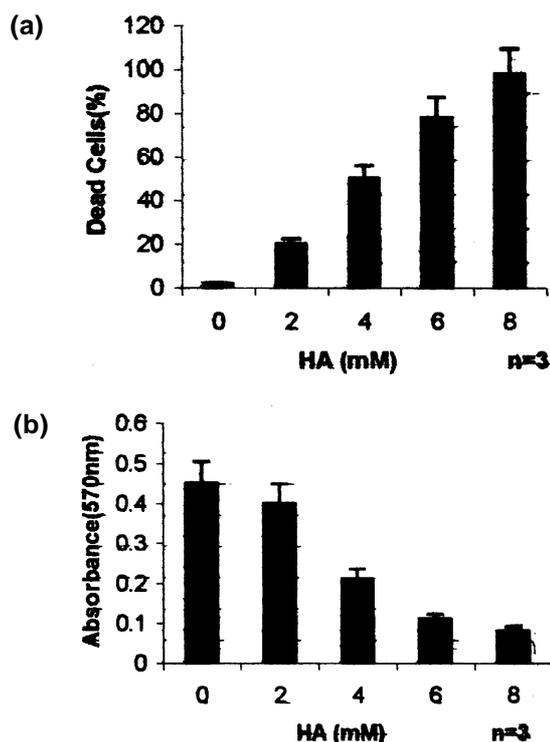
Figure 1. Dose-dependent induction of apoptosis in *D. discoideum* cells by *in situ* generation of H_2O_2 upon catalase inhibition. *D. discoideum* cells were treated with increasing concentrations (0–8 mM) of hydroxylamine (HA) for 1 h and assayed for dead as well as viable cells after 24 h. (**a**) Percent of dead cells by Trypan blue exclusion method. (**b**) Assay of viable cells by MTT reduction method.

Trypan blue exclusion method (figure 2a) and assessed by the reduction of MTT (figure 2b). The AD_{50} was found to be 0·45 mM. No significant change in specific activity of acid phosphatase was observed in the cell free supernatant of cells subjected to cumene H_2O_2 stress and apoptotic bodies were not released at 24 h of post treatment (table 1).

3.3 *Induction of cell death in* D. discoideum *by NO stress*

Cell death due to NO stress was induced by treating *D. discoideum* cells at pH 7·5 with different concentrations of SNP. The percentage of dead cells increased from 25% to 98% with increase in SNP concentration from 0·2 mM to 2 mM (24 h post treatment) as shown by Trypan blue exclusion and the MTT reduction assay (figure 3). The AD_{50} dose of SNP was found to be 1 mM (figure 3). No vesicles resembling apoptotic bodies were released at 24 h SNP post treatment. No significant change in specific activity of acid phosphatase was observed in the cell free supernatant of cells subjected to SNP (table 1).

3.4 *Enzymatic antioxidant status in* D. discoideum *under different stress conditions*

We have studied the enzymatic antioxidant status by monitoring catalase, SOD and glutathione peroxidase activities in *Dictyostelium* cells exposed to oxidative stress, NO stress and nutrient stress (starvation). The cells were exposed to AD_{50} doses of different oxidants i.e. HA, cumene H_2O_2 and SNP, and at 4 h of post treatment, the enzymatic antioxidant status was assessed and the results are shown in figure 4. Cells subjected to 4 mM HA showed about 50% inhibition in the catalase activity, confirming that oxidative stress was generated. No significant change was observed in SOD activity compared to control cells. However, cells exposed to 4 mM hydroxylamine showed 35% increase in glutathione peroxidase

Table 1. Effect of oxidative stress on acid phosphatase specific activity* in cell free supernatant of *D. discoideum* cells.

Treatment	Specific activity (IU/mg protein)
Control	126·1 ± 14·49
Cumene H_2O_2	148·7 ± 9·262
HA	142·3 ± 10·17
SNP	125·3 ± 11·26

*Values are expressed as mean ± SE of three independent experiments.

(GSH-Px) activity compared to control cells ($P < 0·001$). The cells subjected to 0·45 mM cumene H_2O_2 did not show significant change in the catalase and SOD activities, whereas 31% increase in its GSH-Px activity ($P < 0·001$) was observed compared to control cells. *Dictyostelium* cells upon 1 mM SNP treatment exhibited no significant change in the activities of SOD and catalase, while a 39% increase in GSH-Px activity ($P < 0·001$) was observed. The cells under nutrient stress showed similar catalase and SOD activities compared to control cells, however, 37% increase in GSH-Px activity was observed ($P < 0·001$).
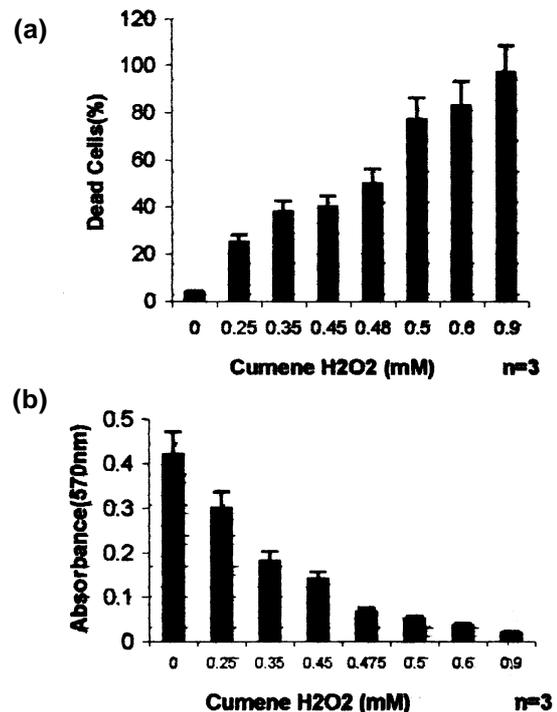
3.5 *Lipid peroxidation status in* D. discoideum *under different stress conditions*

Oxidative damage of polyunsaturated fatty acids (PUFA) was measured by determining thiobarbituric acid reacting substances (TBARS) in *D. discoideum* cells under nutrient stress. Cells were also monitored at 4 h after treatment with AD_{50} doses of different oxidants. Interestingly, no significant change in lipid peroxidation levels was obser-
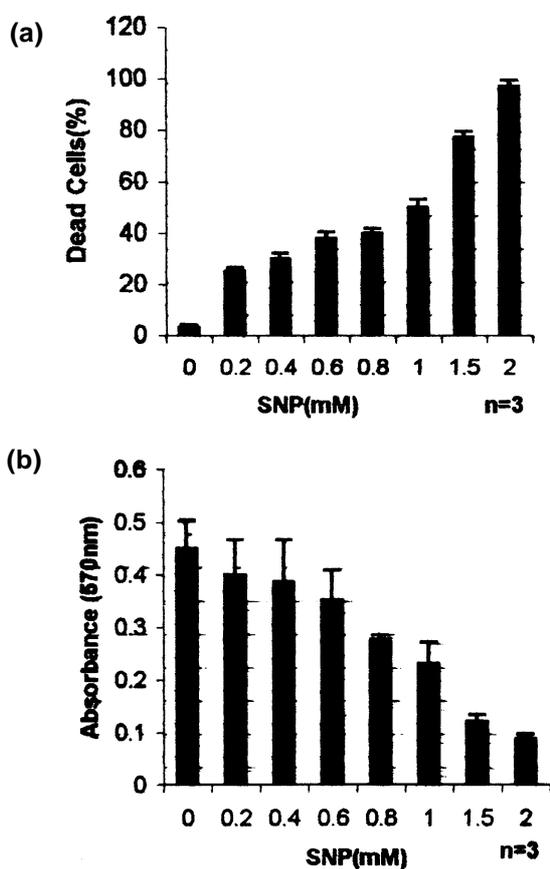

Figure 2. Dose-dependent induction of apoptosis in *D. discoideum* cells by cumene H_2O_2 treatment. *D. discoideum* cells were treated with increasing concentrations (0–0·9 mM) of cumene H_2O_2 for 1 h and assayed for dead and viable cells after 24 h. (**a**) Percent of dead cells by Trypan blue exclusion method. (**b**) Assay of viable cells by MTT reduction method.

ved in *D. discoideum* under starvation, HA, cumene H_2O_2 and SNP stress conditions (figure 5).

4. Discussion

Living systems are exposed to increasing threats of oxidative stress caused by a plethora of oxygen-derived free radicals, which may be generated metabolically or otherwise. Oxidative stress constitutes one of the major stimuli which can induce apoptosis in cells *in vitro* as well as *in vivo* (Thompson 1995; Suzuki *et al* 1997). PCD can be induced by oxidative stress and this is further supported by studies where apoptosis was inhibited by antioxidants (Buttke and Sandstorm 1994; Mohan *et al* 2003). Exposure of low doses of H_2O_2 induces apoptosis in a variety of cell types (Lennon *et al* 1991; Sah *et al* 1999). Nitric oxide has also been implicated as an inducer of

apoptosis in macrophages and monocytes (Albina *et al* 1993). However, the effect of oxidative stress on *D. discoideum* development and morphogenesis still remains to be elucidated. The cellular slime mould, *D. discoideu*m, widely used in studies of cell and developmental biology (Loomis 1975; Alexander and Rossomando 1992), is an evolutionarily ancient organism and can be considered as a choice for the study of apoptosis in a eukaryote that has both unicellular and multicellular phases. *D. discoideum* cells are unusually resistant to UV-light, gamma-radiation and DNA-damaging chemicals (Yu *et al* 1998; Garcia *et al* 2000); however, the basis for this is not well understood. An attempt has been made in this study to develop *D. discoideum* as a model for oxidative-stress-induced apoptosis and to investigate the plausible mechanism of high resistance observed in *D. discoideum* to oxidative stress.

Figure 3. Dose-dependent induction of apoptosis in *D. discoideum* cells by sodium nitroprusside (SNP) treatment. *D. discoideum* cells, suspended in HL-5 medium, pH 7·5 were treated with increasing concentrations (0–2 mM) of SNP for 1 h; cells were shifted to HL-5 medium, pH 6·5 and assayed for dead and viable cells after 24 h. (**a**) Percent of dead cells by Trypan blue exclusion method. (**b**) Assay of viable cells by MTT reduction method.
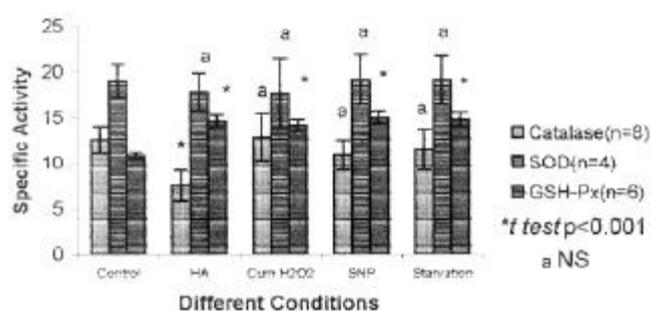
Figure 4. Enzymatic antioxidant status of *D. discoideum* under different stress conditions. *D. discoideum* cells were subjected to AD_{50} doses of hydroxylamine, cumene H_2O_2, sodium nitroprusside and to nutrient stress for 4 h. Cells were harvested and resuspended in KK_2 buffer. Cell lysis was carried out by sonication, and the activity of catalase, SOD and GSH-Px antioxidant enzymes and protein concentration were estimated in the cell lysate according to the standard methods.
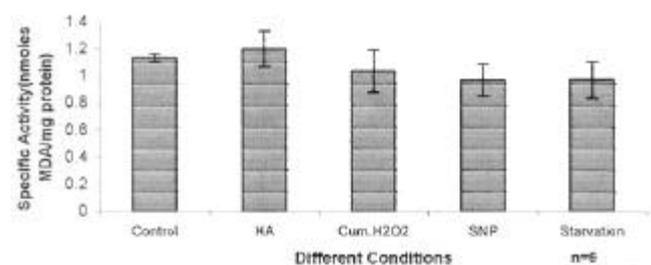
Figure 5. Lipid peroxidation levels of *D. discoideum* under different stress conditions. *D. discoideum* cells were subjected to AD_{50} doses of hydroxylamine, cumene H_2O_2, sodium nitroprusside and to nutrient stress for 4 h. Cells were harvested and resuspended in KK_2 buffer. Cell lysis was carried out by sonication, and lipid peroxidation levels and protein concentration were estimated in the cell lysate according to the standard methods.

Our results demonstrate that *D. discoideum* cells could be induced to undergo PCD by exogenous addition of cumene H_2O_2, *in situ* generation of H_2O_2 by hydroxylamine and NO generation by SNP. During necrosis – unlike apoptotic cell death – rupture of plasma membrane and organelles takes place leading to release of intracellular and intraorganellar contents into the medium. No significant change in specific activity of acid phosphatase was observed in the cell free supernatant of cells subjected to oxidative stress (table 1) suggesting that the cells exhibit apoptotic death but not necrosis (Nirmala and Puvanakrishnan 1996). Our unpublished results also indicate that *Dictyostelium* cells under oxidative stress exhibit apoptotic death since externalization of phosphatidylserine would be observed as early as 4 h without propidium iodide staining (Annexin V positive and PI negative). Interestingly, no vesicles resembling apoptotic bodies were released from *Dictyostelium* cells at 24 h of HA, cumene H_2O_2 and SNP treatment. However, Amoult *et al* (2001) and Tatischeff *et al* (2001) reported release of apoptotic corpses in the *Dictyostelium* cell death which was induced by conditioned medium or stationary phase cell death. The AD_{50} doses for HA, cumene H_2O_2 and SNP reported to be 4 mM, 0·45 mM, 1 mM respectively. Hasnain *et al* (1999) reported AD_{50} dose of HA in *Sf-9* insect cells as 2·2 mM. Verkerk and Jongkind (1992) showed that doses of H_2O_2 above 80 μM causes 100% death in vascular endothelial and smooth muscle cells. Del Carlo and Loeser (2002) have reported that treatment of SNP resulted in 100% apoptotic cell death at concentrations of 0·5 mM in chondrocytes. Our results on the other hand show that higher doses of oxidants are required for *D. discoideum* cells to achieve significant death compared to other eukaryotic cells and therefore further support the fact that *D. discoideum* is unusually resistant to oxidative stress.

To address the biochemical events associated with high resistance to oxidative stress, in *D. discoideum*, the activity of catalase, SOD and GSH-Px antioxidant enzymes were measured. SOD activity did not show any change in cells subjected to oxidative, NO and nutrient stress (figure 4). Since, catalase activity was reduced to 50% in the cells subjected to AD_{50} dose of hydroxylamine, it suggests that *D. discoideum* catalase is inhibited by hydroxylamine and leads to *in situ* generation of H_2O_2. No significant induction of catalase activity was observed in cumene H_2O_2-treated cells, SNP-treated cells and cells under starvation compared to that of control cells, corroborating the earlier reports by Garcia *et al* (2000). However, catalase activity of *D. discoideum* cells is reported (Madigan and Katz 1989) to be 2 to 5 times higher than other organisms, and therefore this could confer high resistance to oxidative stress. *D. discoideum* has two types of catalases i.e. Cat A and Cat B which are temporally and spatially regulated. While Cat A is constitutively expressed, Cat B is found only in pre-spore cells and is expressed 10–12 h before development (Garcia *et al* 2000), possibly offering protection to the spores from oxidative stress during prolonged dormancy. Ours is the first report on the GSH-Px activity in *D. discoideum* cells. Induction of GSH-Px could also confer high resistance to oxidative stress. Thus, presence of high levels of catalase throughout the *D. discoideum* life cycle, and significant induction of GSH-Px could confer higher resistance of *D. discoideum* to oxidative stress. ROS seem to play a role in morphogenesis and development of *D. discoideum* (Tao *et al* 1997), and GSH-Px might scavenge the deleterious ROS that are generated during development.

Lipid peroxidation, oxidative damage of polyunsaturated fatty acids, in membrane phospholipids is one of the multiple toxic effects of oxidative stress that is related to several pathological conditions (Zimniak *et al* 1977). In our studies, lipid-peroxidation, the marker of oxidative stress, showed no significant change in *D. discoideum* under any stress condition (figure 5). *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 either monounsaturated or with two double bonds and hence are less susceptible to lipid peroxidation compared to polyunsaturated fatty acids. In the current study, induction in GSH-Px activity and non significant changes in lipid peroxidation (LPO) levels can be the best explained for the high resistance of *D. discoideum* to oxidative stress.

The objective of establishing *D. discoideum* as a model to study oxidative stress-induced apoptosis was to investigate the effect of oxidative stress on growth and development and also the evolutionary aspects of cell death. *D. discoideum* is one of the most anciently diverged, currently surviving, eukaryotes (Christen *et al* 1991; Field *et al* 1988). Its phylogenetic position is believed to lie after the divergence of the kingdom plantae, and before individualization of the kingdoms Animalia and Fungi (Baldauf and Doolittle 1997). Demonstration of a common cell death mechanism between this organism and some of the higher eukaryotes would throw light on the origin and evolutionary changes of PCD.

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