

**Reactive Oxygen Species Mediate Caspase Activation and Apoptosis
Induced by Lipoic Acid in Human Lung Epithelial Cancer Cells
through Bcl-2 Downregulation**

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Abbreviations: LA, α -lipoic acid; DHLA, dihydrolipoic acid; ROS, reactive oxygen species; H₂O₂, hydrogen peroxide; O₂^{•-}, superoxide anion; OH[•], hydroxyl radical; GPx, glutathione peroxidase; SOD, superoxide dismutase; MnTBAP, Mn(III)tetrakis(4-benzoic acid) porphyrin chloride; DCF-DA, dichlorofluorescein diacetate; DHE, dihydroethidium bromide; NAC, *N*-acetyl cysteine; LAC, lactacystin; ROT, rotenone; DPI, diphenylene iodonium.

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ABSTRACT

The antioxidant α -lipoic acid (LA) is a naturally-occurring compound that has been shown to possess promising anticancer activity due to its ability to preferentially induce apoptosis and inhibit proliferation of cancer cells relative to normal cells. However, the molecular mechanisms underlying the apoptotic effect of LA are not well understood. We report here that LA induced reactive oxygen species (ROS) generation and a concomitant increase in apoptosis of human lung epithelial cancer H460 cells. Inhibition of ROS generation by ROS scavengers or by overexpression of antioxidant enzymes glutathione peroxidase (GPx) and superoxide dismutase (SOD) effectively inhibited LA-induced apoptosis, indicating the role of ROS, especially hydroperoxide and superoxide anion, in the apoptotic process. Apoptosis induced by LA was found to be mediated through the mitochondrial death pathway which requires caspase-9 activation. Inhibition of caspase activity by pan-caspase inhibitor (z-VAD-fmk) or caspase-9-specific inhibitor (z-LEHD-fmk) completely inhibited the apoptotic effect of LA. Likewise, the mitochondrial respiratory chain inhibitor rotenone potently inhibited the apoptotic and ROS inducing effects of LA, supporting the role of mitochondrial ROS in LA-induced cell death. LA induced downregulation of mitochondrial Bcl-2 protein through peroxide-dependent proteasomal degradation and overexpression of the Bcl-2 protein prevented the apoptotic effect of LA. Together, our findings indicate a novel pro-oxidant role of LA in apoptosis induction and its regulation by Bcl-2, which may be exploited for the treatment of cancer and related apoptosis disorders.

INTRODUCTION

α -Lipoic acid (LA) is a naturally-occurring essential co-enzyme in mitochondrial multi-enzyme complexes catalyzing the oxidative decarboxylation of α -keto acids such as pyruvate, α -ketoglutarate, and branched-chain α -keto acid (Packer et al., 1995; Bilaska and Wlodex, 2005). LA has been shown to combat oxidative stress by quenching a variety of intracellular reactive oxygen species (ROS) (Suzuki et al., 1991; Bilaska and Wlodex, 2005). In addition to ROS scavenging, LA has also been shown to be involved in the recycling of other cellular antioxidants including vitamins C and E, and glutathione (Biewenga et al., 1997). LA has been demonstrated to be effective in preventing pathology in various experimental models in which ROS have been implicated, such as ischemia-reperfusion injury (Coombes et al., 2000), diabetes (Kocak and Karasu, 2002; Da Ros et al., 2005), diabetic neuropathy (Vincent et al., 2005), neurodegeneration (Pirlich et al., 2002), hypertension (de Champlain et al., 2004; Vasdev et al., 2005), radiation injury (Demir et al., 2005), and HIV activation (Patrick, 2000). On the other hand, LA has been reported to possess pro-oxidant activities (Dicter et al., 2002; Gorolska et al., 2003; Cakatay et al., 2005). For examples, LA dose dependently increases intramuscular ROS production and stimulates glucose uptake into adipocytes by increasing intracellular oxidant levels (Dicter et al., 2002). In cancer cells, ROS also play a crucial role in cell growth and apoptosis regulation. LA and its reduced form dihydrolipoic acid (DHLA) have been shown to inhibit proliferation and induce apoptosis of several cancer and transformed cell lines, while being less active toward normal non-transformed cells (Sen et al., 1999; Pack et al., 2001; Mark et al., 2003; Wenzel et al., 2005). However, it is unclear whether LA induces apoptosis

through pre-existing apoptosis machinery or through the induction of a signaling molecule. Furthermore, the role of ROS and the identity of specific ROS involved in the apoptotic process remain to be established.

The role of ROS as intermediates for apoptosis signaling is well supported by numerous investigations, including 1) antioxidants inhibit apoptosis induced by a variety of apoptotic agents (Chan et al., 2005; Izeradjene et al., 2005; Rayner et al., 2006), 2) ROS is elevated in cells undergoing apoptosis (Alexandre et al., 2006), 3) exogenous ROS can induce apoptosis in various cell types (Conde de la Rosa et al., 2005; Rayner et al., 2006), and 4) inhibition of intracellular antioxidant levels promotes apoptotic cell death (Pirlich et al., 2002). Because LA has been reported to act both as a pro- and anti-oxidant, its role in apoptosis and mechanisms of action are unclear. In transformed human colon cancer HT29 cells, LA was shown to increase mitochondrial respiration and superoxide anion ($O_2^{\bullet-}$) generation with a concomitant increase in apoptosis, the effects that were not observed in normal non-transformed colonocytes (Wenzel et al., 2005). Likewise, LA was shown to induce apoptosis of several tumor cell lines including Jurkat, FaDu, and Ki-v-Ras-transformed mesenchymal cells with minimal effect on non-transformed cell lines (Mark et al., 2003). LA also potentiated Fas-mediated apoptosis of human leukemic T-cells through redox regulation without having an effect on peripheral blood monocytes from healthy humans (Sen et al., 1999). These studies support the potential utility of LA as an anticancer agent and the role of ROS in cancer cell death by LA. Because of its therapeutic potential and the widespread use of LA as a nutritional supplement, we sought to determine its apoptosis activity and the underlying mechanisms of action.

The specific questions addressed in this study are 1) whether LA-induced apoptosis is mediated through an ROS-dependent mechanism, 2) if so, what specific ROS are involved in this process and what are their cellular sources, 3) what specific pathway(s) of apoptosis is activated by LA and whether this process is caspase-dependent. Using a variety of biochemical and gene manipulation approaches, we found that ROS play a critical role in LA-induced apoptosis of human lung epithelial cancer H460 cells. This process is mediated through the mitochondrial death pathway via caspase-9 activation, which is negatively regulated by the anti-apoptotic Bcl-2 protein. Downregulation of Bcl-2 protein by mitochondrial ROS, especially hydroperoxide, is a key regulatory event controlling LA-induced cell death. This finding on ROS-mediated Bcl-2 downregulation and caspase activation provides new insights into the pro-oxidant role of LA and its mechanisms of apoptotic cell death that may be exploited in cancer treatment.

MATERIALS AND METHODS

Reagents and Antibodies

All chemicals employed in this study were analytical grade. α -Lipoic acid (LA), dihydrolipoic acid (DHLA), *N*-acetyl cysteine (NAC), rotenone (ROT), diphenylene iodonium (DPI), sodium formate (NaF), and lactacystin (LAC), were obtained from Sigma Chemical Inc. (St. Louis, MO). Cell permeable SOD mimetic, Mn(III)tetrakis (4-benzoic acid) porphyrin (MnTBAP), was purchased from Calbiochem (La Jolla, CA) and catalase was from Boehringer Mannheim (Indianapolis, IN). The oxidative probes

dichlorofluorescein diacetate (DCF-DA) and dihydroethidium bromide (DHE), and the apoptosis dye Hoechst 33342 were from Molecular Probes (Eugene, OR). Caspase-8/FLICE fluorometric substrate (IETD-AFC), caspase-9/Mch6 fluorometric substrate (LEHD-AFC), caspase-8 inhibitor (z-IETD-fmk), caspase-9 inhibitor (z-LEHD-fmk), and pan-caspase inhibitor (z-VAD-fmk) were from Alexis Biochemicals (San Diego, CA). The transfecting agent Lipofectamine was from Invitrogen (Carlsbad, CA). Monoclonal antibody against Bcl-2 was from Santa Cruz Biotechnology (Santa Cruz, CA) and peroxidase-conjugated secondary antibodies and monoclonal β -actin antibody were from Sigma Chemical Inc. (St. Louis, MO).

Cell Culture

The human lung epithelial cell line NCI-H460 was obtained from the American Type Culture Collection (Rockville, MD). The cells were cultured in RPMI-1640 medium (Invitrogen, Carlsbad, CA) supplemented with 5% fetal bovine serum (FBS) and 2 mM L-glutamine, and 100 units/ml penicillin/ streptomycin. Cell cultures were maintained in a humidified atmosphere of 5% CO₂ at 37°C. Cells were passaged at confluent densities by the use of a solution containing 0.05% trypsin and 0.5 mM EDTA (Invitrogen, Carlsbad, CA).

Caspase Activity and Apoptosis Assays

Caspase activity was determined by fluorometric assay using the enzyme substrate IETD-AMC for caspase-8 and LEHD-AMC for caspase 9, which are specifically cleaved by the respective enzymes at the Asp residue to release the fluorescent leaving group, amino-4-

methyl coumarin (AMC). Cell extracts containing 20 μg protein were incubated with 100 mM 4-(2-hydroxyethyl)-1-piperazine ethanesulfonic acid (HEPES) containing 10% sucrose, 10 mM dithiothreitol, 0.1% 3-[(3-chloamidiopropyl)-1] propane sulfonate, and 50 μM caspase substrate in a total reaction volume of 0.25 ml. The reaction mixture was incubated for 2 h at 37°C. At the end of incubation, the liberated fluorescent group AMC was determined fluorometrically (RF-531PC spectrofluorometer, Shimadzu, Japan) at the excitation and emission wavelengths of 380 nm and 460 nm, respectively. Apoptosis was determined by incubating the cells with 10 $\mu\text{g}/\text{ml}$ Hoechst 33342 for 30 min at 37°C and scoring the percentage of cells having intensely condensed chromatin and/or fragmented nuclei by fluorescence microscopy (Carl Zeiss Axiovert, Göttingen, Germany) using a Pixera software (n = 300).

ROS Detection

Intracellular hydroperoxide and superoxide anion production were determined by flow cytometry using dichlorofluorescein diacetate (DCF-DA) and dihydroethidium bromide (DHE) as fluorescent probes. Cells ($1 \times 10^6/\text{ml}$) were incubated with the probes (10 μM) for 30 min at 37°C, after which they were washed, resuspended in phosphate buffered saline (PBS), and analyzed for fluorescence intensity using FACS Caliber (Becton-Dickinson, Rutherford, NJ) at the excitation and emission wavelengths of 488 nm and 538 nm respectively for DCF fluorescence measurements, and at 488 nm and 610 nm for DHE measurements. The median fluorescence intensity was quantitated by CellQuest software (Becton-Dickinson) analysis of the recorded histograms.

Plasmids and Stable Transfection

The Bcl-2, GPx, and SOD1 plasmids were generously provided by Dr. Christian Stehlik (West Virginia University Cancer Center, Morgantown, WV). Authenticity of all plasmid constructs was verified by DNA sequencing. Stable transfectants of Bcl-2, GPx, and SOD were generated by culturing H460 cells in a 6-well plate until they reached 80% confluence. One microgram of cytomegalovirus-neo vector and 15 μ l of Lipofectamine reagent with 2 μ g of Bcl-2, GPx, SOD, or control pcDNA3 plasmid were used to transfect the cells in the absence of serum. After 10 h, the medium was replaced with culture medium containing 5% FBS. Approximately 36 h after the beginning of the transfection, the cells were digested with 0.03% trypsin and the cell suspensions were plated onto 75-ml culture flasks and cultured for 24-28 d with G418 selection (400 μ g/ml). Stable transfectants were identified by Western blot analysis and were cultured in G418-free RPMI medium for at least two passages before each experiment.

Western Blotting

Cell extracts are performed by incubating the cells in lysis buffer containing 20 mM Tris-HCl (pH 7.5), 1% Triton X-100, 150 mM sodium chloride, 10% glycerol, 1 mM sodium orthovanadate, 50 mM sodium fluoride, 100 mM phenylmethylsulfonyl fluoride, and a commercial protease inhibitor mixture (Roche Molecular Biochemicals) for 30 min on ice. After insoluble debris was pelleted by centrifugation at 14,000g for 15 min at 4°C, the supernatants were collected and assayed for protein content using a bicinchoninic acid assay kit (Pierce Biotechnology, Rockford, IL). Equal amount of proteins per sample (20 μ g) were resolved on a 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-

PAGE) and transferred onto 0.45- μ m nitrocellulose membrane (Pierce). The transferred membranes were blocked for 1 h in 5% non-fat dry milk in TBST (25 mM Tris-HCl pH 7.4, 125 mM NaCl, 0.05% Tween-20) and incubated with appropriate primary antibodies at 4°C overnight. Membranes were washed three times with TBST for 10 min, incubated with peroxidase-labeled isotype-specific secondary antibodies for 1 h at room temperature. The immune complexes were detected by chemiluminescence (Supersignal[®] West Pico, Pierce) and quantified by imaging densitometry using analyst/PC densitometry software (Bio-Rad, Richmond, CA). Mean densitometry data from independent experiments were normalized to result in cells in the control. The data were presented as the mean \pm S.D. and analyzed by the Student's *t* test.

RESULTS

LA and DHLA Induce Apoptosis and Caspase Activation in H460 Cells

LA and its reduced form dihydrolipoic acid (DHLA) have been reported to induce apoptosis in some cancer and transformed cell lines (Pack et al., 2001; Mark et al., 2003; Wenzel et al., 2005); however their mechanisms of apoptosis induction are unclear. To determine the apoptosis mechanisms and to test whether these compounds can also induce apoptosis in human lung cancer epithelial cells, we first characterized the apoptosis response to LA and DHLA treatment in H460 cells. The cells were treated with various concentrations of LA or DHLA (0-100 μ M), and apoptosis was determined by Hoechst nuclear fragmentation and caspase activity assays. Figs. 1A and 1B show that both LA and DHLA significantly induced cell apoptosis over control level with the effect of the former being more

pronounced. The apoptotic cells exhibited shrunken nuclei, DNA fragmentation, and intense nuclear fluorescence. Caspase activity assays show that LA, and to a lesser extent DHLA, induced caspase-9 activation but had no significant effect on caspase-8 activity (Fig. 1C). Since caspase-9 serves as the apical caspase of the intrinsic (mitochondrial) pathway of apoptosis while caspase-8 represents the apical caspase of the extrinsic (death receptor) pathway (Salvesen and Dixit, 1997; Green and Reed, 1998; Wallach et al., 1999), the results suggest that the mitochondrial death pathway is the major pathway of apoptosis induction by LA and DHLA. Control experiments using pan-caspase inhibitor (z-VAD-fmk) and caspase-9-specific inhibitor (z-LEHD-fmk) showed complete inhibition of the apoptotic effect of LA and DHLA by the inhibitors (Fig. 1D), supporting the role of caspases and in particular caspase-9 in the apoptotic process.

Effect of Antioxidants on LA- and DHLA-Induced Apoptosis

To test whether the apoptosis-inducing effect of LA and DHLA is associated with their pro-oxidant activity, cells were treated with LA or DHLA in the presence or absence of various known antioxidants, including NAC (general antioxidant), catalase (H_2O_2 scavenger), MnTBAP ($\text{O}_2^{\bullet-}$ scavenger), sodium formate (OH^{\bullet} scavenger), and apoptosis was determined by Hoechst and caspase activation assays. The results show that all tested antioxidants were able to inhibit apoptosis induced by LA or DHLA (Fig. 2A), indicating that multiple ROS are involved in the apoptotic process. The potent inhibitory effects of catalase and MnTBAP further indicate that H_2O_2 and $\text{O}_2^{\bullet-}$ play an important role in the process. Subsequent studies using GPx and SOD overexpressed cells confirm this finding. Similar results were obtained for the inhibition of caspase-9 activation by these antioxidants (data

not shown). These results also suggest that LA and DHLA induce apoptosis through a similar ROS-dependent mechanism.

LA and DHLA Induce ROS Generation

To provide a relationship between the apoptotic response and ROS generation under the test conditions, flow cytometric analysis of cellular ROS was performed in treated cells using the fluorescent probe DCF-DA and DHE, which detect hydroperoxide and $O_2^{\bullet-}$, respectively. Fig. 2B shows that LA and DHLA were able to induce intracellular peroxide production in a dose-dependent manner, as indicated by the increase in DCF fluorescence intensity. The effect of LA was more pronounced than DHLA and was inhibited by the addition of catalase, indicating the specificity of peroxide detection in the test system. Likewise, the general antioxidant NAC strongly inhibited the fluorescence signal, whereas the SOD mimetic MnTBAP showed minimal effect. DHE analysis of treated cells also indicates the formation of $O_2^{\bullet-}$ by LA and DHLA treatment. The DHE signal was inhibited by the addition of MnTBAP and NAC (Fig. 2C). Interestingly, catalase also inhibited this signal, suggesting that LA-induced $O_2^{\bullet-}$ generation may involve peroxide formation, possibly through the reaction of peroxide with cellular trace metals such as Fe(III), i.e., $H_2O_2 + Fe(III) \rightarrow Fe^{2+} + 2H^+ + O_2^{\bullet-}$ (Halliwell and Gutteridge, 1999) or through the reaction of peroxide with thiols to form $O_2^{\bullet-}$ via peroxidase-catalyzed reaction (Harman et al., 1986). Alternatively, DHE may detect some form of peroxides that can be blocked by catalase.

Mitochondrial ROS are Responsible for LA-Induced Apoptosis

To determine the cellular source of ROS induced by LA, cells were treated with LA in the presence or absence of diphenylene iodonium (DPI), a specific inhibitor of NADPH oxidase (Freeman B and Crapo, 1982; Irani et al., 1997), or rotenone, a mitochondrial electron transport chain interrupter (Irani et al., 1997; Chen et al., 2003), and their effects on apoptosis and ROS generation were examined. The results show that rotenone strongly inhibited LA-induced apoptosis and ROS generation, whereas DPI had weak effects (Figs. 3A-3C). These results indicate that mitochondria are the primary source of ROS production induced by LA and that these ROS are involved in the apoptotic process. The differential inhibitory effect of rotenone on superoxide and peroxide generation could reflect the non-specific effect of this compound on ROS and the varying rates of reaction between the ROS, their probes, and the compound. The role of specific ROS in LA-induced apoptosis could be more conclusively addressed using specific ROS inhibitors or antioxidant enzymes, as earlier demonstrated.

GPx and SOD Overexpression Inhibits LA-Induced Apoptosis and ROS Generation

To confirm the role of ROS in LA-induced apoptosis, cells were stably transfected with the antioxidant enzyme GPx, SOD, or control plasmid, and their effects on enzyme expression, ROS generation, and apoptosis were determined. Western blot analysis of the transfected cells showed an increase in antioxidant enzymes in the corresponding transfected cells as compared to mock transfected control (Fig. 4A). Apoptosis assays showed a decrease in apoptotic response to LA treatment in GPx- and SOD-transfected cells but not in mock-transfected cells (Fig. 4B). Flow cytometric analysis of ROS generation by DCF fluorescence also showed a substantial reduction in LA-induced fluorescence in GPx-

transfected cells, as compared to mock and SOD-transfected cells (Fig. 4C). DHE fluorescence measurements showed strong inhibition of LA-induced fluorescence in SOD and GPx-transfected cells relative to mock-transfected cells (Fig. 4D), the results that are consistent with our earlier MnTBAP and catalase data.

Bcl-2 Overexpression Protects Cells from LA-Induced Apoptosis

LA-induced apoptosis was earlier shown to be mediated through the mitochondrial caspase-9 activation pathway (Fig. 1). Since this pathway is known to be regulated by the anti-apoptotic Bcl-2 protein (Green and Reed, 1998), we evaluated whether this protein is involved in the regulation of apoptosis by LA. Cells were stably transfected with Bcl-2 or control plasmid, and the effect on LA-induced apoptosis was determined. Western blot analysis of the transfected cells showed an increase in Bcl-2 expression in Bcl-2-transfected cells compared to mock-transfected control (Fig. 5A). Apoptosis assays showed a strong inhibition of LA-induced apoptosis by the Bcl-2 overexpression at various treatment concentrations of LA (Fig. 5B). These results indicate the role of Bcl-2 as a negative regulator of LA-induced apoptosis and further support the role of mitochondria in the death signaling process.

Effect of LA Treatment on Bcl-2 Expression

To provide a mechanistic insight into the regulation of LA-induced apoptosis by Bcl-2, the expression level of Bcl-2 in response to LA treatment was determined by Western blot analysis. Figs. 6A and 6B show that treatment of the cells with LA caused a dose- and time-dependent decrease in Bcl-2 expression levels. Since previous studies have shown that Bcl-

2 is downregulated primarily through proteasomal degradation pathway (Breitschopf et al., 2000; Sharma et al., 2005; Chanvorachote et al., 2006), we tested whether such degradation is involved in the downregulation of Bcl-2 by LA. Cells were treated with LA in the presence or absence of lactacystin, a highly specific proteasome inhibitor, and its effect on Bcl-2 expression was determined. Fig. 6C shows that lactacystin completely inhibited Bcl-2 downregulation induced by LA. This result was confirmed by the observation that another proteasome inhibitor, MG132, also inhibited LA-induced Bcl-2 downregulation (data not shown). This finding indicates that proteasomal degradation is a key mechanism of LA-induced Bcl-2 downregulation.

Effect of Antioxidants on Bcl-2 Expression

Having demonstrated the role of Bcl-2 in LA-induced apoptosis, we next investigated the potential regulation of Bcl-2 by ROS. ROS have previously been shown to mediate TNF- α -induced proteasomal degradation of Bcl-2 in human umbilical vein endothelial cells (Breitschopf et al., 2000). To test whether ROS might also mediate the effect of LA on Bcl-2, cells were treated with LA in the presence or absence of antioxidants and their effect on Bcl-2 expression was determined. Fig. 7A shows that treatment of the cells with NAC or catalase completely inhibited LA-induced Bcl-2 downregulation, whereas MnTBAP treatment showed no inhibitory effect. These results were confirmed in GPx- and SOD-overexpressed cells. Fig. 7B shows that GPx overexpression completely inhibited LA-induced Bcl-2 downregulation, whereas SOD overexpression was ineffective. These results indicate that hydroperoxide is the primary ROS responsible for LA-induced Bcl-2 downregulation.

DISCUSSION

The present study demonstrated the role of ROS in LA-induced apoptosis of human lung epithelial H460 cells. LA has been shown to act as a pro-oxidant (Dicter et al., 2002; Gorolska et al., 2003; Cakatay et al., 2005) as well as anti-oxidant (Suzuki et al., 1991; Bilska and Wlodex, 2005), depending on cell type and cellular oxidative status. The anti-oxidant role of LA is commonly associated with cells under oxidative stress and this action of LA has been attributed to its ability to regenerate other cellular antioxidants such as vitamin C and E, and glutathione (4). The pro-oxidant role of LA is generally observed under non-oxidative stress conditions, which is also supported by this study. In human colon cancer HT29 cells, LA was shown to act a pro-oxidant by increasing mitochondrial $O_2^{\bullet-}$ generation (Wenzel et al., 2005). The colon cancer cells were also shown to possess a lower antioxidant status and are more susceptible to LA-induced apoptosis as compared to normal non-transformed cells, thus providing a basis for the selective effect of LA on cancer cells.

Consistent with the previous finding, our results showed that LA was able to induce $O_2^{\bullet-}$ generation in human lung epithelial H460 cells (Fig. 2C). We further showed that LA also induced peroxide generation in these cells (Fig. 2B). Similar ROS-inducing effects were observed with DHLA. Inhibition of ROS generation by the antioxidant NAC, catalase, and MnTBAP effectively inhibited apoptosis induced by LA and DHLA, indicating the role of ROS, especially H_2O_2 and $O_2^{\bullet-}$, in this process. The results also suggest a similar ROS-dependent apoptogenic mechanism of LA and DHLA. The role of H_2O_2 and $O_2^{\bullet-}$ was

confirmed in cells overexpressing GPx and SOD, both of which showed strongly reduced apoptotic responses to LA treatment (Fig. 4B).

We also studied the mechanism by which ROS mediate the apoptotic effect of LA and DHLA. LA and related short-chain fatty acids have been suggested to induce apoptosis through Fas death signaling pathway (Sen et al., 1999; Hara et al., 2000). Fas is a cell surface receptor which upon activation by Fas ligand or agonistic Fas antibody induces apoptosis via caspase-8 activation (Lenardo et al., 1999; Nagata, 1999). However, our results show that LA and DHLA were unable to induce caspase-8 activation in H460 cells (Fig. 1C). We previously showed that H460 cells are susceptible to Fas-mediated apoptosis through caspase-8 activation (Chanvorachote et al., 2005). Therefore, the inability of LA and DHLA to activate caspase-8 suggests that their apoptotic effect is not mediated through the Fas signaling pathway. These results are consistent with a previous report showing that Fas-deficient Jurkat cells retained their sensitivity to LA-induced apoptosis (Mark et al., 2003). We also showed that LA and DHLA induce apoptosis through the mitochondrial death pathway (Fig. 1C). This induction of apoptosis involves mitochondrion-dependent activation of caspase-9, which can be inhibited by caspase-9-specific inhibitor (z-LEHD-fmk) or pan-caspase inhibitor (z-VAD-fmk) (Fig. 1D). LA- and DHLA-induced caspase-9 activation was also found to be inhibited by the ROS scavenger NAC (data not shown). These results are consistent with previous reports showing the presence of procaspase-9 in the mitochondria which is released and subsequently activated in the cytoplasm during apoptosis (Salvesen and Dixit, 1997; Green and Reed, 1998).

The generation of ROS induced by LA occurs primarily in the mitochondria since the mitochondrial respiratory chain inhibitor rotenone effectively inhibited this generation (Fig. 3). Rotenone has previously been shown to inhibit mitochondrial ROS in other cell systems (Irani et al., 1997; Chen et al., 2003). DPI, a known inhibitor of cellular NADPH oxidase (Freeman and Crapo, 1982; Irani et al., 1997), showed a weak effect on LA-induced ROS generation, indicating the less important role of this enzyme system in the ROS production.

Bcl-2 is a key regulator of the mitochondrial pathway that prevents apoptosis by preserving mitochondrial permeability transition (Fiers et al., 1999). We showed that overexpression of Bcl-2 strongly inhibited LA-induced apoptosis (Fig. 5), further supporting the role of mitochondria in LA-induced cell death. Exposure of the cells to LA caused a dose- and time-dependent downregulation of Bcl-2 (Figs. 6A & 6B). Such downregulation is mediated by the proteasomal pathway since inhibition of this pathway by the proteasome inhibitor lactacystin completely inhibited the effect of LA on Bcl-2 (Fig. 6C). We also showed that this downregulation is ROS-dependent since co-treatment of the cells with antioxidant NAC or catalase completely inhibited the downregulation effect of LA on Bcl-2 (Fig. 7A). The $O_2^{\cdot-}$ scavenger MnTBAP failed to inhibit this effect, indicating that peroxide, but not $O_2^{\cdot-}$, is the primary ROS responsible for LA-induced Bcl-2 downregulation. Gene transfection studies using GPx and SOD overexpressed cells confirmed these results and indicate the critical role of peroxide in this process (Fig. 7B). The observation that MnTBAP and SOD failed to inhibit Bcl-2 downregulation while exhibiting a protective effect on LA-induced apoptosis suggests that other $O_2^{\cdot-}$ -mediated Bcl-2-independent mechanisms may be involved in the apoptotic effect of LA.

Signal transduction leading to apoptotic cell death has been of great interest in biomedical and pharmaceutical research mainly because successful apoptotic agents could be used to treat cancer. The therapeutic potential of LA in cancer treatment has been demonstrated in several studies (Sen et al., 1999; Pack et al., 2001; Mark et al., 2003; Wenzel et al., 2005). Previously, chemotherapeutic agents such as doxorubicin, cisplatin, vincristine, and the alkaloid taxol have commonly been used as anti-tumor agents. However, at high concentrations these drugs are toxic to cells and cause adverse side-effects. In contrast, LA is an endogenous agent that has been widely used as a dietary supplement. It is known to increase cellular glutathione levels, regulate cellular redox balance and help protect against diabetic complications (Sen et al., 1997; Da Ros et al., 2005; Kocak and Karasu, 2002). Our finding on the apoptotic effect of LA in human lung cancer cells supports its potential utility as an agent for the treatment of lung cancer.

In summary, our data provide evidence that ROS play an important role in LA-induced apoptosis in human lung epithelial H460 cells. The mechanism by which LA induces apoptosis involves the following steps. LA induces a rapid generation of ROS which is required for subsequent activation of apoptosis by LA, since the inhibition of these ROS by antioxidants prevented the apoptotic effect. ROS generation by LA occurs mainly in the mitochondria since this production is inhibited by the mitochondrial respiratory chain inhibitor rotenone. Apoptosis induced by LA is mediated through the mitochondrion-dependent caspase-9 activation pathway which is negatively regulated by the anti-apoptotic Bcl-2 protein. LA induces downregulation of Bcl-2 through a process that

involves H₂O₂-mediated proteasomal degradation. H₂O₂ may represent a common regulator of Bcl-2 function that controls apoptotic cell death induced by various physiologic and pathologic stimuli. In demonstrating the role of this oxidative species in LA-induced cell death, we document a novel mechanism of apoptosis induction by LA, which may be exploited in the treatment of cancer and related apoptosis disorders.

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FOOTNOTES

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LEGENDS FOR FIGURES

Fig 1. LA and DHLA induce apoptosis and caspase activation in human lung epithelial H460 cells. A, Subconfluent (90%) monolayers of H460 cells were treated with varying concentrations of LA or DHLA (0-100 μ M) for 24 h and the cells were analyzed for apoptosis by Hoechst 33342 assay. B, Fluorescence micrographs of treated cells stained with the Hoechst dye. Apoptotic cells exhibited shrunken and fragmented nuclei with bright nuclear fluorescence. (Original magnification, x400) C, Fluorometric assays of caspase activity in control, LA- and DHLA-treated cells (100 μ M). Cell lysates (50 μ g protein) were prepared and analyzed for caspase-8 and -9 activity using the fluorometric substrate IETD-AMC and LEHD-AMC, respectively. D, Cells were similarly treated with LA or DHLA in the presence or absence of caspase-9 inhibitor z-LEHD-fmk (10 μ M) or pan-caspase inhibitor z-VAD-fmk (10 μ M), and caspase activity was determined. Data are mean \pm S.D. ($n = 4$). * $p < 0.05$ versus non-treated control. # $p < 0.05$ versus treated control.

Fig. 2. Effects of antioxidants on LA- and DHLA-induced apoptosis and ROS generation. A, Subconfluent (90%) monolayers of H460 cells were either left untreated or pretreated with NAC (100 μ M), catalase (1,000 U/ml), MnTBAP (100 μ M), or sodium formate (10 mM) for 1 h. The cells were then treated with LA or DHLA (100 μ M) for 24 h and analyzed for apoptosis by Hoechst assay. B and C, Flow cytometric analysis of ROS production in H460 cells. Cells were treated with varying doses of LA or DHLA (0-100 μ M) and DCF and DHE fluorescence intensities were determined as described under

Materials and Methods. Plots show relative fluorescence intensity over non-treated control at the peak response time of 1 h after treatment. In antioxidant treatment experiments, cells were pretreated for 1 h with NAC (100 μ M), catalase (1,000 U/ml), or MnTBAP (100 μ M), and then treated with LA (100 μ M) for another hour. Values are mean \pm S.D. ($n \geq 3$). * $p < 0.05$ versus non-treated control, # $p < 0.05$ versus treated control.

Fig. 3. Effects of diphenylene iodonium and rotenone on LA-induced apoptosis and ROS generation. A, H460 cells were either left untreated or pretreated with the indicated concentrations of DPI or rotenone for 1 h. The cells were then treated with LA (100 μ M) and analyzed for apoptosis after 24 h. B and C, Flow cytometric measurements of DHE and DCF fluorescence intensities. Cells were pretreated for 1 h with DPI (1 μ M) or rotenone (1 μ M), followed by LA treatment (100 μ M) for another 1 h. Plots show relative fluorescence intensity over non-treated control. Values are mean \pm S.D. ($n \geq 3$). * $p < 0.05$ versus non-treated control, # $p < 0.05$ versus LA-treated control.

Fig. 4. Effects of GPx and SOD overexpression on LA-induced apoptosis and ROS generation. A, H460 cells were stably transfected with GPx, SOD, or control pcDNA3 plasmid as described under *Materials and Methods*. Cell extracts were prepared and separated on 10% polyacrylamide-SDS gels, transferred, and probed with GPx or SOD antibody. β -actin was used as a loading control. B, Transfected cells were treated with LA (100 μ M) for 24 h and analyzed for apoptosis by Hoechst 33342 assay. C and D, Transfected cells were treated with LA (100 μ M) and analyzed for DCF and DHE

fluorescence intensities at 1 h post-treatment. Plots show relative fluorescence intensity over non-treated control. Values are mean \pm S.D. ($n \geq 3$). * $p < 0.05$ versus non-treated control, # $p < 0.05$ versus LA-treated control.

Fig. 5. Bcl-2 overexpression inhibits LA-induced apoptosis. A, H460 cells were stably transfected with Bcl-2 or control pcDNA3 plasmid as described under *Materials and Methods*. Cell extracts were prepared and separated on a 10% polyacrylamide-SDS gel, transferred, and probed with Bcl-2 antibody. Blots were reprobed with β -actin antibody to confirm equal loading of samples. B, Transfected cells were treated with varying doses of LA (0-100 μ M) for 24 h and apoptosis was determined by Hoechst 33342 assay. Plots are mean \pm S.D. ($n = 4$). * $p < 0.05$ versus mock transfected controls.

Fig. 6. Effect of LA on Bcl-2 expression. A, H460 cells were treated with LA (100 μ M) for various times and analyzed for Bcl-2 expression by Western blots using anti-Bcl-2 antibody. B, Dose effect of LA (0-100 μ M) on Bcl-2 expression determined at 9 h post-treatment. C, Effect of proteasome inhibitor on LA-induced Bcl-2 downregulation. Cells were treated LA (100 μ M) in the presence or absence of lactacystin (LAC) (10 μ M) and Bcl-2 expression was determined. Immunoblot signals were quantified by densitometry and mean data from independent experiments were normalized to the result obtained in cells in the absence of LA (control). Plots are mean \pm S.D. ($n = 4$). * $p < 0.05$ versus non-treated control, # $p < 0.05$ versus LA-treated control.

Fig. 7. Effect of antioxidants on Bcl-2 expression. A, H460 cells were either left untreated or pretreated with NAC (100 μ M), catalase (1,000 U/ml), or MnTBAP (100 μ M) for 1 h, followed by LA treatment (100 μ M) for 9 h. Cell lysates were then prepared and analyzed for Bcl-2 expression by Western blotting. B, GPx, SOD, or mock transfected cells were treated with LA (100 μ M) for 9 h and Bcl-2 expression was similarly determined. Blots were reprobbed with β -actin antibody to confirm equal loading of samples. Densitometry was performed to determine the relative expression of Bcl-2 in treated cells compared to non-treated cells. Plots are mean \pm S.D. ($n = 4$). * $p < 0.05$ versus non-treated control, # $p < 0.05$ versus LA-treated control.

Figure 1

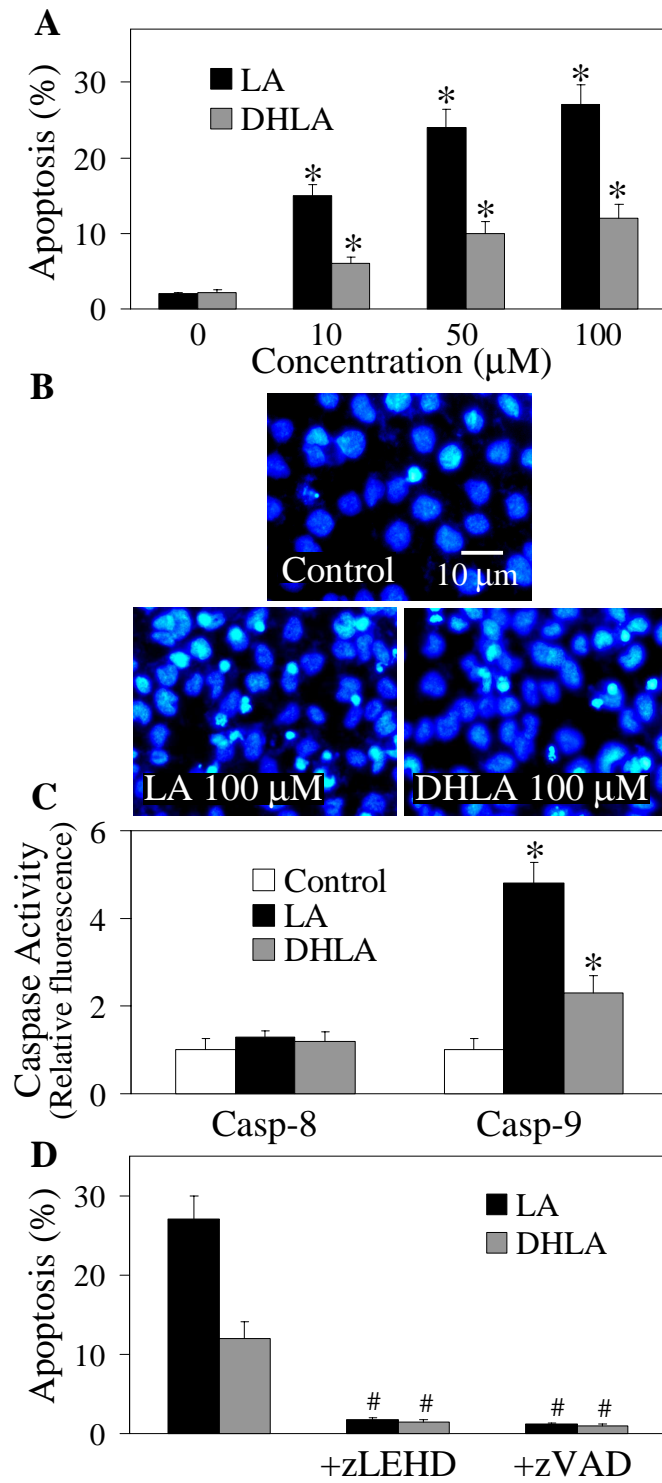


Figure 2

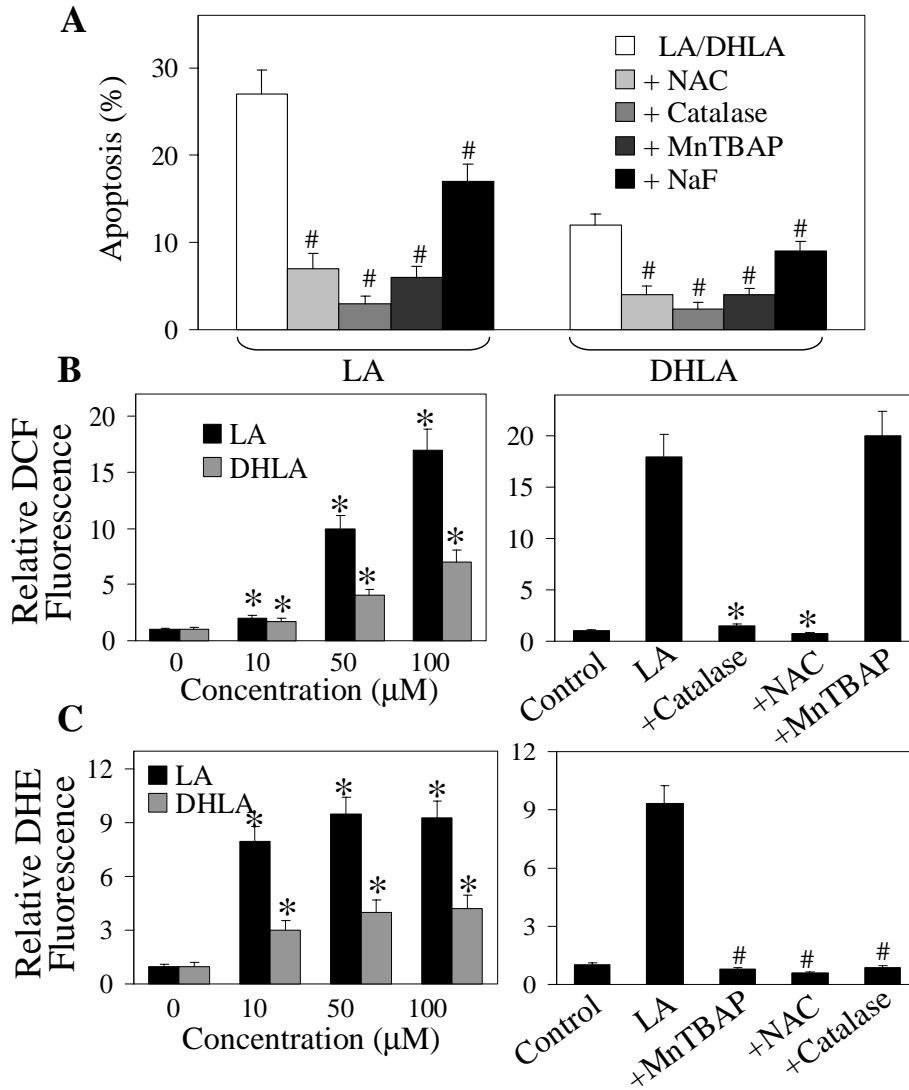


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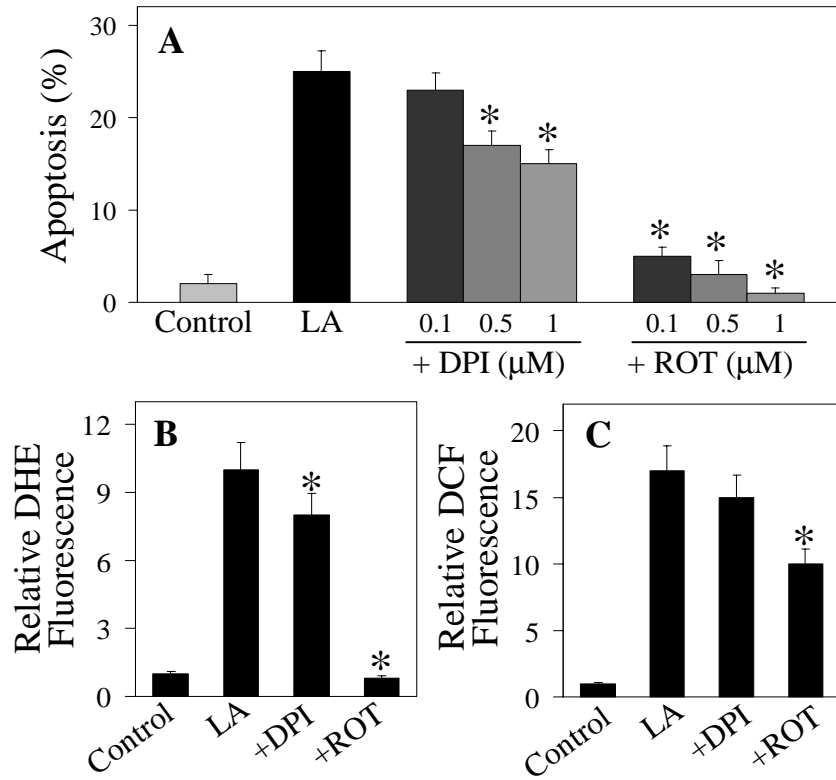


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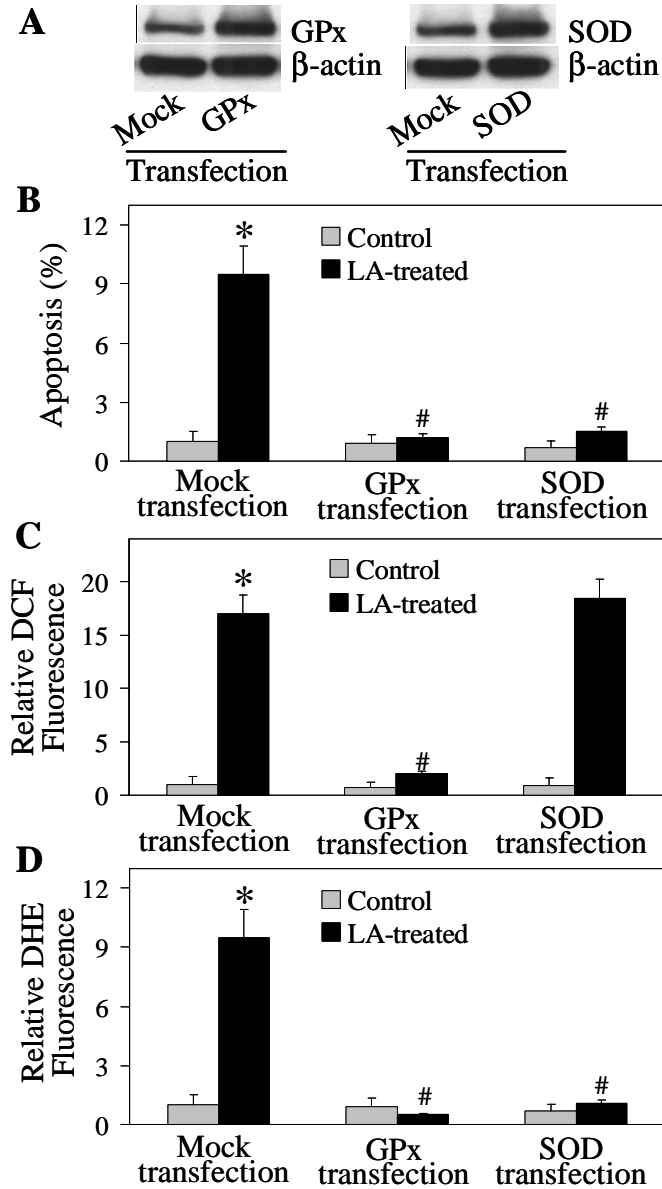


Figure 5

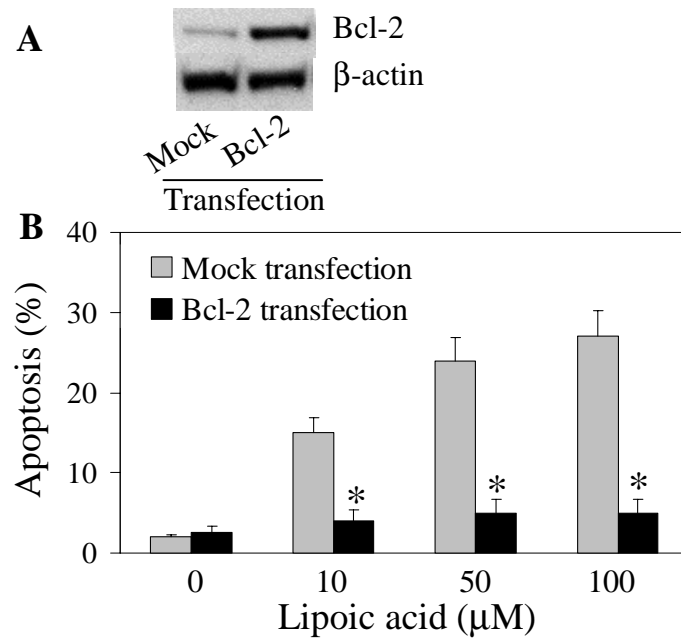


Figure 6

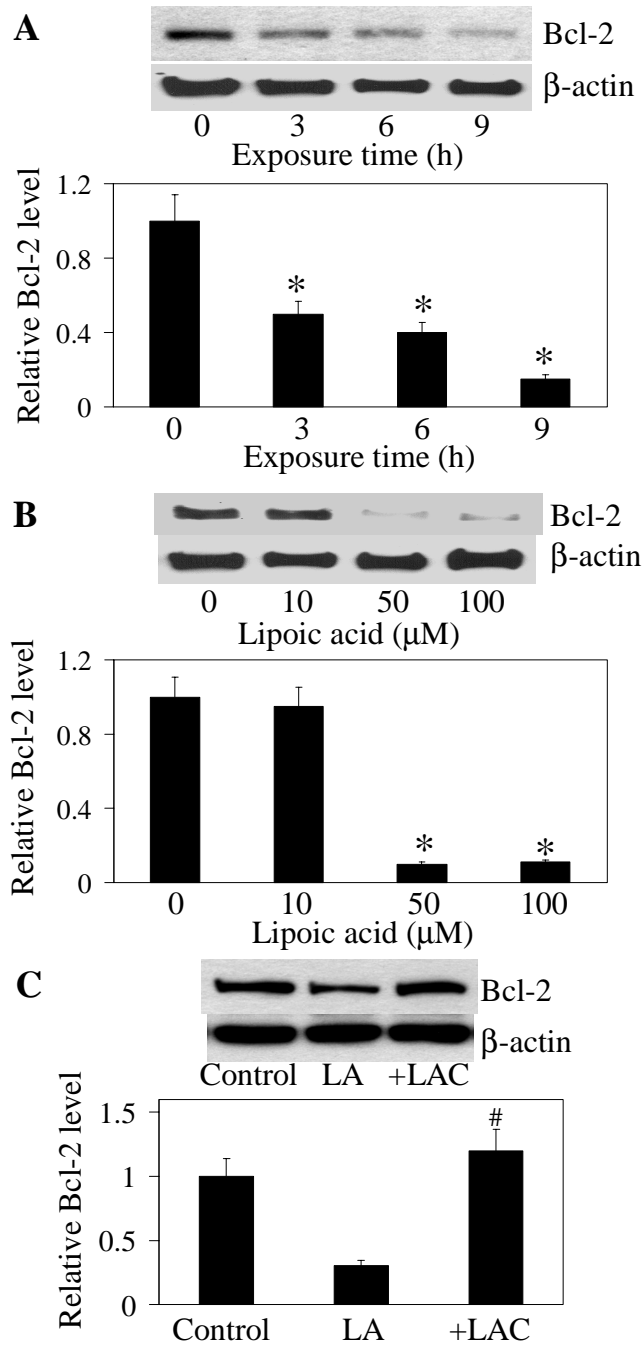


Figure 7

