Apopdbsis or programmed cell death is an essential physiological process that plays a critical role in controlling the number of cells in development and throughout an organism's life by removal of cells at the appropriate time. However, apoptosis is also involved in a wide range of pathological conditions such as cardiovascular diseases, cardiomyopathies, neurodegenerative diseases and immunological diseases (Zimmermann et al., 2001; Khoynezad et al., 2004).

3.1 APOPTOSIS IN THE CARDIOMYOCYTES

Gottlieb et al. (1994) first described apoptosis in myocardial infarction. Apoptosis was expected to have only a limited role in the healthy heart, as the parenchymal cells and terminally differentiated adult cardiomyocytes rarely proliferate, if at all. However, extensive studies now indicate that apoptosis is deeply involved in the pathology of almost all types of heart diseases, including ischemic-related ailments (Yaoita et al., 2000; Takemura and Fujiwara, 2004). It has been observed that the apoptotic cells were dispersed among the normal myocardial cells in the periphery of the infarct area, while in the central zone, where necrosis of cardiomyocytes was evident, a mixture of necrozed cells and end-labelled apoptotic cells were observed (Ziori et al., 2003). Apoptosis is characterized by cell shrinkage, chromatin condensation and internucleosomal cleavage of genomic DNA (Zimmermann et al., 2001). ROS are known to be potent inducers of apoptosis (Gottlieb et al., 1994). High nitric oxide levels produced by inducible nitric oxide synthase, contribute to progressive cardiac failure by causing apoptosis (Wildhirt et al.,
1995). Extensive production of these highly reactive free radicals may mediate an apoptotic signaling pathway that leads to cell death. Two major molecular signaling pathways lead from a triggering event to the activation of an initiator caspase-cysteine protease that degrade specific intracellular substrates and thereby promote apoptosis (Figure 3.1). The first depends on the participation of mitochondria and the second involves death receptors, such as the Tumour Necrosis Factor (TNF) receptor and the Fas receptor.

3.2 THE MITOCHONDRIAL PATHWAY

The main pathway involves the release of mitochondrial intermembrane protein cytochrome c, apoptosis inducing factor (AIF) and probably other factors into the cytosol (Green and Reed, 1998). Cytochrome c, when complexed with apoptotic protease factor 1 (Apaf-1), activates the most upstream caspase on this pathway, procaspase 9, forming the proapoptosome (Li et al., 1997). Recently a second mitochondrial activator of caspase has been discovered (Du et al., 2000), a new protein termed second mitochondrial derived activator of caspase, SMAC is released from mitochondria into cytosol and acts by binding to inhibitor of apoptosis proteins (IAP). The mitochondrial pathway is regulated by IAP and another group of proteins. This group comprises of two classes which include, antiapoptotic intracellular proteins (such as Bcl-2 family proteins) and proapoptotic proteins such as Bax (Green and Reed, 1998; Deveraux and Reed, 1999).
Figure 3.1 Two major signalling pathways of apoptosis
3.3 THE DEATH RECEPTOR PATHWAY

This alternate pathway for activation of apoptosis is best studied in literature, but is thought to play only a secondary role in cardiomyocyte apoptosis (Kubota et al., 1997). The death receptor pathway involves binding of Fas ligand, TNF-α to their receptors such as FADD (Fas-associated death domain), thus activating most upstream pro-caspase 8, leading to activation of caspase 3 (Zheng et al., 1995). TNF receptor (TNFR1) needs an adapter protein TNFR-1-associated death domain (TRADD). TNF-related apoptosis-inducing ligand (TRAIL)/Apo-2L have receptors like TRAIL-R1/DR4, TRAIL-R2/DR5 (Zimmermann et al., 2001). The inhibitors of death receptor pathway include FADD-like interleukin-1β-converting enzyme (FLICE)-inhibitory protein (FLIP), caspase homologue (CASH) and FADD-like antiapoptotic molecule-1 (FLAME-1) (Srinivasula et al., 1997).

3.4 CYCLOPHOSPHAMIDE INDUCED APOPTOSIS: ROLE OF ANTIOXIDANTS

CP induced apoptosis may be through both or one of the two major signaling pathways of apoptosis. It is known to activate the mitochondrial/caspase 9 pathway of cell death (Schwartz and Waxman, 2001). The second pathway of Fas/Fas-ligand interactions has also been implicated in the induction of the apoptotic cell death in thymus (Wang and Cai, 1999). In vivo blockade of the Fas-Fas Ligand pathway inhibits CP induced diabetes in NOD (Non Obese Diabetic) mice (Mahiou et al., 2001). Acrolein a metabolite of CP is an α,β unsaturated aldehyde, also present in cigarette smoke, automobile exhaust, overheated cooking oil. Acrolein induces only very modest levels of apoptosis at low doses while causing
almost exclusively oncosis/necrosis at higher doses by modulation of caspases activity (Kern and Kehrer, 2002). Tanel and Averill-Bates (2005) have reported that acrolein, a toxic end product of endogenous LPO induces apoptosis through the mitochondria, though it could also cause apoptosis by alternative or complementary mechanisms.

Increase in the highly reactive free oxygen radicals have been reported to play a role in the pathogenesis of CP cardiotoxicity (Lee et al., 1996). Acrolein-induced cell death, mainly necrosis, is accompanied by the accumulation of cellular ROS through mitochondria as its initial target (Luo et al., 2005). These ROS are also known to be potent inducers of cardiac apoptosis (Gottlieb et al., 1994). Amifostine protected peripheral lymphocytes from the apoptotic damage induced by chemotherapy consisting of three cycles of cisplatin, adriamycin, and CP in patients (Provinciali et al., 1999). In rat thymocytes exposed to methylprednisolone or etoposide, inducers of apoptosis, preincubation with DHLA, which is a potent antioxidant inhibited apoptosis at an early stage of cell shrinkage and chromatin fragmentation (Bustamante et al., 1995). In vitro studies using H9c2 cardiac cell line have been designed to evaluate the antiapoptotic and cytoprotective role of LA in cardiac cells treated with 4-hydroperoxycyclophosphamide which breaks down to 4-hydroxycyclophosphamide the activated derivative of CP.

3.5 MATERIALS AND METHODS

3.5.1 CHEMICALS

The prodrug 4-hydroperoxycyclophosphamide (4-HC) which spontaneously breaks down to 4-hydroxycyclophosphamide in aqueous
solution, was procured from Squarix Biotechnology, GmbH, Germany. DL-α-Lipoic acid and bovine serum albumin were procured from Sigma Chemicals, St Louis, MO, USA. The plastic culture wares were bought from TPP, Switzerland. Dulbecco’s Modified Eagle’s Medium (DMEM), FBS and other tissue culture reagents were obtained from Seromed®-Biochrom AG, Germany. All other chemicals and solvents used were of highest purity and analytical grade.

3.5.2 CULTURE AND DRUG EXPOSURE

H9c2 cardiac cell line was procured from National Center for Cell Sciences, Pune, India. The cells were maintained in DMEM with 4 mM L-glutamine adjusted to contain 1.5 g/l sodium bicarbonate and 4.5 g/l glucose and 10% FBS. The cells were maintained in growth conditions of 37°C and 5% carbon dioxide. Cells were exposed to 20 to 100 μM concentrations of 4-HC for 1 h. Thereafter the cells were placed in drug free medium for 24 h. Another set of cells were treated with 50 μM to 300 μM concentrations of LA and 40 μM 4-HC for 1 h followed by incubation in drug free medium for 24 h. Solvent control and drug controls were also included. After 24 h, all cells were harvested for experimental studies.

3.5.3 DETERMINATION OF CELL VIABILITY BY TRYPAN BLUE EXCLUSION ASSAY

Cell death was determined by counting the proportion of cells unable to exclude the dye trypan blue. The culture medium was removed and cells were collected by trypsinisation. The cells were centrifuged and again resuspended in phosphate buffered saline (PBS). Trypan blue solution (0.5%
trypan blue in DMEM containing 0.1% BSA) was added to the cells and incubated for 15-20 min. The solution was removed and cells were again washed with PBS to remove excess dye solution. The number of unstained viable cells and the blue colored non-viable cells were counted using a haemocytometer. The percentage of viable/nonviable cells were calculated using the formula.

\[
\text{Percentage of viable/nonviable cells} = \frac{\text{No. of unstained / stained cells}}{\text{Total number of cells}} \times 100
\]

3.5.4 LDH LEAKAGE

LDH was assayed in the culture medium aspirated from each well by the method of King (1965a) as described in section 2.4.4.1. The enzyme activity is expressed as units/h/10^6 cells.

3.5.5 MEASUREMENT OF INTRACELLULAR ROS ACCUMULATION

H9c2 cells were incubated in the presence of optimized concentrations of 4-HC and/or LA for 1 h followed by incubation in drug free medium for 24 h. 2',7'-dichlorofluorescin diacetate (DCF-DA) was used to detect intracellular ROS levels in H9c2 cells (Cao et al., 2003). DCF-DA is a widely used probe for detecting cellular ROS. DCF-DA is cell membrane permeable. Once inside cells, it is hydrolysed by cellular esterases to form DCF, which is trapped intracellularly due to its membrane impermeability. DCF then reacts with intracellular ROS and is oxidized to form the fluorescent product, 2,7-dichlorofluorescein. Briefly, H9c2 cells were rinsed once with PBS after 24 h and then incubated with 10 μM DCF-DA in DMEM at 37°C for 30 min. Then, the cells were washed once with PBS and lysed in 3 ml of
ice-cold 10 mM Tris–HCl buffer (pH 7.4), containing 0.2% SDS. The cell lysates were collected and centrifuged at 2000 x g for 5 min at 4°C. The fluorescence of the supernatants was measured at an excitation wavelength of 495 nm and an emission wavelength of 525 nm.

3.5.6 ASSAY OF NITRITE AND NITRATE

The concentration of NO in the system was measured by the determination of total nitrate and nitrite concentrations in the sample (R&D Systems, Minneapolis, USA). 100 µl of medium was aspirated from each well and the concentration of NO in the system was measured by the determination of total nitrate and nitrite concentrations in the sample as described in section 2.4.8.

3.5.7 FLUORESCENT STAINING OF MITOCHONDRIA

Mitochondria of H9c2 cells were stained with Rhodamine 123 (Park et al., 2003). Rhodamine 123 is a fluorescent lipophilic cationic dye that specifically accumulates in the mitochondria of living cells and is recognized as an indicator of mitochondrial membrane potential. Depolarisation of mitochondrial membrane potential results in dequenching of Rhodamine 123 fluorescence (Kindmark et al., 2001). Cells were stained with 5 µg/ml Rhodamine 123 at 37°C. After incubation at 37°C, cells were washed thrice with PBS and were observed at 530 nm under Nikon fluorescence microscope.
3.5.8 ANALYSIS OF THE EXPRESSION OF CYTOCHROME C AND Bcl-2 BY WESTERN BLOTTING

Immunoblotting was performed as described by Towbin et al. (1979). Cells were collected, washed twice with ice cold Phosphate Buffered Saline (PBS) and lysed with 50 mM Tris HCl (pH 8.0), containing 150 mM NaCl, 1% Triton X-100, 100 μg/ml phenylmethylsulfonyl fluoride and 1μg/ml aprotinin. This was followed by incubation for 30 min in ice and then centrifuged at 10000 x g for 20 min at 4°C. Supernatant was collected for western blotting of Bcl-2.

For analysis of cytochrome c, the cells for lysate preparation were washed twice with ice-cold PBS and collected by centrifugation at 1000 x g for 10 min at 4°C. The cell pellets were washed once with ice-cold PBS and resuspended in lysis buffer (20 mM HEPES pH 7.5, 10 mM KCl, 1.5 mM MgCl₂, 1.0 mM sodium EDTA, 1.0 mM sodium EGTA, 1.0 mM DTT, 0.1 mM PMSF, and 250 mM sucrose) supplemented with protease inhibitors (10 μg/ml leupeptin, 10 μg/ml aprotinin and pepstatin A). The cells were then homogenized in a glass homogenizer, and the nuclei and cell debris were removed by centrifugation at 1000 x g for 15 min at 4°C. The supernatants were further centrifuged at 10,000 x g for 15 min at 4°C, and the resulting mitochondrial pellets removed. The supernatants created from the 10,000 x g centrifugation were centrifuged once again at 100,000 x g for 1 h at 4°C, and the supernatant was collected and designated the cytosolic fraction for western blotting analysis of cytochrome c (Kim et al., 2005).

Samples processed for Bcl-2 and cytochrome c were boiled and equal amounts of protein were separated by SDS PAGE on 12% gel. Proteins were
transferred to nitrocellulose membranes. Membranes were incubated in blocking solution (5% skimmed milk in PBS) overnight at 4°C. Then the membrane was washed thrice with PBS. Primary antibody (Bcl-2 and cytochrome c) raised in rabbit (Cell Signalling Technology, Danvers, MA, USA) was added in the concentration of 1 μg/ml in PBS containing 1% BSA and rocked gently at room temperature for 1 h. The blot was washed thrice with PBS for 5 min each. Goat anti-rabbit secondary antibody conjugated with either horseradish peroxidase (HRP) or alkaline phosphatase (ALP) (1:1000) for Bcl-2 and cytochrome c respectively in PBS containing 1% BSA was added and allowed to hybridize for 1 h at room temperature. The bands were detected using 3,3-diaminobenzidine tetrahydrochloride (DAB) and 0.01% H₂O₂ in 0.05 M Tris-HCl buffer for HRP and Nitro Blue Tetrazolium (NBT) and 1,2 bischlorophenolindophenol for ALP.

3.5.9 IMMUNOCYTOCHEMISTRY

Cells grown on coverslips were fixed in 3% paraformaldehyde in PBS, followed by incubation in 80% ethanol in PBS for 30 min for permeabilisation. The cells were washed thrice with PBS and then incubated in 2% BSA in PBS for 60 min at room temperature. A fresh solution of 0.3% H₂O₂ in PBS was used to treat the cells for 30 min at 37°C to inhibit endogenous peroxidase activity. After washing the cells again with PBS, primary antibody (1:100) caspase 3 active (Cell signaling Technology, Danvers, MA, USA) and rabbit cleaved α-fodrin (150 kDa) raised in rabbit (Cell signaling Technology, Danvers, MA, USA) was added and the cells were incubated overnight at 4°C. The cells were then rinsed with PBS and incubated with appropriate goat anti-rabbit HRP conjugated secondary
antibodies, diluted at 1:1000 in PBS for 30 min at room temperature. After three rinses with PBS, 1 ml of DAB reagent was added (Govindaiah et al., 2002). Harris haemotoxylin solution was added and the cells were mounted with anti-fade mounting solution and examined under light microscope, Nikon Eclipse E400, model 115, Japan.

3.5.10 PROPIDIUM IODIDE STAINING

Propidium iodide staining was done according to the method of Ekhterae et al. (1999). Cells were grown in a six-well plate, trypsinised, pelleted in microfuge tubes and resuspended in 50 µl of PBS. 5 µl RNase (1 mg/ml) were added to each tube, and 5 µl propidium iodide (25 µg/ml in PBS) was added and incubated at 37°C for 1 h. Fluorescence was visualized under a Nikon fluorescence microscope.

3.5.11 ANALYSIS OF DNA FRAGMENTATION: AGAROSE GEL ELECTROPHORESIS

Cells were washed twice with ice-cold PBS and resuspended in lysis buffer containing 10 mM Tris-HCl (pH 8.0), 20 mM EDTA and 0.5% Triton X-100 and incubated for 30 min at 4°C. The DNA of H9c2 cells were isolated by the manufacturer’s instructions provided with the kit (DNA-zol reagent kit) and dissolved in TE buffer (10 mM Tris and 1 mM EDTA, pH 8.0). Agarose gel electrophoresis was carried out for the analysis of DNA fragmentation (Yokozawa and Dong, 2001). The DNA samples (1 µg) were electrophoresed on 1% agarose gel using TBE buffer (0.89 M Tris, 0.89 M boric acid and 20 mM EDTA, pH 8.3) at 40 V for 5 h. Then the
gel was stained with ethidium bromide (10 mg/ml) and viewed under UV-transilluminator and photographed.

3.5.12 STATISTICAL ANALYSIS

The results are expressed as mean values ± S.D for three experiments. Differences between groups were assessed by ANOVA using the SPSS (Statistical Package for Social Sciences) software package for Windows. Post hoc testing was performed for inter-group comparisons using the Least Significance Difference (LSD) test; significance at $P$-values < 0.001, < 0.01, < 0.05 have been given respective symbols in the tables.

3.6 RESULTS AND DISCUSSION

CP is a prodrug that has to be activated by the liver mixed function oxidase system. CP is metabolized to yield phosphoramid mustard and acrolein, both of which represent active forms of the drug (Dollery, 1999). The 4-hydroperoxy form of cyclophosphamide yields 4-hydroxycyclophosphamide and acrolein in vitro as the parent drug in vivo (Dorr and Lagel, 1994). H9c2 myocardiac cells are a stable cell line derived from embryonic rat ventricle (Hong, 2002). It is a continuous cell line of cardiac myoblasts (Wang et al., 1998).

Neonatal myocytes were equi-sensitive to both 4-HC and acrolein, though acrolein is a non specific toxin (Dorr and Lagel, 1994). 4-HC dose-dependently decreased the viability of cells (Figure 3.2). The determination of cell viability by trypan blue exclusion assay revealed that about 49.3% of the cells remained viable after the administration of 40 μM 4-HC. This dose of 4-HC was chosen for further studies. LA at a concentration of 200 μM
Figure 3.2  Dose dependent effect of 4-hydroperoxycyclophosphamide on H9c2 cells

![Graph showing dose dependent effect of 4-hydroperoxycyclophosphamide on H9c2 cells. The x-axis represents Concentration of 4-HC (μM) and the y-axis represents % of non-viable cells.]

Figure 3.3  Dose dependent effect of lipoic acid on 4-hydroperoxycyclophosphamide treated H9c2 Cells

![Graph showing dose dependent effect of lipoic acid on 4-hydroperoxycyclophosphamide treated H9c2 Cells. The x-axis represents Concentration of LA (μM) and the y-axis represents % cell viability.]

All the experiments were done in triplicates. Average of three values are represented in the graph.
increased the number of viable cells and restored cell viability (89.67%) in the H9c2 cells exposed to 4-HC (Figure 3.3). Loss of viability in the myocytes was also observed as LDH leakage in 4-HC administered cells, indicating its cardiotoxicity to H9c2 cells (Figure 3.4). The activity of LDH was increased by 67.92% in 4-HC cells while, LA administration restored LDH activity to near normalcy. Further investigations were carried out to assess the contribution of apoptosis in this observed loss of cell viability.

Incubation of H9c2 cells with 4-HC resulted in a dramatic intracellular accumulation of ROS as indicated by a significant increase \( (P < 0.001) \) in DCF fluorescence (Figure 3.5). DCF detects the intracellular production of \( \mathrm{H}_2\mathrm{O}_2 \), (Ubezio and Civoli, 1994) other hydroperoxides, hydroxyl radical, and nitrogen radicals, but is not oxidized by superoxide anion (Yuan et al., 1993). Transient depletion in non-enzymic antioxidant GSH accompanied the toxic effects of CP in cardiac myocytes (Dorr and Lagel, 1994). CP administration to \textit{in vivo} models also resulted in the development of oxidative stress (Sulkowska and Sulkowski, 1997; Sudharsan et al., 2005a). ROS such as \( \mathrm{O}_2^- \), \( \mathrm{H}_2\mathrm{O}_2 \) and \( \cdot \mathrm{OH} \) radicals are involved in reperfusion injuries after myocardial ischemia (Shao et al., 2002). \( \mathrm{H}_2\mathrm{O}_2 \) is produced from the reaction of superoxide anion and dismutase of cells. Other sources include NADPH oxidase, D-amino acid oxidase and uric acid oxidase. It causes membrane damage to release arachidonic acid, which is responsible for the prolonged damage of cells and also reacts with metal ions to form hydroxyl radicals (Gao et al., 2001). ROS can cause cellular damage by direct oxidation of biomolecules and can also modulate signaling pathways involved in programmed cell death or apoptosis and survival (Duranteau et al., 1998; von Harsdorff et al., 1999). LA significantly reduced \( (P < 0.001) \) the ROS levels
Figure 3.4 Effect of 4-hydroperoxycyclophosphamide and lipoic acid on LDH leakage in H9c2 cells

All the experiments were done in triplicates. Average of three values are represented in the graph. Comparisons are made between: a - Group I and Groups II, III and IV; b - Groups II and Group IV; The symbol *** represents statistical significance at $P < 0.001$. 
Figure 3.5  Effects of lipoic acid on intracellular ROS accumulation in H9c2 cells following incubation with 4-hydroperoxycyclophosphamide

All the experiments were done in triplicates. Average of three values are represented in the graph. Comparisons are made between: a - Group I and Groups II, III and IV; b - Group II and Group IV; The symbol *** represents statistical significance at $P < 0.001$. 
in 4-HC cells. Cao et al. (2003) showed that simultaneous induction of key cellular antioxidants and phase 2 enzymes by LA in H9c2 cardiomyocytes may be an important mechanism underlying the protective effects of LA in various forms of oxidative injury.

4-HC-induced ROS can result in mitochondrial dysfunction preceding myocyte death. In control cells, mitochondria were mainly located in cytosol with punctuated form (Figure 3.6 a), whereas exposure of H9c2 cells to 4-HC for 24 h caused a dramatic change in mitochondrial membrane potential, shown as diffuse and unquenched fluorescence form, which was located in the cytosol and nucleus (Figure 3.6b). CP has been reported to induce apoptosis through the mitochondrial pathway/caspase 9 pathway (Schwartz and Waxman, 2001). Apoptosis is accompanied by the signs of mitochondrial dysfunction, including loss of the inner mitochondrial transmembrane potential (Park et al., 2003). Cytochrome c release appears to depend on the opening of the mitochondrial permeability pore, which is associated with a breakdown of the electrochemical gradient on the inner membrane of mitochondria (Ferrari, 2002). The components of the permeability transition pore are the adenine dinucleotide transporter and the voltage-dependent anion channel that come together at the inner and outer mitochondrial membrane contact sites and form a large channel (Bernardi et al., 1994). This pore allows ions and small molecules to freely pass with limited selectivity, but responds to stimuli that control the mitochondrial transmembrane potential ($\Delta \psi_m$) such as pH, cellular Ca$^{2+}$ concentration and redox status of mitochondrial matrix (Kim et al., 2005). CP has been reported to induce cardiotoxicity in rats by increasing inner membrane permeability to calcium (Al-Nasser, 1998). DHLA causes a remarkable suppression of mitochondrial
Figure 3.6 Effect of 4-hydroxybenzocyclooctene and ligustilide on mitochondrial membrane potential in HepG2 cells (450x).

(a) In control cells, mitochondrial fluorescence was mainly located in cytosol with punctuated form.
(b) In 4-EC treated cells, diffuse and unquenched fluorescence was located in the cytosol and nucleus.
(c) Mitochondrial fluorescence in LA treated cells was similar to control cells.
(d) Cells treated with LA and 4-EC show mitochondrial fluorescence located in the cytosol with punctuated form.
superoxide production in rat heart. It penetrates phospholipid membranes and interferes with the radical generating machinery of redox-cycling ubisemiquinone and thus reduces superoxide release (Schonheit et al., 1995). LA thus improves mitochondrial function through scavenging free radicals.

ROS open the mitochondrial transition pore and favour the release of proapoptotic factors such as cytochrome c (Skarka and Ostadal, 2002). Key players in the control of mitochondrial integrity are members of the Bcl-2 family. While the exact mechanism by which these proteins act as agonists or antagonists in driving mitochondrial permeability is controversial, various models have been proposed. Evidence suggests that the Bcl-2 family members comprising of both pro- and antiapoptotic proteins Bax, Bcl-2, Bcl-XL, and Bid are more abundant at the PTP and thus may control the $\Delta\psi_m$ by altering mitochondrial permeability (Kim et al., 2005).

Bcl-2 present on the mitochondrial outer membrane stabilizes the membrane permeability, thus preserving mitochondrial integrity and suppressing the release of cytochrome c (Yang et al., 1997). It has been implicated in modulating mitochondrial calcium homeostasis and proton flux (Zhu et al., 1999). In 4-HC treated cells the expression of Bcl-2 protein was reduced in western blots, while it was near control levels in LA and 4-HC treated cells (Figure 3.7). In $H_2O_2$ mediated apoptosis, the expression level of Bcl-2 was markedly decreased (Park et al., 2003). Bcl-2 protein is a key inhibitor of apoptosis (Reed, 1994). Loss of Bcl-2 causes transient membrane depolarization and cytochrome c release (Cook et al., 1999). In the mitochondria, Bcl-2 forms heterodimers with Bax (Jia et al., 1999; Von-Haradorn et al., 1999). Bax is a pore-forming cytoplasmic protein, that in
Figure 3.7 Effect of 4-hydroperoxycyclophosphamide and lipoleic acid on Bcl-2 expression in H9c2 cells

Lane 1  Lane 2  Lane 3  Lane 4  Lane 5

97.4 kDa
66 kDa
43 kDa
29 kDa
26.1 kDa
14.3 kDa

Lane 1: Protein molecular weight marker
Lane 2: Control
Lane 3: 4-HC
Lane 4: LA
Lane 5: 4-HC + LA
response to an enhanced oxidative stress, translocates to the outer mitochondrial membrane, influences its permeability and induces cytochrome c loss (Crompton, 2000). The ratio of antiapoptotic Bcl-2 and the proapoptotic protein Bax determines the apoptotic fate of the cell (Spallarossa et al., 2004). The decrease of antiapoptotic protein Bcl-2 expression and proteolytic cleavage of nuclear proteins-lamin B and poly(ADP-ribose) polymerase-1 (PARP-1) were observed in leukemic cells of patients treated with combination chemotherapy comprising of CP (Rogalinska et al., 2004).

A decline in the expression of Bcl-2 was coupled with the cytosolic release of cytochrome c from the mitochondria in 4-HC cells (Figure 3.8). However, when cells were simultaneously exposed to both 4-HC and LA, the levels of cytochrome c were similar to that of control cells. Oxidised phospholipids such as cardiolipin seems to be essential for the formation of the mitochondrial permeability transition pore while oxidized phosphatidyl serine contributes to externalization and recognition by receptors on phagocytes. Specific interactions of cytochrome c with each of these phospholipids result in the conversion and activation of cytochrome c, transforming it from an innocuous electron transporter into a calamitous peroxidase capable of oxidizing the activating phospholipids and triggering the cascade of apoptosis (Kagan et al., 2004). Peroxynitrite and high calcium concentration induce the release of cytochrome c present in the intermembrane space (Bortuaite et al., 1999).

Peroxynitrite is formed from the reaction of NO with superoxide anion as discussed in chapter 2. CP produces an increase in NOx levels in plasma and urine of rats and also induces the expression of iNOS (Xu et al., 2001).
Figure 3.3  Effect of 4-hydroperoxycyclophosphamide and lipase acid on cytochrome c expression in cytosol of H9c2 cells

Lane 1  Lane 2  Lane 3  Lane 4  Lane 5

97.4 kDa
66 kDa
43 kDa
29 kDa
20.1 kDa
14.3 kDa

Lane 1: Protein molecular weight marker
Lane 2: Control
Lane 3: 4-HC
Lane 4: LA
Lane 5: 4-HC + LA
The levels of NO metabolites, nitrate and nitrite increased 3.54-fold after exposure to 4-HC (Figure 3.9). NO displays bifunctional capacity. It appears that high levels of NO produced by iNOS promote apoptosis while basal levels of NO production from eNOS protect cardiomyocytes from apoptosis. NO has been shown to regulate mitochondrial permeability transition, cytochrome c release and Bax/Bcl-2 ratio (Balakirev et al., 1997; Razavi et al., 2005). The high Ca^{2+} concentration and NO metabolites after CP administration can trigger the release of cytochrome c from the mitochondria which is an early event in the apoptotic pathway of cell death. At the mitochondrial level, acrolein caused a decrease in membrane potential, followed by the liberation of cytochrome c (Tanel and Averill-Bates, 2005).

In the cytosol, it triggers to assemble caspase-9 with Apaf-1 to form apoptosome which activates caspase-3 that degrade specific intracellular substrates and thereby promote the apoptotic programmes (Kluck et al., 1997; Yang et al., 1997). As reported in chapter 2, NO suppressing properties of both LA and DHLA (Packer et al., 2001) is beneficial in reducing the apoptotic changes.

Activation of caspase cascades is critical in the initiation of apoptosis in diverse biological systems (Budihardjo 1999). The expression of activated caspase-3 in the cytosol of H9c2 cells was increased after 4-HC administration (Figure 3.10). Active caspase-3 immunohistochemistry revealed apoptotic cells after 4-HC administration (Jezernik et al., 2003). Caspases (cysteine aspartate-specific proteases) exhibit primary substrate specificity for aspartic acid residues, a very uncommon substrate site preference (Cohen, 1997; Earnshaw et al., 1999). The most prevalent caspase in the cell is caspase-3, a cytosolic protease. Caspase-3 is of particular interest
Figure 3.9  Effect of 4-hydroperoxycyclophosphamide and lipoic acid on nitric oxide metabolites released from H9c2 cells

All the experiments were done in triplicates. Average of three values are represented in the graph. Comparisons are made between: a - Group I and Groups II, III and IV; b - Group II and Group IV; The symbol *** represents statistical significance at $P < 0.001$. 
Figure 3.10: Immunocytochemical detection of active caspase-3 expression in H9c2 cells (300x)

(a) Control cells do not show active caspase-3 expression
(b) Cells show active caspase-3 expression in the cytosol of H9c2 cells after 4-HC treatment
(c) LA drug treated cells do not show caspase-3 expression
(d) H9c2 cells treated with LA and 4-HC do not exhibit expression of active caspase-3.
as it appears to be a common downstream apoptosis effector for the mitochondrial and death receptor pathway (Nicholson and Thornberry, 1997). Activation of caspase-3 requires proteolytic processing of its inactive zymogen form by cleavage at specific aspartate residues (Thornberry and Lazebnik, 1998). It is a major "effector caspase" that causes the apoptotic phenotype by cleavage or degradation of several important substrates including cytoskeletal proteins, enzymes involved in signal transduction, cell-cycle proteins and nuclear DNA-repairing proteins (Wang, 2000). The antioxidant LA protected against the loss of glutathione in hepatocytes exposed to TNF and Actinomycin D, completely prevented mitochondrial damage, caspase activation, cytochrome c release, and apoptosis (Pierce et al., 2000).

High and low molecular DNA fragmentation is caused by the action of caspase-3 on a complex of caspase activated DNase (CAD)/DNA fragmentation factor (DF) F40, a nuclease and iCAD/DFF45, its inhibitor (Liu et al., 1997; Enari et al., 1998). This corroborates with our results where increase in caspase-3 expression has been associated with DNA fragmentation in 4-HC treated cells (Figure 3.11). Propidium iodide staining revealed significant nuclear condensation and fragmentation in cells treated with 4-HC (Figure 3.12). Morphologically the onset of apoptosis is characterized by shrinkage of the cell and nucleus and condensation of nuclear chromatin into sharply delineated masses that become marginated against the nuclear membranes. Later on the nucleus progressively condenses and breaks up (karyorrhexis). These processes depend on caspase mediated degradation of nuclear lamins A and B, and proteins involved in the regulation of chromatin structure such as nuclear mitosis associated protein (NuMa) (Saraste and
Figure 3.11  Effect of 4-hydroxycyclophosphamide and lipid acid on DNA fragmentation in MDA cells

Lane 1: DNA ladder
Lane 2: Control
Lane 3: 4-HC
Lane 4: LA
Lane 5: 4-HC + LA
Figure 3.13 Propidium iodide staining of HEp-2 cells treated with 4-hydroperoxycyclophosphamide and epidermal growth factor (EGF)

(a) Control cells do not show apoptotic nuclei (b) 4-HC treated cells show nuclear fragmentation and condensation (c) LA drug control cells do not show apoptotic nuclei (d) LA treatment during 4-HC exposure prevented nuclear apoptotic changes.
Pulki, 2000). Agarose gel electrophoresis of cellular DNA showed ladder pattern of DNA fragmentation. Endogenous DNases cut the internucleosomal regions into double stranded DNA fragments of 180-200 base pairs (Wyllie, 1980). Internucleosomal DNA fragmentation and activation of caspase are the biochemical hallmarks of apoptosis (Saraste and Pulki, 2000). LA prevented CP induced DNA fragmentation in the cardiomyocytes. Previously, in rat thymocytes exposed to methylprednisolone or etoposide, inducers of apoptosis, preincubation with DHLA inhibited apoptosis at an early stage of cell shrinkage and chromatin fragmentation (Bustamante et al., 1995).

Caspase activation may also be responsible for cytoskeletal alterations that can result in cell shrinkage and negatively affect tension development (Chen and Chien, 1999), two important events for the development of cardiomyopathies. α-fodrin is one of the primary targets cleaved by caspases during apoptosis. The expression of cleaved α-fodrin (150 kDa) was increased in 4-HC treated cells, which was near normal in LA and 4-HC treated cells (Figure 3.13). α-Fodrin (nonerythroid spectrin) is a part of the membrane skeleton and expressed in the majority of mammalian cells. α-fodrin has been shown to associate with membrane ion channels and pumps. α-fodrin is a 240 kDa protein forming a heterodimer with either β-fodrin, a 235 kDa molecule that is homologous to α-fodrin, or with β-spectrins. These heterodimers can self-associate into tetramers. The tetramers are anchored to the plasma membrane and bind to actin, calmodulin and microtubules (Ulbricht et al., 2003). Fodrin is cleaved into 145/150 kDa fragments and 120 kDa fragment by the cytosolic cysteine proteases calpains.
(a) Control cells do not show expression of cleaved α-fodrin (b) Cells show cleaved α-fodrin (150 kD) expression in the cytosol of H9c2 cells after 4-HC treatment (c) LA drug control cells do not show expression of cleaved α-fodrin (d) LA and 4-HC
and by caspase-3 (Wang et al., 2001). Cleavage of α-fodrin leads to membrane malfunction, blebbing and cell shrinkage (Janicke et al., 1998).

CP treatment induces apoptosis in other normal tissues including hair follicles, bone marrow, testis, thymus and urothelium (Jezernik et al., 2003; Lopez and Luderer, 2004). Catecholamines, atrial natriuretic peptides, angiotensin II have been shown to induce apoptosis in cultured myocytes (Ferrari, 2002). The apoptotic changes triggered in in vivo conditions may also be due to an increase in norepinephrine. Such abnormal increase in norepinephrine has been reported after CP administration (Hanaki et al., 1990). Pretreatment with amifostine, a phosphorylated aminothiol afforded chemoprotection against CP triggered apoptotic phosphatidyl externalization in normal bone marrow cells (Mazur et al., 2002).

LA was found to be beneficial in preventing the apoptotic changes induced by 4-HC. The use of specific antioxidants like LA which can act as water and lipid soluble antioxidant can prevent oxidative stress and apoptosis. LA at low micromolar concentrations was effective in inhibiting ROS generation by xanthine/xanthine oxidase system in H9c2 cells (Cao et al., 2003). Using an assay for DHLA that minimizes oxidative loss of DHLA during cell extraction and sample work-up, Jones et al. (2002) found that cultured human endothelial cells readily take up and reduce LA to DHLA, and that the mechanism of acute NADPH-dependent reduction involves thioredoxin reductase rather than glutathione reductase. The DHLA generated from LA reduction by these cells enhances their ability to reduce extracellular ferricyamide, to recycle DHA to ascorbate, and to consume ROS generated by menadione. These antioxidant effects can prevent the free radical mediated
cell death. Moreover LA was found to protect cardiac mitochondria from CP-mediated toxicity in rats. The depletion of intracellular GSH seen in apoptotic thymocytes was also prevented in the presence of DHLA, suggesting that its ability to maintain a reducing environment within the cell was indeed responsible for its inhibition of cell death (Bustamante et al., 1995). This corroborates with another report where LA inhibited oxidative stress and retinal capillary cell apoptosis induced by advanced glycation end products (Kowlur, 2005).