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The natural antioxidant alpha-lipoic acid induces p27^{Kip1}-dependent cell cycle arrest and apoptosis in MCF-7 human breast cancer cells

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ABSTRACT

Unlike normal cells, tumor cells survive in a specific redox environment where the elevated reactive oxygen species contribute to enhance cell proliferation and to suppress apoptosis. Alpha-lipoic acid, a naturally occurring reactive oxygen species scavenger, has been shown to possess anticancer activity, due to its ability to suppress proliferation and to induce apoptosis in different cancer cell lines. Since at the moment little information is available regarding the potential effects of alpha-lipoic acid on breast cancer, in the present study we addressed the question whether alpha-lipoic acid induces cell cycle arrest and apoptosis in the human breast cancer cell line MCF-7. Moreover, we investigated some molecular mechanisms which mediate alpha-lipoic acid actions, focusing on the role of the PI3-K/Akt signalling pathway. We observed that alpha-lipoic acid is able to scavenge reactive oxygen species in MCF-7 cells and that the reduction of reactive oxygen species is followed by cell growth arrest in the G1 phase of the cell cycle, via the specific inhibition of Akt pathway and the up-regulation of the cyclin-dependent kinase inhibitor p27^{kip1}, and by apoptosis, via changes of the ratio of the apoptotic-related protein Bax/Bcl-2. Thus, the anti-tumor activity of alpha-lipoic acid observed in MCF-7 cells further stresses the role of redox state in regulating cancer initiation and progression.

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1. Introduction

Alpha-lipoic acid (thioctic acid, 5-(1,2-dithiolan-3-yl)pentanoic acid,) is a naturally occurring antioxidant synthesized in small amounts by plants and animals, including humans, which functions as an essential co-factor for several mitochondrial multi-enzyme complexes involved in energy metabolism (Biewenga et al., 1997; Packer et al., 1995, 1997). In addition to these physiological functions, due to its ability to scavenge reactive oxygen species and to regenerate other endogenous antioxidants (Packer et al., 1997, 2001; Somchit et al., 2004), alpha-lipoic acid is utilized in clinical conditions with oxidative stress, like diabetes, liver and neurodegenerative diseases (Gualandri et al., 2003; Packer et al., 1995). In cells, alpha-lipoic acid is reduced to yield the active form, dihydrolipoic acid, which scavenges various reactive oxygen species and regenerates other endogenous antioxidants (Biewenga et al., 1997; Packer et al., 1997, 2001).

Previous studies indicated that intracellular redox balance is linked to cellular growth control and that the redox state plays a crucial role in carcinogenesis. Moreover, it has been shown that dietary intake of antioxidants may be chemopreventive and may improve the efficacy of chemotherapy (Drisko et al., 2003; Kovacic and Jacintho, 2001). Unlike normal cells, tumor cells survive in a specific redox environment where the elevated reactive oxygen species, which have been indicated as critical signalling molecules (Kamata and Hirata, 1999), contribute to enhance cell proliferation and to suppress apoptosis (Fruehauf and Meyskens, 2007). In addition, the fluctuation of the intracellular redox state has been shown to affect cell proliferation and apoptosis via modulation of the serine-threonine kinase Akt, a critical enzyme involved in cell survival pathways (Kennedy et al., 1997; Torii et al., 2006).

Over the last years, considerable attention has been focused on the potential anticancer effects of different naturally occurring compounds, such as resveratrol, curcumin and genistein (Kuo et al., 2002; Li et al., 2005; Rao et al., 2005), in order to identify and validate novel strategies for cancer therapy, to overcome resistance and side effects of drugs presently utilized in the clinical therapy and to improve patient outcome. Among these molecules, alpha-lipoic acid has gained considerable attention since it was able to induce cell cycle arrest and apoptosis in different cancer cell lines (Simbula et al., 2007; van de Mark

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et al., 2003; Wenzel et al., 2005), while it exerted protective effects in normal cells (Pierce et al., 2000; Piotrowski et al., 2001). Since at the moment little information is available regarding the potential effects of alpha-lipoic acid on breast cancer, which is worldwide a major cause of mortality in women, in the present study we addressed the question whether alpha-lipoic acid induces cell cycle arrest and apoptosis in the human breast cancer cell line MCF-7. Moreover, we investigated the role of the phosphatidylinositol 3-kinase (PI3-K)/Akt signalling pathway in such effects.

2. Materials and methods

2.1. Reagents

A stock solution was prepared by dissolving alpha-lipoic acid (Istituto Biochimico Pavese Pharma, Pavia, Italy) in absolute ethanol. The PI3-K inhibitor LY-294,002 (Sigma-Aldrich, Milan, Italy) was dissolved in dimethyl sulfoxide. Primary antibodies, including monoclonal anti-Akt, anti-Bax, anti-Bcl-2, anti-$p27^{kip1}$, polyclonal anti-phospho-Akt (pAkt, Ser 473) and anti-rabbit and anti-mouse IgG secondary antibodies conjugated with horseradish peroxidase were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). All other reagents were from Sigma-Aldrich, unless otherwise indicated.

2.2. Cell culture

MCF-7 human breast cancer cells (American Type Culture Collection, Rockville, MD) were grown in RPMI 1640 (Biochrom, Berlin, Germany) supplemented with 1 mM sodium pyruvate, 100 U/ml penicillin, 100 µg/ml streptomycin, 2 mM glutamine, 1 mM non essential aminoacids and 10% fetal bovine serum (Gibco, Grand Island, NY). The medium was replaced at 3-day intervals. Subconfluent cells were routinely harvested with 0.05% trypsin/0.02% EDTA (Biochrom). Treatments were performed with increasing doses of alpha-lipoic acid (0.25–2.5 mM), selected according to previous studies involving tumor clones (Choi et al., 2009; Shi et al., 2008; van de Mark et al., 2003).

2.3. MTT assay

Cell growth was evaluated by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (Promega Italia, Milan, Italy). The assay was performed by the addition of a premixed optimized dye solution. After a 4-h incubation at 37 °C, solubilization/stop solution mix was added to solubilize the formazan product and the absorbance at 570 nm was recorded.

2.4. BrdU assay

MCF-7 cells were seeded in a 96-well plate in a final volume of 200 µl per well and incubated at 37 °C for 24 h for cell attachment. Then, the culture medium was replaced by adding 180 µl of experimental medium and 200 µl of 100 µM 5′-bromo-2′deoxyuridine (BrdU labeling solution, Delfia cell proliferation kit, Perkin Elmer, Milan, Italy) and the incubation was carried on for further 24 h. Cells were subsequently fixed (fix solution, 100 µl per well) for 30 min and then incubated, at room temperature, for further 120 min with a primary monoclonal antibody against BrdU (0.5 µg/ml) conjugated with Europium (Eu; Anti-BrdU-Eu working solution). Finally, after adding a specific inducer solution (200 µl/well), the Eu-fluorescence was measured in a time-resolved fluorometer. To exclude any unspecific binding of both BrdU and anti-BrdU Eu, for every experiment a row without cells (only medium) has been analyzed.

2.5. Western blot assay

Cells were collected in 100 µl lysis buffer containing 1% protease inhibitor cocktail. The cell suspension was kept on ice for 20 min and then centrifuged at 15,000 × *g* for 15 min at 4 °C. Protein concentration was quantified with the BCA Protein Assay kit (Pierce, Rockford, IL). 50 µg protein samples/lane and molecular mass marker (GE Healthcare, Milan, Italy) were separated on a sodium dodecylsulfate-polyacrylamide gel. Blots were blocked with 5% dry milk in tris-buffered saline/0.1% tween-20 and then incubated overnight with a diluted solution of the primary antibody. The incubation with a HRP-conjugated antibody was performed at room temperature for 2 h. Immunoreactivity was detected by the SuperSignal West Pico Substrate solution (Pierce) and X-ray films.

2.6. Cell cycle analysis

Cells were collected and resuspended at the concentration of 1×10^6 cells/ml in a DNA-staining solution containing 0.1% Nonidet P-40, 0.5 mg/ml RNAse (type IIIA) and 25 g/ml propidium iodide (PI) and incubated for 30 min at 37 °C in the dark. DNA content was analysed by flow cytometry (CyFlow, Partec Italia, Milan, Italy).

2.7. Cell apoptosis analysis

A Vybrant Apoptosis Assay Kit #2 (Molecular Probes, Leiden, The Netherlands) was used. Cells were harvested, washed, incubated with Alexa Fluor 488 annexin-V fluorescein isothiocyanate (FITC) and PI, and incubated for 15 min at room temperature. After treatment with both probes, apoptotic cells showed green fluorescence, dead cells showed red and green fluorescence, while living cells showed little or no fluorescence. Cells were analyzed by flow cytometry.

2.8. Measurement of intracellular reactive oxygen species

Intracellular reactive oxygen species levels were measured using the non fluorescent compound 2′,7′-dichlorofluorescein-diacetate (DCFH-DA), according to Jiao (Jiao and Zhao, 2002). Cells were incubated for 30 min at 37 °C with 20 µM DCFH-DA which passes through the cell membrane and once inside the cell is converted to the non fluorescent derivate dichlorofluorescein, which in turn remains inside the cell and reacts with intracellular reactive oxygen species to produce the fluorescent dye 2′,7′-dichlorodihydrofluorescein (DCF). The fluorescence was recorded at 490 nm excitation and 530 nm emission using a spectrophotofluorimeter.

2.9. Statistical analysis of biological assays

Statistical analysis was performed using the Prism statistical analysis package (GraphPad Software, San Diego, CA). Data are expressed as mean ± S.D. or S.E.M. Differences between treatment groups were evaluated by ANOVA, followed by post hoc Dunnett's test and considered significant at $P<0.05$.

3. Results

3.1. Alpha-lipoic acid inhibits cell growth

MCF-7 cells were treated for 24, 48 and 72 h with increasing doses of alpha-lipoic acid (0.25–2.5 mM) and subjected to MTT assay. Alpha-lipoic acid was found to inhibit cell growth at each time point tested and the magnitude of this suppression was seen as early as after 24-h exposure to 0.5 mM alpha-lipoic acid (−13%); at the same time point, 1 and 2.5 mM alpha-lipoic acid reduced cell growth by about 41%. Increasing doses of alpha-lipoic acid (0.25, 0.5, 1 and 2.5 mM) yielded a parallel reduction of cell growth (by 17, 60, 73 and

80% after 48 h and by 43, 63, 86 and 94% after 72 h, respectively) (Fig. 1A).

3.2. Alpha-lipoic acid induces cell cycle arrest and increases $p27^{kip1}$ expression

To elucidate whether the growth inhibitory effect of alpha-lipoic acid was due to cell cycle arrest, we evaluated cell cycle distribution by flow cytometry. After 24 h, alpha-lipoic acid caused a significant dose-dependent accumulation in the G1 phase of the cell cycle, reaching a plateau at the doses of 2 and 2.5 mM, and a parallel reduction in the S and G2 phases (Fig. 1B). The reduced DNA synthesis induced by alpha-lipoic acid was also confirmed by the BrdU assay, which indicated a significant inhibitory effect on cell proliferation starting at the dose of 1 mM (Fig. 1C). The expression of the cyclin-dependent kinase inhibitor $p27^{kip1}$, which is involved in the arrest of the cell cycle in the G1 phase, was also evaluated. $p27^{kip1}$ levels, not detectable in basal condition, have been found to be increased after 24-h exposure to 0.5–2.5 mM alpha-lipoic acid, with a maximal expression at the dose of 2 mM (Fig. 1D).

3.3. Alpha-lipoic acid induces apoptosis and reduces the expression of Bcl-2

MCF-7 cells were treated for 24 h with different alpha-lipoic acid concentrations and then were subjected to annexin-V FITC/PI flow cytometry analysis to determine cell apoptosis. Alpha-lipoic acid was found to promote both early and late apoptosis in a dose-dependent manner (Fig. 2A and B). The expression of the apoptosis-related proteins Bax and Bcl-2 was also evaluated by Western blot. After 24 h, alpha-lipoic acid dose-dependently down-regulated the expression of the anti-apoptotic protein Bcl-2, whereas it did not affect the pro-apoptotic protein Bax (Fig. 2C).

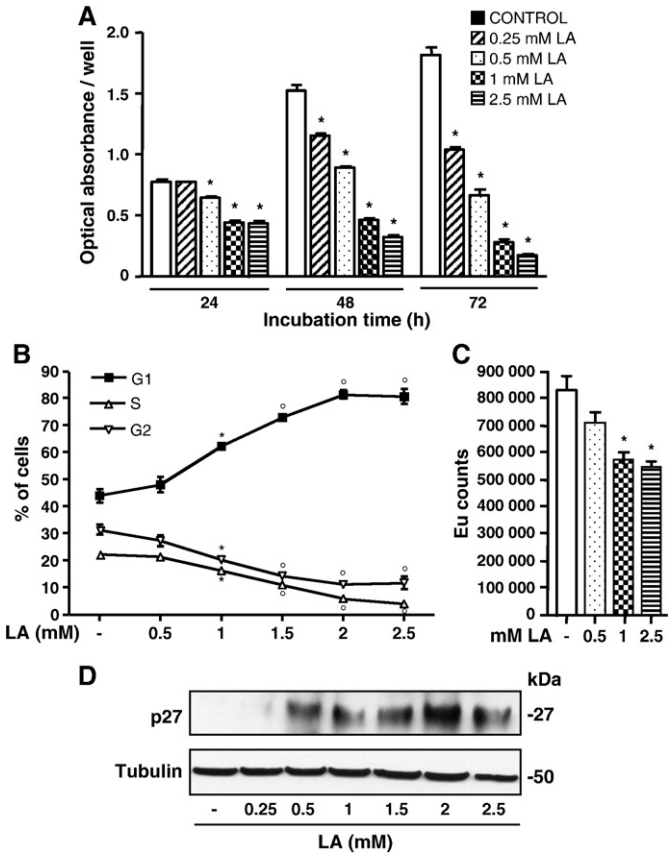


Fig. 1. Alpha-lipoic acid effects on MCF-7 cell growth and cell cycle. A) Alpha-lipoic acid inhibits MCF-7 cell growth. Cell viability was assessed by MTT assay, after 24, 48 and 72 h. One of three independent experiments is shown. Values are mean percent of cells ± S.D., $n = 3$; $^{*}P < 0.01$ *vs.* control. B) Effect of 24-h alpha-lipoic acid treatment on MCF-7 cell cycle. Values are mean ± S.E.M. of three experiments; $^{*}P < 0.05$; $^{\circ}P < 0.01$ *vs.* control. C) Evaluation of cell proliferation by BrdU assay. One experiment is shown. Values are mean ± S.D., $n = 8$; $^{*}P < 0.01$ *vs.* control. D) 50 μg protein extract/lane was analyzed by Western blot with anti-$p27^{kip1}$ and anti-tubulin antibodies. One representative experiment is shown.

3.4. Alpha-lipoic acid down-regulates Akt signalling pathway

The involvement of Akt in alpha-lipoic acid-mediated effects was also examined. After 24 h, alpha-lipoic acid reduced the levels of pAkt in a dose-dependent fashion, with a complete turn off of the signal starting at the dose of 1.5 mM. A parallel down-regulation of the constitutive Akt was also observed. Tubulin expression indicated that each sample has been loaded equally (Fig. 3A). To verify whether the down-regulation of the pAkt signal may be responsible for some of the effects exerted by alpha-lipoic acid, we utilized the PI3-K/Akt inhibitor LY-294,002, able to block Akt phosphorylation, to study the correlation among pAkt, cell cycle and apoptosis. After 24 h, LY-294,002 abolished pAkt signal, as expected, stimulated $p27^{kip1}$ expression, whereas it did not affect the levels of Akt and Bcl-2 protein (Fig. 3B). A significant reduction of proliferating cells (Fig. 3C) and a parallel arrest in the G1 phase of the cell cycle were also observed (Fig. 3D). Relative to apoptosis, after 24 h, LY-294,002 did not appear to increase the number of apoptotic cells (Fig. 3E).

3.5. Alpha-lipoic acid reduces reactive oxygen species

The accumulation of reactive oxygen species in MCF-7 cells was measured by a converting reaction of DCFH-DA to DCF after exposure of cells to different alpha-lipoic acid concentration for 6 h, a time preceding the occurrence of the anti-proliferative and pro-apoptotic effects observed. The decrease in DCF fluorescence intensity suggested that alpha-lipoic acid, in the range of doses form 0.5 to 2.5 mM, is able to scavenge intracellular reactive oxygen species in our *in vitro* system; to be noted, exposure to 2.5 mM alpha-lipoic acid reduced intracellular reactive oxygen species level by about 52% (Fig. 4).

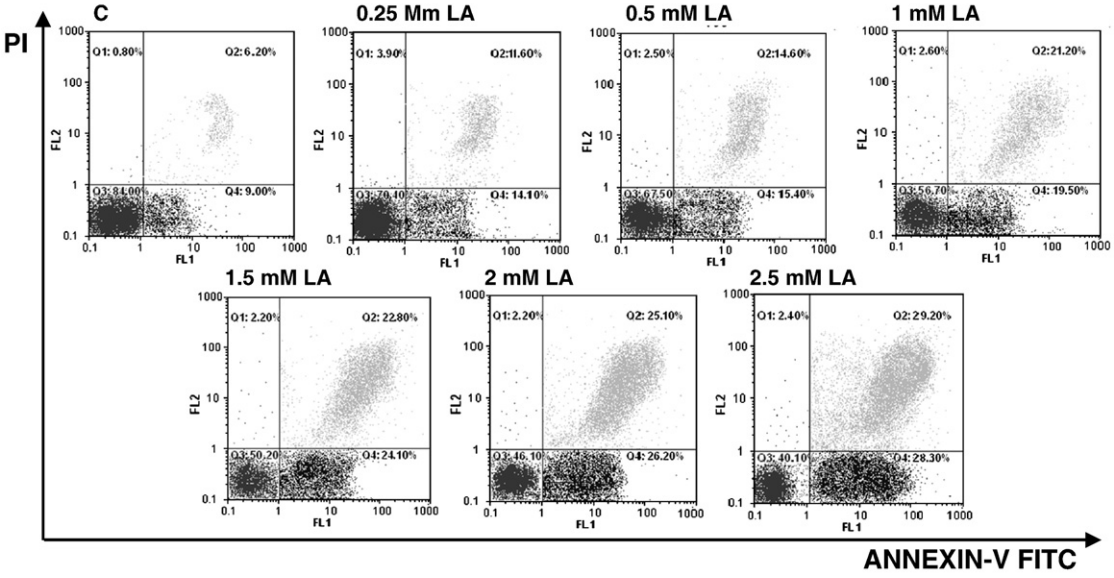
4. Discussion

It is well established that uncontrolled cellular growth, as a consequence of defects in cell cycle and apoptotic machinery, is responsible for the development of most cancers. Therefore, agents that can modulate cell cycle and apoptosis may be useful in the management and therapy of tumors (McDonald and El-Deiry, 2000; Owa et al., 2001). Epidemiological investigation and laboratory studies have indicated that different compounds developed from natural sources play an important role in the treatment of many cancers (Kim, 2008; Xian et al., 2007). Among these molecules, alpha-lipoic acid is of particular interest, since it has been shown to possess anti-tumor activity in different human cancer cells without affecting normal cells (McDonald and El-Deiry, 2000; Wenzel et al., 2005).

In the present study, we showed that alpha-lipoic acid is able to scavenge reactive oxygen species in the human breast cancer cell line MCF-7 and that the reduction of reactive oxygen species is temporally followed by cell growth arrest, via the specific inhibition of Akt pathway and the up-regulation of the cyclin-dependent kinase inhibitor $p27^{kip1}$, and apoptosis, via changes of the ratio of the apoptotic-related proteins Bax/Bcl-2.

At present, the role of reactive oxygen species in cancer is a controversial issue since both stimulation and suppression of reactive oxygen species have been reported to induce cancer cell growth arrest and apoptosis. Compared with their normal counterparts, malignant cells have increased levels of reactive oxygen species, which have been shown to be involved both in cancer initiation and progression.

A



B

	Q3 (living cells)	Q4 (early apoptotic cells)	Q2 (late apoptotic cells)
C	85.10±0.64	8.47±0.32	5.80±0.26
0.25mM	70.97±0.35[a]	13.80±0.17[b]	11.87±0.43[c]
0.5 mM	66.83±0.41[a]	16.47±0.54[b]	14.80±0.12[c]
1 mM	57.57±0.49[a]	18.50±0.53[b]	21.70±0.26[c]
1.5 mM	50.70±0.26[a]	23.77±0.18[b]	22.73±0.12[c]
2 mM	48.83±0.37[a]	26.03±0.27[b]	24.90±0.38[c]
2.5 mM	39.87±0.12[a]	27.93±0.20[b]	29.87±0.34[c]

C

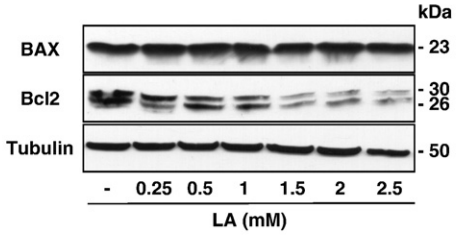


Fig. 2. Effect of 24-h alpha-lipoic acid treatment on MCF-7 cell apoptosis. A) One representative cytometry analysis is shown. Cell population $FITC^-/PI^-$, $FITC^+/PI^-$, and $FITC^+/PI^+$ were regarded as living (Q3), early apoptotic (Q4) and late apoptotic/necrotic cells (Q2). B) The number of living, early and late apoptotic/necrotic cells is expressed as mean ± S.D. $n = 3$. a, $P<0.001$ *vs.* living control cells; b, $P<0.001$ *vs.* apoptotic control cells; c, $P<0.001$ *vs.* late apoptotic/necrotic control cells. C) 50 μg protein extract/lane was analyzed by Western blot with anti-Bax, anti-Bcl-2 and anti-tubulin antibodies. One representative experiment is shown.

Apart from its role as a causative factor in carcinogenesis through reactive oxygen species-induced mutagenesis, redox dysregulation contributes to malignant transformation and progression through reactive oxygen species-mediated mitogenic signalling and redox modulation of apoptotic and survival pathways (Trachootham et al., 2009). Paradoxically, reactive oxygen species, apart from being involved in proliferative, anti-apoptotic and metastatic processes, over a certain threshold level, may also exert cytotoxic and pro-apoptotic functions that would limit tumorigenicity and malignant progression (Fruehauf and Meyskens, 2007). Thus, cancer cells become well adapted to survive under a persistent intrinsic increased oxidative stress (Trachootham et al., 2009). Nevertheless, it is still unclear whether a decrease or a deficit in antioxidants is a major cause of increased reactive oxygen species stress in tumors. In fact, both a reduced and an elevated expression of certain reactive oxygen species-scavenging enzymes and of antioxidant molecules has been observed in different primary cancer cells and also in primary cancer tissues (Oltra et al., 2001; Skrzydlewska et al., 2001). It follows that, if antioxidant compounds scavenge the excess of reactive oxygen species, the oxidative stress-responsive genes can be suppressed and, consequently, cancer cell proliferation is inhibited. On the other hand, pro-oxidant compounds, by increasing oxidative stress to a cytotoxic level, may also be useful in promoting cancer cell death (Trachootham et al., 2009).

Relative to alpha-lipoic acid, some works indicated that the molecule does not have antioxidant effects, or even it may be a pro-oxidant agent able to induce apoptosis by increasing reactive oxygen species generation, whereas other reports suggested that alpha-lipoic acid reduces cell growth by acting as an antioxidant (Moini et al., 2002; Moungjaroen et al., 2006; Shi et al., 2008; Simbula et al., 2007; Wenzel et al., 2005). At the same time, previous reports which evaluated the effects of other antioxidants on MCF-7 cells indicated the ability of these molecules to inhibit cell growth and promote apoptosis by either increasing or suppressing reactive oxygen species level (Filomeni et al., 2007; Jin et al., 2010; Lin et al., 1999). Our results suggest that in MCF-7 cells alpha-lipoic acid exerts antioxidant effect and that the reduction of reactive oxygen species is temporally followed by growth arrest and apoptosis. Probably, the biochemical properties of alpha-lipoic acid, the experimental conditions and the redox state of the cell models may be considered in order to explain such differences among studies. On the latter, it thus should be noted that the use of antioxidants, and among them of alpha-lipoic acid, in cancer therapy may be both a positive and a deleterious strategy since the final effect may strongly depend on the level of reactive oxygen species of cancer cells (Trachootham et al., 2009). In fact, in cells with low/moderate increased reactive oxygen species alpha-lipoic acid, both playing as an antioxidant molecule, which scavenges reactive oxygen species, or as a

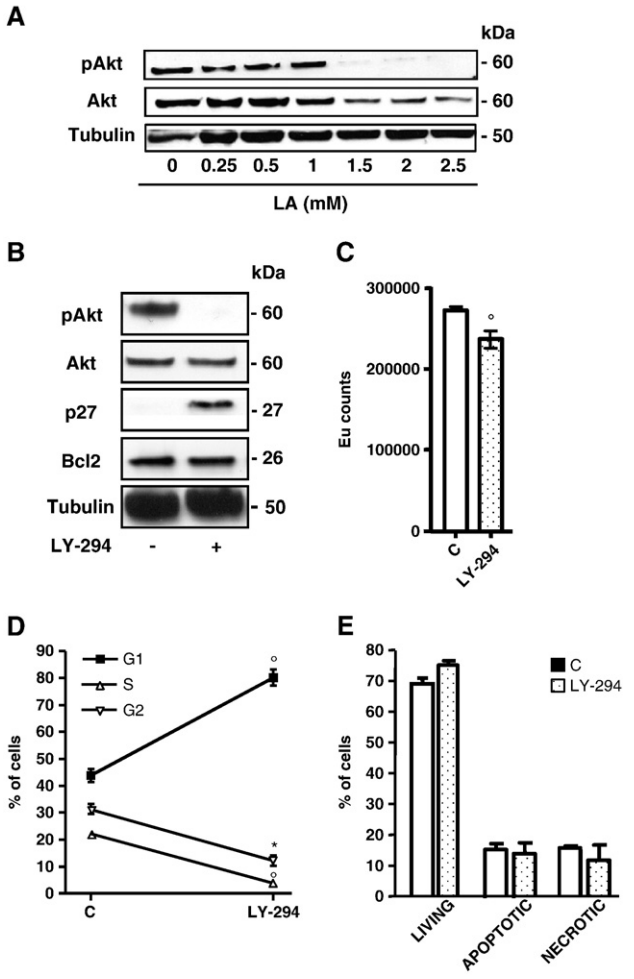


Fig. 3. Role of Akt pathway in alpha-lipoic acid-mediated effects. A) 50 µg protein extract/lane was analyzed by Western blot with anti-pAkt, anti-Akt and anti-tubulin antibodies. One representative experiment is shown. B) After treatment with LY-294,002 (10 µM, 24 h), 50 µg protein extract/lane was analyzed by Western blot with anti-pAkt, anti-Akt, anti-$p27^{kip1}$, anti-Bcl-2 and anti-tubulin antibodies. One representative experiment is shown. C) Cell proliferation was assessed by BrdU assay after 24-h treatment with LY-294,002. One representative experiment is shown. Values are mean ± S.D., $n=8$; °$P<0.01$ *vs.* control. D) Cells were treated with LY-294,002 for 24 h, labeled with PI and analysed by flow cytometry. Values are mean percent of cells ± S.E.M., $n=3$; *$P<0.05$; °$P<0.01$ *vs.* control. E) Cells were treated with LY-294,002 for 24 h, stained with annexin-V FITC/PI and analysed by flow cytometry. Values are mean percent of cells ± S.E.M., $n=3$.

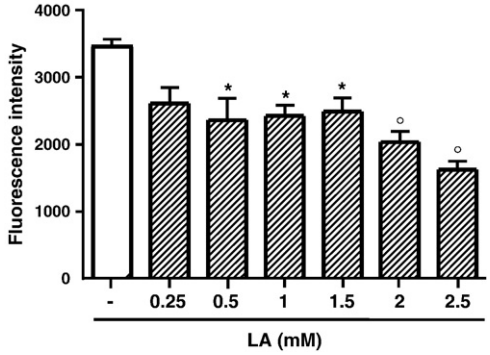


Fig. 4. Effect of alpha-lipoic acid (LA) treatment on reactive oxygen species level. Cells were treated with alpha-lipoic acid for 6 h, loaded with DCFH-DA and the fluorescence was recorded at 490 nm excitation and 530 nm emission. Values are mean ± S.D., $n=6$. *$P<0.05$; °$P<0.001$ *vs.* control.

pro-oxidant agent, which increases reactive oxygen species to a toxic level, may be useful since it could promote cell cycle arrest and apoptosis. On the contrary, the use of alpha-lipoic acid, as well as of other antioxidants, in cells with very high levels of reactive oxygen species requires special considerations. In fact, promoting reactive oxygen species accumulation, alpha-lipoic acid may enhance cancer cell cytotoxicity, whereas reducing reactive oxygen species may prevent oxidative damage and also reduce the reactive oxygen species-antitumoral activity of different anticancer drugs. Our observation that in MCF-7 cells alpha-lipoic acid acts as an antioxidant agent able to reduce cell growth might mainly depends on the redox state of this cell line. It has been suggested that Akt signalling pathway is essential for cell survival and that the expression of a constitutively active Akt or an increased activity of the PI3-K/Akt pathway leads to multidrug resistance in human breast cancer (Jin et al., 2003; Knuefermann et al., 2003). In the present study, we observed that alpha-lipoic acid inhibits Akt phosphorylation and also reduces the level of the constitutive Akt protein. By the use of LY-294,002, we showed that in MCF-7 the down-regulation of Akt phosphorylation was strongly associated to $p27^{kip1}$ expression and cell cycle arrest. Since the down-regulation of pAkt levels induced by alpha-lipoic acid was also associated to $p27^{kip1}$ expression and G1 cell cycle arrest, we concluded that the effect of alpha-lipoic acid on MCF-7 cell cycle progression was mediated, at least in part, by Akt phosphorylation. Previous results suggested that reactive oxygen species could improve Akt phosphorylation, leading to cell proliferation, while antioxidants, reducing reactive oxygen levels, inhibit Akt activation and cell growth (Dong-Yun et al., 2003). Accordingly, it is possible that the ability of alpha-lipoic acid to scavenge reactive oxygen species may lead to down-regulation of Akt phosphorylation, which in turn regulates cell growth and controls $p27^{kip1}$ expression. Our additional observation that alpha-lipoic acid treatment reduced Akt expression is an agreement with some reports indicating that the overall expression of this kinase may be modulated by different agents which modify the intracellular redox balance and/or induce apoptosis (Hortelano et al., 2000; Martin et al., 2002; Rovin et al., 2002). In our experimental condition we did not observe any correlation among pAkt levels and the induction of apoptosis. Thus, the possibility for alpha-lipoic acid to affect apoptosis via degradation of Akt or by affecting signalling pathways different from Akt may exist.

5. Conclusions

In conclusion, the anti-tumor activity of alpha-lipoic acid observed in MCF-7 cells further stresses the role of redox state in regulating cancer initiation and progression and indicates that Akt represents one of the critical signalling nodes operating downstream of alpha-lipoic acid effects.

Acknowledgments

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