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Oxalomalate, a competitive inhibitor of NADP$^+$-dependent isocitrate dehydrogenase, regulates lipid peroxidation-mediated apoptosis in U937 cells

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Abstract
Membrane lipid peroxidation processes yield products that may react with DNA and proteins to cause oxidative modifications. Recently, we demonstrated that the control of cytosolic redox balance and the cellular defense against oxidative damage is one of the primary functions of cytosolic NADP$^+$-dependent isocitrate dehydrogenase (IDPc) through to supply NADPH for antioxidant systems. The protective role of IDPc against lipid peroxidation-mediated apoptosis in U937 cells was investigated in control and cells pre-treated with oxlalomalate, a competitive inhibitor of IDPc. Upon exposure to 2,2′-azobis (2-amidinopropane) hydrochloride (AAPH) to U937 cells, which induces lipid peroxidation in membranes, the susceptibility to apoptosis was higher in oxalomalate-treated cells as compared to control cells. The results suggest that IDPc plays an important protective role in apoptosis of U937 cells induced by lipid peroxidation-mediated oxidative stress.

Keywords: *Lipid peroxidation, isocitrate dehydrogenase, oxalomalate, apoptosis, antioxidant enzyme*

Introduction

Numerous diseases, including cancer, cardiovascular disease, and neurodegenerative disease, are associated with oxidative damage of biological macromolecules.[1,2] In biological membranes, lipid peroxidation is frequently a consequence of free radical attack. The peroxidation of unsaturated fatty acids of cells produces many reactive species such as free radicals, hydroperoxides, and carbonyl compounds, which may cause damage to proteins and DNA and inducing alterations in cell proliferation, cell cycle progression, and apoptosis.[3] It has also been assumed that the decomposition of hydroperoxides mediated by catalytic transition metal ions may form much more toxic breakdown products such as alkoxy radicals (RO$^{\cdot}$), peroxy radicals (ROO$^{\cdot}$), hydroxyl radicals ($^{\cdot}$OH), and reactive aldehydes, including malondialdehyde (MDA) and 4-hydroxynonenal (HNE).[4,5] It is possible that in complex biological systems, oxygen free radicals and reactive aldehydes may cause protein and DNA damage and consequently apoptotic cell death indirectly by initiating lipid peroxidation, since polyunsaturated side chains of membrane lipids are especially susceptible to free radical-initiated oxidation.[3,6]

Biological systems have evolved an effective and complicated network of defense mechanisms which enable cells to cope with lethal oxidative environments. These defense mechanisms involve antioxidant enzymes, such as superoxide dismutases (SOD), which catalyze the dismutation of O_2^- to H_2O_2 and O_2,[7] catalase, and peroxidases which remove hydrogen peroxide and hydroperoxides.[8] The isocitrate dehydrogenases (ICDHs; EC1.1.1.41 and EC1.1.1.42) catalyze oxidative decarboxylation of isocitrate to α-ketoglutarate and require either NAD$^+$ or NADP$^+$, producing NADH and NADPH, respectively. NADPH is an essential

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reducing equivalent for the regeneration of reduced glutathione (GSH) by glutathione reductase and for the activity of NADPH-dependent thioredoxin system,[9] both are important in the protection of cells from oxidative damage. Therefore, cytosolic ICDH (IDPc) may play an antioxidant role during oxidative stress.

In the present study the role of IDPc in the lipid peroxidation-mediated apoptosis was investigated using the control and the oxalomalate-treated human premonocytic U937 cells. Oxalomalate, a tricarboxylic acid (α-hydroxy-β-oxalosuccinic acid) formed in *in vitro* and *in vivo* by condensation of oxaloacetate and glyoxylate, has been known to be a potent competitive inhibitor of IDPc.[10] The results revealed that IDPc has an important protective role in lipid peroxidation-mediated apoptosis, presumably, through acting as an antioxidant enzyme.

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Materials and methods

Materials

RPMI 1640, fetal bovine serum (FBS), penicillin–streptomycin were obtained from GIBCO-BRL (Rockville, MD). Oxalomalate, β-$NADP^+$, isocitrate, propidium iodide (PI), 4′. 6-diamidino-2-phenylindole (DAPI), and 2,2′-azobis (2-amidinopropane) hydrochloride (AAPH) were obtained from Sigma Chemical Co. (St Louis, MO). 2′,7′-Dichlorofluoroscin diacetate (DCFH-DA), *t*-butoxycarbonyl-Leu-Met-7-amino-4-chloromethylcoumarin (CMAC), dihydrorhodamine 123 (DHR 123), and diphenyl-1-pyrenylphosphine (DPPP) were purchased from Molecular Probes (Eugene, OR). Antibodies against Bcl-2, Bax, lamin B, cleaved caspase-3, and cleaved poly (ADP-ribose) polymerase (PARP) were purchased from Santa Cruz (Santa Cruz, CA).

Cell culture

Human premonocytic U937 cells (American Type Culture Collection, Rockville, MD) were grown in RPMI 1640 culture medium supplemented with 10% (v/v) FBS, penicillin (50 units/ml), and 50 μg/ml streptomycin at 37°C in a 5% CO_2–95% air humidified incubator.

Enzyme assay

Cells were collected at 10,000*g* for 10 min at 4°C and were washed once with cold PBS. Briefly, cells were homogenized with a Dounce homogenizer in sucrose buffer (0.32 M sucrose, 10 mM Tris–Cl, pH 7.4). Cell homogenates were centrifuged at 1000*g* for 5 min, and the supernatants were further centrifuged at 15,000*g* for 30 min. The resulting supernatants were used as the cytosolic fractions. Protein concentration was determined by the method of Bradford using the reagents purchased from Bio-Rad. The supernatants were added by 1/10 volume of 10X PBS containing 1% Triton-X100, which finally made the solution 1X PBS containing 0.1% Triton-X100. The supernatants were used to measure the activities of several cytosolic enzymes.[11] The activity of ICDH was measured by the production of NADPH at 340 nm. The reaction mixture for ICDH activity contained 50 mM MOPS, pH 7.2, 5 mM threo-DS-isocitrate, 35.5 mM triethanolamine, 2 mM NAD^+, 1 mM ADP, 2 mM $MgCl_2$, and 1 μg/ml rotenone. One unit of ICDH activity is defined as the amount of enzyme catalyzing the production of 1 μmol of NADPH/min. Catalase activity was measured with the decomposition of hydrogen peroxide, which was determined by the decrease in absorbance at 240 nm. SOD activity in cell extracts was assayed spectrophotometrically using a pyrogallol assay, where one unit of activity is defined as the quantity of enzyme which reduces the superoxide-dependent color change by 50%. Glutathione reductase activity was quantified by the GSSG-dependent loss of NADPH as measured at 340 nm. Glucose 6-phosphate dehydrogenase (G6PD) activity was measured by following the rate of $NADP^+$ reduction at 340 nm using the procedure described. Glutathione peroxidase activity in the crude extracts was measured by the standard indirect method based on NADPH oxidation by *t*-BOOH in the presence of excess glutathione and glutathione reductase, as previously described.

Immunoblot analysis

Proteins were separated on 10–12.5% SDS-PAGE, transferred to nitrocellulose membranes, and subsequently subjected to immunoblot analysis using appropriate antibodies. Immunoreactive antigen was then recognized by using horseradish peroxidase-labeled anti-rabbit IgG and an enhanced chemiluminescence detection kit (Amersham Pharmacia Biotech).

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To determine the portion of apoptotic cells, cells were analyzed with PI staining.[12] U937 cells were collected at 2000*g* for 5 min and washed once with cold PBS, fixed in 70% ethanol, decant ethanol by centrifuge and stained with 1 ml of solution containing 50 mg/ml PI, 1 mg/ml RNase A, 1.5% Triton X-100 for at least 1 h in the dark at 4°C. Labeled nuclei were subjected to flow cytometric analysis and then gated on light scatter to remove debris, and the percentage of nuclei with a sub-G_1 content was considered apoptotic cells.

Cellular GSH levels

After U937 cells were exposed to 1 mM AAPH for 16 h, cells were incubated with 5 μM CMAC cell tracker for 30 min. The images of CMAC cell tracker fluorescence by GSH was analyzed by the Zeiss Axiovert 200

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inverted microscope at fluorescence DAPI region (excitation, 351 nm; emission, 380 nm).[13]

Measurement of intracellular ROS

Intracellular peroxide production was measured using the oxidant-sensitive fluorescent probe DCFH-DA with confocal microscopy.[14] Cells were grown at 2×10^6 cells per 100-mm plate containing slide glass coated with poly-L-lysine and maintained in the growth medium for 24 h. Cells were treated with 10 μM DCFH-DA for 15 min and exposed to AAPH. Cells on the slide glass were washed with PBS and a cover glass was put on the slide glass. DCF fluorescence (excitation, 488 nm; emission, 520 nm) was imaged on a laser confocal scanning microscope (DM/R-TCS, Leica) coupled to a microscope (Leitz DM REB). To evaluate the levels of mitochondrial reactive oxygen species (ROS) U937 cells in PBS were incubated for 20 min at 37°C with 5 μM DHR 123 and cells were double-stained with 100 nM MitoTracker Red. Cells were washed, resupended in complete growth media, and ionizing radiation was applied to the cells. The cells were then incubated for an additional 40 min. DHR 123 and MitoTracker Red fluorescence were visualized by a fluorescence microscope.

Lipid peroxidation

Lipid peroxidation was also estimated by using a fluorescent probe DPPP as described by Okimoto *et al.*[15] After U937 cells (1×10^6 cells/ml) were incubated with 5 μM DPPP for 15 min in the dark, cells were exposed to ionizing radiation. The images of DPPP fluorescence by reactive species were analyzed by the Zeiss Axiovert 200 inverted microscope at fluorescence DAPI region (excitation, 351 nm; emission, 380 nm).

Quantitaion of relative fluorescence

The averages of fluorescence intensity from fluorescence images were calculated as described.[16]

Replicates

Unless otherwise indicated, each result described in this paper is representative of at least three separate experiments.

Results

To study the relationship between IDPc activity and lipid peroxidation-mediated apoptotic cell death, control U937 cells and U937 cells pre-treated with 3 mM oxalomalate for 3 h were exposed to oxidant known to induce lipid peroxidation. Measurement of ICDH activity revealed that U937 cells pre-treated with 3 mM oxalomalate for 3 h contained 25–30% less activity compared to that of the untreated control (Figure 1A). We evaluated the effect of IDPc inhibition by oxalomalate on the NADPH level. The total NADPH in oxalomalate-treated cells was 15–20% lower than that in control cells. Because cellular antioxidants act in a concerted manner as a team, it is important to investigate whether the modulation in cellular IDPc activity caused concomitant alterations in the activity of other major antioxidant enzymes. Reduced IDPc activity by oxalomalate did not significantly alter the activities of antioxidant enzymes such as SOD, catalase, G6PD, glutathione peroxidase and glutathione reductase with or without exposure to AAPH (Figure 1B), suggesting that decreased IDPc activity did not affect the activities of other enzymes involved in antioxidation.

AAPH undergoes spontaneous thermal decomposition producing carbon-centered radicals at a constant rate in the presence of oxygen, these radicals can attack membrane polyunsaturated fatty acids and

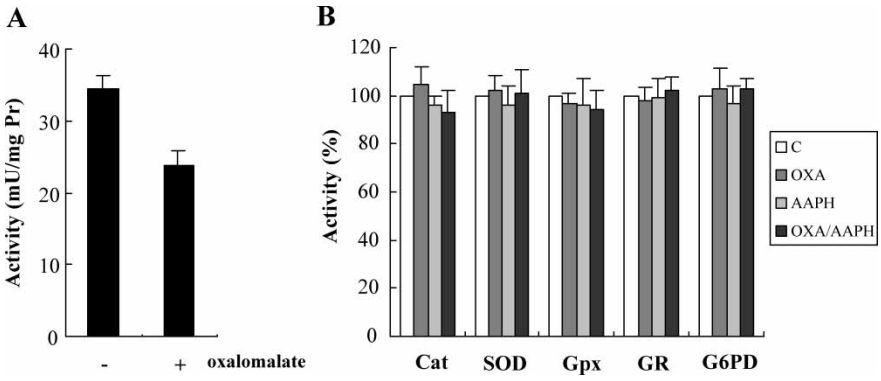

Figure 1. (A) Activity of IDPc in U937 cells untreated and treated with oxalomalate. U937 cells were untreated or treated with 3 mM oxalomalate for 3 h. Results are shown as the means ± S.D. of five separate experiments. (B) Activity of antioxidant enzymes in U937 cells untreated and treated with oxalomalate. Activity of untreated cells is expreseed as 100%. Open and shaded bars represent activities in the cells unexposed and exposed to oxalomalate. Results are shown as the means ± S.D. of three separate experiments. Cat, catalase; SOD, superoxide dismutase; Gpx, glutathione peroxidase; GR, glutathione reductase; G6PD, glucose 6-phosphate dehydrogenase. C, control cells; AAPH, AAPH-treated cells; OXA, oxalomalate-treated cells; AAPH/OXA, oxalomalate-treated and AAPH-treated cells.

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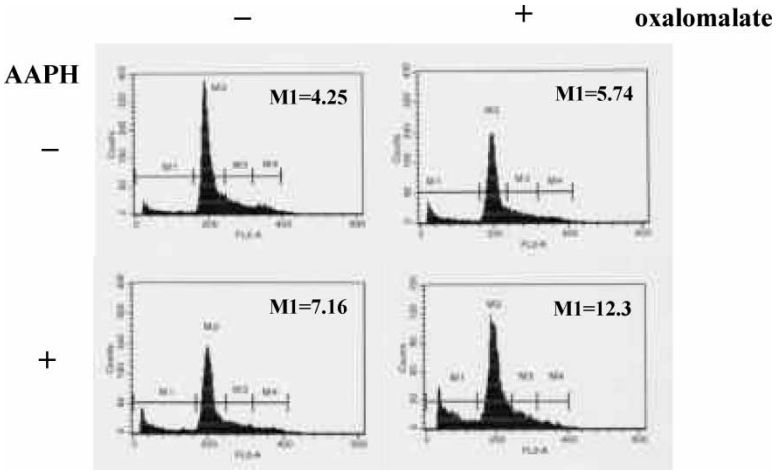

Figure 2. AAPH-induced apoptosis in U937 cells. Cell cycle analysis with cellular DNA content was examined by flow cytometry. The sub-G_1 region (presented as "M1") includes cells undergoing apoptosis. The number of each panel refers to the percentage of apoptotic cells.

initiate lipid peroxidation chain reaction.[17] Exposure of U937 cells to 1 mM AAPH for 16 h caused shrinkage of the cell and plasma membrane blebbing that was apparent by light microscopy (data not shown). Figure 2 shows a typical cell cycle plot of U937 cells that were untreated or treated with 1 mM AAPH for 16 h. Apoptotic cells were estimated by calculating the number of subdiploid cells in the cell cycle histogram. When cells were exposed to AAPH, apoptotic cells were increased markedly in oxalomalate-treated cells as compared to control cells.

To investigate the role of IDPc in cellular defense against high lipid peroxidation-mediated oxidative stress, we determined the cellular redox status in U937 cells unexposed or exposed to AAPH. GSH is one of the most abundant intracellular antioxidants and determination of changes in its concentration provides an alternative method of monitoring oxidative stress within cells. It has been shown that GSH sensitive fluorescent dye CMAC can be employed as a useful probe to evaluate the level of intracellular GSH.[13] Cellular GSH levels in oxalomalate-treated U937 cells exposed to AAPH were significantly decreased (Figure 3). These results strongly suggest that diminished ICDH activity by oxalomalate resulted in the perturbation of cellular antioxidant mechanisms by the depletion of GSH presumably through the decrease in NADPH generation. The levels of intracellular peroxides in U937 cells were measured by confocal microscopy with the oxidant-sensitive probe DCFH-DA. Deacylation by esterase to dichlorofluoroscin occurs within the cells and the nonfluorescent dichlorofluoroscin is, subsequently, oxidized in the presence of intracellular hydroperoxides and peroxides to highly fluorescent dichlorofluorescein.[18] As shown in Figure 3, DCF fluorescence intensity was significantly increased in cells treated with oxalomalate as compared to that of the control upon exposure to AAPH. The levels of intracellular peroxides in the mitochondria of U937 cells were evaluated by confocal microscopy with the oxidant-sensitive probe DHR 123. As shown in Figure 3, the intensity of fluorescence was significantly increased in the oxalomalate-treated cells compared to that of the control upon exposure to AAPH. The data strengthen the conclusion that decreased activity of IDPc induces apoptosis by increasing the steady-state level of intracellular oxidants upon exposure to AAPH. We determined whether the change in cellular IDPc activity correlated with

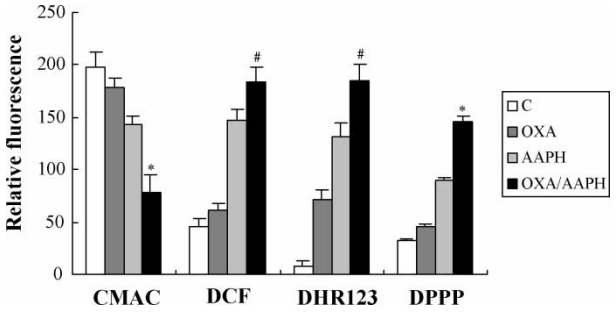

Figure 3. Lipid peroxidation-mediated modulation of cellular redox status and lipid peroxidation of U937 cells. Control and oxalomalate-treated U937 cells were exposed to 1 mM AAPH for 16 h. To determine the effect of lipid peroxidation on GSH levels in U937 cells, fluorescence image of CMAC-loaded cells were obtained under microscopy. Measurement of *in vivo* molecular oxidation by DCF fluorescence was obtained under laser confocal microscopy. To determine the effect of lipid peroxidation on mitochondrial ROS generation, DHR 123 was employed. DHR 123 fluorescence was visualized by a fluorescence microscope. In order to visualize lipid peroxidation in U937 cells, cells (1×10^6 cells/ml) were stained with 5 μM DPPP for 15 min. Fluorescence images were obtained under microscopy. The averages of fluorescence intensity were calculated as described.[16] Each value represents the mean ± S.D. from three independent experiments. C, control cells; AAPH, AAPH-treated cells; OXA, oxalomalate-treated cells; AAPH/OXA, oxalomalate-treated and AAPH-treated cells. $^{\#}P < 0.05$ and $^{\star}P < 0.01$ compared with AAPH-treated cells not exposed to oxalomalate.

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the change in DPPP fluorescence as an indicator of lipid peroxidation. Recently, it has been shown that DPPP is a suitable fluorescence probe to monitor lipid peroxidation within cell membrane specifically. DPPP reacts with lipid hydroperoxides stoichiometrically to give highly fluorescent product DPPP oxide.[15] When exposed to 1 mM AAPH for 16 h, DPPP fluorescent intensity was increased markedly in U937 cells pre-treated with oxalomalate as compared to the untreated control (Figure 3).

Caspase-3 activation in U937 cells was assessed by immunoblot analysis of lysates from cells that had been exposed to AAPH. As shown in Figure 4, the appearance of the apoptotic-cleaved product of caspase-3 was identified in U937 cells treated with AAPH, and the cleavage was more pronounced in oxalomalate-treated cells when compared to that in control cells. AAPH also induced the formation of fragments which represents proteolytic fragments of PARP and lamin B, indicates an oncoming apoptotic process. The cleaved products of PARP and lamin B increased markedly in oxalomalate-treated cells compared to control cells upon exposure to AAPH. Taken together, lipid peroxidation-mediated cleavage of procaspase-3 into the active form of caspase-3 and caspase-3 induces degradation of PARP or lamin B. The results also indicate that IDPc exhibits a protective effect on the lipid peroxidation-mediated apoptosis. The role of mitochondrial pathway of apoptosis in the lipid peroxidation-mediated death of U937 cells were examined by immunoblot analysis of the abundance of Bcl-2, an antiapoptotic protein, and of Bax, an proapoptotic protein. As shown in Figure 4, the abundance of Bcl-2 in U937 cells was significantly decreased in oxalomalate-treated cells as compared to that of control cells when exposed to AAPH. The amount of Bax was

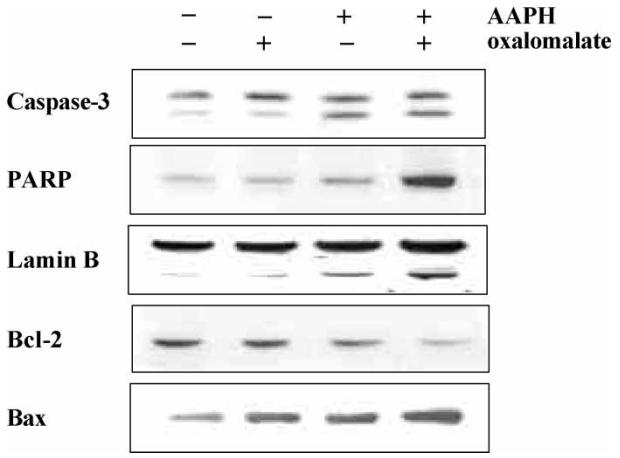

Figure 4. Immunoblot analysis of various apoptosis-related proteins in U937 cells untreated or treated with 1 mM AAPH for 16 h. Cell extracts were subjected to 10–12.5% SDS-PAGE and immunoblotted with antibodies against cleaved caspase-3, cleaved PARP, lamin B, Bcl-2, and Bax.

increased after treatment with AAPH, and it was significantly increased in oxalomalate-treated cells as compared to that of control cells.

Discussion

It has been proposed that lipid peroxidation is a continual process in living aerobic cells, is maintained at a low level, and can be prevented from entering into the autocatalytic phase by protective enzymes and antioxidants.[19] Chemical and physical agents that enhance membrane free radical reactions may accelerate this process beyond the capabilities of the protective systems, and thus cause widespread lipid peroxidation.[1] Lipid peroxidation of polyunsaturated fatty acid produces ROS and toxic aldehydes such as 4-HNE and MDA. The ROS and aldehydes then contribute to the tissue damage that results by modifying critical biomolecules.

When cells are grown in air, NADPH must be used to maintain a level of GSH as well as reduced thioredoxin to combat oxidative damage. Glutathione reductase converts GSSG to GSH in the cell using NADPH as a reductant.[20] The oxidized form of thioredoxin, with a disulfide bridge between the half-cystines, is reduced by NADPH in the presence of a flavoprotein, thioredoxin reductase.[21] Reduced thioredoxin may provide reducing equivalents to several enzymes including thioredoxin peroxidases and methionine sulfoxide reductase, presumably involving the defense against oxidative stress. Recently, a family of antioxidant proteins from eucaryotes and procaryotes are purified and these proteins remove hydrogen peroxide using hydrogen provided by the NADPH-dependent thioredoxin system, and thus are called thioredoxin peroxidases.[22,23] Reduced thioredoxin may also provide reducing power to methionine sulfoxide reductase which can reactivate proteins damaged by previous oxidation of their methionine residues.[24]

The pentose phosphate pathway is considered to be a major source of cellular reducing power, with G6PD catalyzing the key NADPH-producing step. It is well documented that this pathway, specifically G6PD, plays a protective role during oxidative stress.[25,26] However, it is possible that other enzymes which generate NADPH may have roles in oxidative stress resistance. ICDH in the cytosol of rat liver has been proven to have an approximately 20 times higher specific activity than G6PD.[27] We recently reported that the control of cellular redox balance and oxidative damage is one of the primary functions of ICDH in cytosol of NIH3T3 cells.[11]

In the present study, we examined the apoptotic pathway initiated by AAPH in U937 cells. A temporal pattern of events was observed, starting from perturbation of redox status reflected by the modulation of intracellular ROS generation and GSH pool, followed by caspase-3 activation, and cleavage of

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caspase target proteins. The inhibition of IDPc activity by oxalomalate significantly deteriorates redox status and enhanced the whole apoptotic pathway. GSH is a well-known antioxidant which is usually present as the most abundant low-molecular-mass thiol in most organisms. It has various functions in the defense against oxidative stress and xenobiotic toxicity.[28] It can act as the electron donor for glutathione peroxidase in animal cells, and also directly reacts with ROS. GSH is readily oxidized to glutathione disulfide (GSSG) by the glutathione peroxidase reaction, as well as the reaction with ROS which may subsequently cause the reduction of GSH level. GSH is readily oxidized to GSSG by the glutathione peroxidase reaction, as well as the reaction with ROS. Reactive aldehydes such as 4-HNE can react with the sulfhydryl group of GSH and subsequently cause the reduction of GSH level.[29] In our study, lipid peroxidation induced by AAPH directly affected the glutathione redox status and the intracellular ROS was increased in the same condition. These results indicate that lipid peroxidation is able to modulate the cellular redox balance presumably by depleting GSH. Consequently, the perturbation of the balance between oxidants and antioxidants leads to a pro-oxidant condition.

In conclusion, IDPc plays an important protective role in AAPH-induced apoptosis of U937 cells may contribute to various pathologies associated with lipid peroxidation-mediated oxidative stress.

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