

Differential Effect of Alpha-lipoic Acid on Healthy Peripheral Blood Lymphocytes and Leukemic Cells

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Introduction

Lipoic acid (LA), also known as alpha-lipoic acid, is a sulfur-containing fatty acid. It is found inside every cell of the body, where it helps to regenerate the energy that keeps cells alive and functioning. LA, an alipoamide, is a constituent of biological membranes and an important cofactor of mitochondrial dehydrogenases. LA is a key part of the process that turns glucose into energy.

LA, unlike other antioxidants that work only in water or fatty tissues, functions in both water and fat. This gives LA a broad spectrum of antioxidant actions. A healthy body makes enough LA to supply its own requirements. However, several medical conditions may be accompanied by low levels of LA.

LA is easily absorbed from the diet. It enters cells from the bloodstream and is readily converted to its reduced form, dehydrolipoic acid (DHLA). Both LA and DHLA act as antioxidants *in vitro* and *in vivo*.¹⁻⁵

The specific effects of LA and DHLA include quenching of reactive oxygen species, such as superoxide radicals and hydroxyl radicals, and chelation of copper, zinc, and iron. It is important in the intracellular recycling of vitamin E through interaction with vitamin C. LA also increases levels of glutathione, a very important antioxidant normally found in cells.⁶

This powerful antioxidant is currently being studied to provide both preventive and therapeutic benefits in

numerous conditions such as diabetes, heart disease, and neurological diseases (Parkinson's and Alzheimer's).⁷⁻¹⁵ For example, LA has been used for decades to treat diabetic peripheral neuropathy. Free radicals (oxidants) are thought to play a role in neuropathy. It has been shown that LA is effective in the prevention of early diabetic glomerular injury and has advantages over high doses of other antioxidants.¹³

Although hundreds of studies over the past years showed how LA energizes metabolism, our studies focused on a new aspect. We analyzed the differential effect of LA on energy metabolism of normal lymphocytes and leukemic lymphocytes and demonstrated inhibition of the energy metabolism of leukemic cells in comparison with normal cells. Analysis was performed for three leukemic cell lines and lymphocytes of healthy subjects. At similar concentrations, lipoic acid was toxic to leukemia cells and non-toxic to blood lymphocytes. Exposure of cells to 200-800 μM of LA was followed by a decrease of ATP production and increased apoptosis of leukemia cells. These experiments may be useful to prove the effectiveness of lipoic acid in the treatment of leukemia.

In addition, we demonstrated that supplementation of lymphocytes under oxidative stress can restore the functional activity of cells and improve the level of mitochondrial functioning and mitochondrial potential. The experiments suggest that lipoic acid has a beneficial effect in pathological conditions involving impairment of the immune system due to oxidative stress.

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Methods

1. Method of ATP Measurements in Cells

Levels of ATP in cells were determined by the CellTiter –GLO Luminescent Cell Viability Assay Kit (Promega Company). This assay generates a luminescence glow type signal produced by a luciferase reaction, which is proportional to the amount of ATP present in the cells. Levels of ATP were proportional to luminescent output and ATP was determined from a standard curve by measuring the level of luminescence for different concentrations of pure ATP (Sigma).

2. Measurements of Mitochondrial Potential

Mitochondrial potential was measured by using the fluorescent potentiometric dye JC-1. JC-1 is able to selectively enter mitochondria and forms aggregates that emit at 595 nm (red-orange range). If the mitochondrial potential is reduced, JC-1 changes to monomers that emit fluorescence at 535 nm (green). The ratio between the red and green signals indicates the mitochondrial potential. Levels of emission were measured by a fluorometer (SPEX Company).

3. Measurements of Apoptosis

Apoptosis (programmed cell death) was measured by Annexin V FITC Kit (Immunotech Coulter Company). In the early phase of apoptosis, the integrity of the cell membrane is maintained, but the cells lose the asymmetry of their membrane phospholipids. Phosphatidylserine (PS), the negatively charged phospholipid in the inner leaflet of the plasma membrane, becomes exposed at the cell surface. Annexin V binds preferentially with high affinity to PS. Apoptotic cells are detected by emission of bounded Annexin V. The signal from Annexin V was detected by the FITC signal detector of a flow-cytometer. Dead cells were separated by staining with propidium iodide.

4. Cell Differentiation

Treatment of leukemia cells with TPA (12-O-tetradecanoylphorbol-13-acetate) may induce differentiation of a number of leukemia cells. The effect of cell differentiation by TPA is associated with the activation of the stress-activated protein kinase, the release of cytochrome C, activation of caspases and other molecules.¹⁶

For differentiation, HL-60 cells were exposed to 32 to 64 nM of TPA. The time of the cell exposure was 24 hours. Differentiation by TPA inhibited cell growth and changed cell morphology. The most evident was an increase in cytoplasm to nucleus ratio. Immuno-fluorescence analysis demonstrated that before differentiation cells were promyelocytes and according to measurements, 90% of cells expressed surface markers of promyelocytes (CD33). After differentiation cells had characteristics of terminally differentiated cells and expressed markers of polymorphonuclear leukocytes (CD3, CD22, CD15 and CD66b).

Results

1. The Effect of Lipoic Acid on Transformed and Differentiated HL-60 Cells

Differentiated and transformed cells were analyzed for levels of ATP, for mitochondrial potential, and mitochondrial mass. Levels of mitochondrial potential and ATP production were compared for TPA treated and untreated cells, which demonstrated increased levels of mitochondrial potential after cell differentiation (25% average difference) and decreased levels of ATP production (28% average difference). Mitochondrial mass for cells after differentiation was measured by flow-cytometer by staining cells with the dye 10-N-nonyl acridine orange (NAO).¹⁷ This dye binds to the cardiolipin of mitochondria and the levels of uptake of this dye do not depend on

the mitochondrial potential. Emission was measured by flow-cytometer (excitation 495 nm, emission 525 nm). The data demonstrated lower values of mass for differentiated cells in comparison with the transformed cells (an average 16% difference).

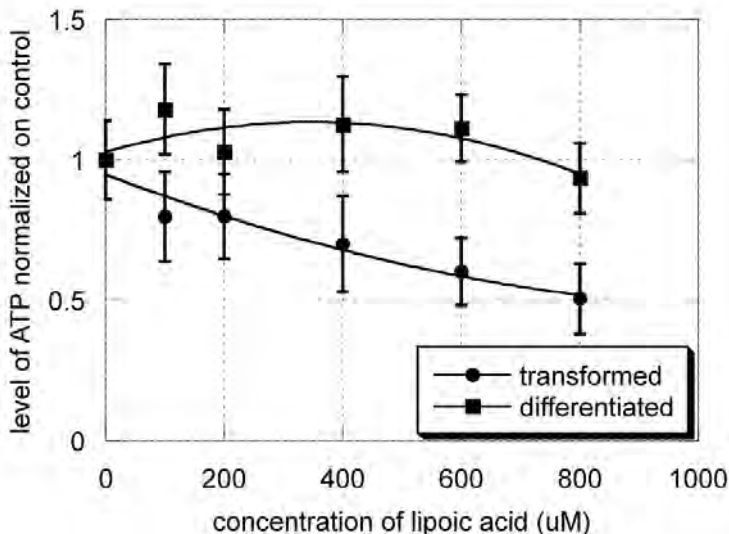
To compare the effect of LA on the level of ATP in differentiated and transformed cells, cells were washed and resuspended at a concentration of 0.5 million cells per mL in Iscove's medium (ATCC). LA was prepared before use in dimethyl sulfoxide (DMSO). The solution was standardized at 333 nm ($\epsilon = 150\text{M}^{-1}\text{cm}^{-1}$) and was added to the medium with cells in a concentration of 100 to 800 μM . Cells were incubated in an atmosphere with 5% CO_2 , 37 $^\circ\text{C}$, and 98% humidity for 24 hours. After incubation the cells were washed by phosphate buffered saline (PBS), counted, and the level of ATP was measured for two different populations.

Results of the experiments are presented in **Figure 1**, (below).

We found that LA had different effects on the level of metabolic activity of transformed and differentiated cells. Supplementation by LA caused inhibition of ATP production in transformed cells. As it is shown in **Figure 1**, concentration of lipoic acid equal to 800 μM inhibited viability of leukemia cells two times. However, the viability of normal differentiated cells was not changed. That effect was not due to the solvent DMSO, as it was shown by the addition of DMSO without LA. Levels of DMSO in medium were less than 0.5%.

The explanation of the difference in the effect of LA on transformed and differentiated cells might be based on the assumption that LA may up-regulate caspase activity and apoptosis in transformed cells by creating a reducing environment.¹⁸

Figure 1. Effect of lipoic acid on the levels of ATP in differentiated and transformed HL-60 Cells (mean \pm SD).



2. The Effect Of Lipoic Acid on Leukemia T Cells and Healthy T Cells and Potentiation of Inhibition of Energy Metabolism in Leukemia Cells by Lipoic Acid

We compared the effect of LA on healthy lymphocytes and T cells and acute pro-myelocytic leukemia cells, chronic myelogenous leukemia, and acute lymphoblastic leukemia T cells. Healthy T cells were separated by the RosetteSep procedure (Stem cell technology) from peripheral blood. The main principle of separation is that blood was mixed with RosetteSep antibody cocktail and unwanted cells were cross-linked to red blood cells (rosetted) with tetrameric antibody complexes. Centrifugation with Ficoll-Paque allowed separation of enriched cells.

In order to determine the potential effect of LA on the level of metabolism of normal and transformed cells, cells were treated with different concentrations of

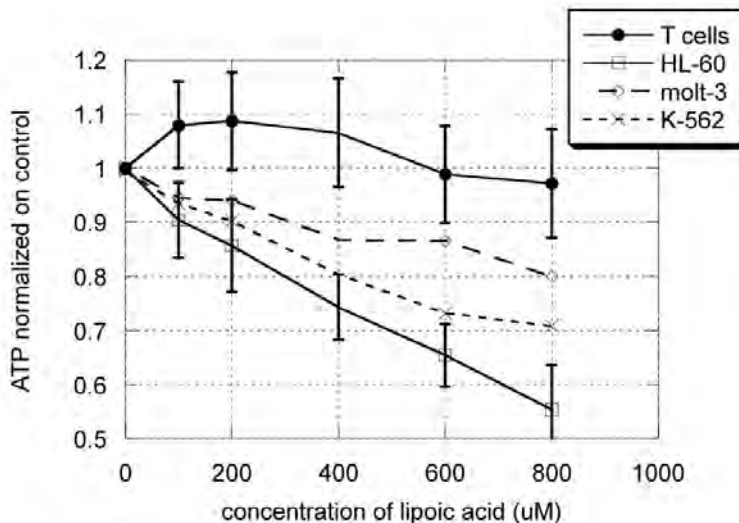
LA. Cells (HL-60- acute pro-myelocytic leukemia, K-562- chronic myelogenous leukemia, Molt-3-acute lymphoblastic leukemia T cells, and healthy T cells) were seeded in 24-well plates with concentrations of 0.5 million cells per mL. Cells were treated by concentrations of LA 100 to 800 μ M for 24 hours.

After exposure, ATP concentrations in the treated cells were compared with control non-treated cells (Figure 2, below).

The data presented in Figure 2 are average values of 5 to 8 experiments. According to these data, treatment of leukemia cells by LA resulted in an inhibition of metabolic activity and ATP concentration in transformed cells. The most pronounced effect was measured for HL-60 cells. The same concentrations of LA did not inhibit ATP production in normal T cells.

Under the same experimental con-

Figure 2. Effect of lipoic acid on the levels of ATP concentrations in healthy and leukemia T cells (means \pm SD).



ditions, we measured the levels of apoptotic cells. For HL-60 cells, the percentage of apoptotic cells was 5%-10% after exposure to 200 μM of LA and increased to 15%-20% after exposure to 800 μM LA. For T cells, the percentage of apoptotic cells was in the range of 1%-4% for a concentration of LA of 100 to 800 μM .

Results of our analysis demonstrated the cytotoxicity of LA to leukemic cells in comparison with healthy T cells. These results may be explained by a previous study of the mechanism of apoptosis.¹⁸ A reduced environment inside the cells is necessary for caspase activity during apoptosis. LA is a redox active chemical, intracellularly reduces to a potent dihydrolipoic acid, and creates a reduced environment in the cells. It potentiates apoptosis of tumor cells or affects the level of energy metabolism of tumor cells.

3. The Effect of Lipoic Acid on Cells Under Oxidative Stress

For many years, LA has been recognized as an antioxidant.⁵ Alpha-lipoic acid, or its reduced form, dehydrolipoate, reacts with reactive oxygen species. In this study, we examined whether in vitro supplementation of cells with LA can protect mitochondrial potential and the level of ATP production against oxidative stress. Enhancement of cellular antioxidant status was performed by preincubation of the cells with LA, which was reduced intracellularly to dihydrolipoic acid. The time of exposure to LA was 4 to 6 hours. After incubation with LA, cells were washed and resuspended in fresh medium. Hydrogen peroxide was added at concentrations of 50 to 200 μM . The concentration of H_2O_2 was standardized at 240 nm ($\epsilon=43.6 \text{ M}^{-1}\text{cm}^{-1}$). After 30 min of exposure, cells were washed and analyzed at the level of ATP and mitochondrial potential. According to our results, the addition of 50 to 200

μM of hydrogen peroxide to the medium with cells resulted in oxidative stress and apoptosis in cells. Addition of 50-200 μM of H_2O_2 decreased the ratio of intensities of emission at 595 nm to 535 nm 1.2 to 3 times in comparison with controls. Examples of the fluorescence emission curves of mitochondrial potential for control cells and cells under oxidative stress are presented in **Figure 3**, (p.88).

Our results proved that free LA, when provided exogenously protects the mitochondrial potential against oxidative stress. For example, data showed that the mitochondrial potential was reduced by 34% to 61% in cells under oxidative stress induced by 100 μM of H_2O_2 . After pretreatment of cells by LA before exposure to hydrogen peroxide, the level of mitochondrial potential was 7% to 29% higher compared with non-treated cells. The level of protection depended on the concentration of LA added in the medium for treatment (**Figure 4**, p.88).

The antioxidant protection by LA before induction of oxidative stress improved levels of ATP production in cells. Lipoic acid supplementation with a concentration of 100 μM resulted in increased ATP production on average in 10%-20% in all experiments.

Conclusion

These findings suggest that LA has different effects on metabolic activity of normal and transformed cells. Treatment of leukemia cells with 100 to 800 μM of LA affected the level of ATP production and mediated apoptosis in leukemia cells. The same treatment of normal T cells did not affect the level of metabolic activity of these cells.

Incubation of blood lymphocytes and leukemic cells with a concentration of 100-800 μM LA has been shown to potentiate apoptosis in the leukemia cells but not in the healthy lymphocytes.

According to these data, LA is pref-

Figure 3. Emission curves for mitochondrial potential for control cells, cells under oxidative stress and cells pretreated by lipoic acid.

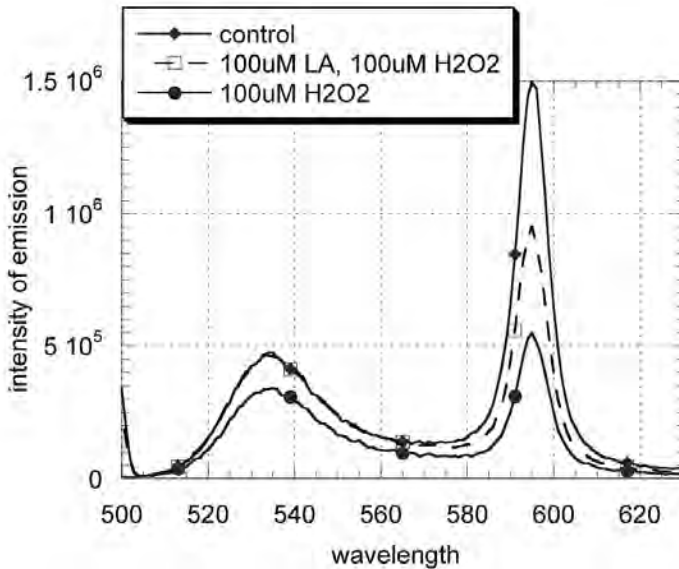
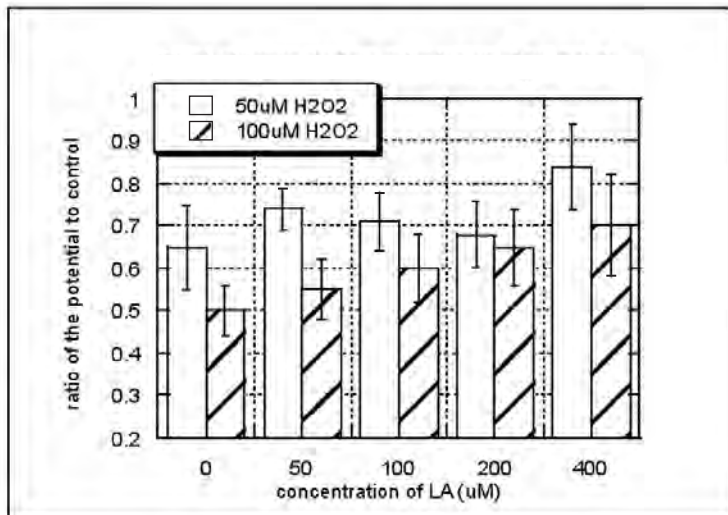


Figure 4. Improvement of mitochondrial potential of cells under oxidative stress treated by lipoic acid.



entially cytotoxic to the leukemic cell lines. These findings suggest that lipoic acid may be considered for the treatment of leukemia.

In addition, we showed that free LA, when provided exogenously, protects mitochondrial potential against oxidative stress. After pre-treatment of cells with 50–400 μM of LA before exposure to 100 μM of hydrogen peroxide, the level of mitochondrial potential was on 7% to 30% higher in comparison with non-treated cells. The level of protection depended on the concentration of LA added in the medium for treatment. The level of ATP production was also improved in 10% to 20% for cells treated by LA before oxidative stress.

Acknowledgement

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References

1. Biewenga G, Guido RM, Haene MM, Bast A: The pharmacology of antioxidant Lipoic acid, *Gen Pharmacol*, 1997; 29(3): 315-331.
2. Griffiths DE, Cain K, Hyams RL: Oxidative phosphorylation: a new biological function for lipoic acid. *Biochem Soc Transact*, 1977; 5(1): 205-207.
3. Kagan VE, Shvedova A, Serbinova E, et al: Dihydrolipoic acid—a universal antioxidant both in the membrane and in the aqueous phase. Reduction of peroxy, ascorbyl, and chromanoxyl radicals: *Biochem Pharmacol*, 1992; 44:1637-1649.
4. Matsugo S, Yan LJ, Han D, et al: Elucidation of antioxidant activity of alpha-lipoic acid toward hydroxyl radical. *Biochem Biophys Res Comm*, 1995; 208: 161-167.
5. Packer L, Witt EH, Tritschler HJ: Alpha-lipoic acid as a biological antioxidant. *Free Rad Biol Med*, 1995; 19: 227-250.
6. Han D, Handelmann G, Marcocci L, et al: Lipoic acid increases de novo synthesis of cellular glutathione by improving cystine utilization. *Biofactors*, 1997; 6: 321-338.
7. Ziegler D, Hanefeld M, Ruhnau KJ, et al: The ALADIN III Study Group. Treatment of symptomatic diabetic polyneuropathy with the antioxidant alpha-lipoic acid: a 7-month multicenter randomized controlled trial (ALADIN III Study). *Diabetes Care*, 1999; 22: 1296-1301.
8. Ziegler D, Hanefeld M, Ruhnau KJ, et al: Treatment of symptomatic diabetic peripheral neuropathy with the anti-oxidant alpha-lipoic acid. A 3-week multicentre randomized controlled trial (ALADIN Study). *Diabetologia*, 1995; 38: 1425-1433.
9. Ziegler D, Gries FA: Alpha-lipoic acid in the treatment of diabetic peripheral and cardiac autonomic neuropathy. *Diabetes*, 1997; 46(suppl 2): S62-S66.
10. Jacob S, Henriksen EJ, Schiemann AL, et al. Enhancement of glucose disposal in patients with type 2 diabetes by alpha-lipoic acid. *Arzneimittelforschung*, 1995; 45: 872-874.
11. Kawabata T, Packer L: Alpha-lipoate can protect against glycation of serum albumin, but not low density lipoprotein. *Biochem Biophys Res Comm*, 1994; 203: 99-104.
12. Nagamatsu M, Nickander KK, Schmelzer JD, et al, Lipoic acid improves nerve blood flow, reduces oxidative stress, and improves distal nerve conduction in experimental diabetic neuropathy. *Diabetes Care*, 1995; 18: 1160-1167.
13. Mona F. Melhem, Patricia A. Craven, Julia Liachenko, and Frederick R. DeRubertis: Alpha-lipoic acid attenuates hyperglycemia and prevents glomerular mesangial matrix expansion in diabetes, *J Am Soc Nephrol*, Jan 2002; 13: 108.
14. Melthem ME, Craven PA, Derubertis FR: Effects of dietary supplementation of α -lipoic acid on early glomerular injury in diabetes mellitus. *J Am Soc Nephrol*, 2001; 12: 124-133.
15. Roy S, Sen CK, Tritschler HJ, Parker L: Modulation of cellular reducing equivalent homeostasis by alpha-lipoic acid.: Mechanisms and implications for diabetes and ischemic injury. *Biochem Pharmacol*, 1997; 53(3): 393-9.
16. Arcangeli A, Carla M, Bene MRD, Becchetti A, Wanke E, and Olivotto M: Polar/Apolar compounds induce leukemia cell differentiation by modulating cell- surface potential. *Proc Natl Acad. Sci, USA*, 1993; 90: 5858-62.
17. Maftan A, Petit JM, Julien R: Specific interactions of the new fluorescent dye 10-N-nonyl acridine orange with inner mitochondrial membrane. *FEBS letters*, 1990; 260(2): 236-240.
18. Sen C.K, Roy S, Parker L. Fas -mediated apoptosis of human Jurkat T cells: intracellular events and potentiation by redox-active alpha-lipoic acid. *Cell Death Differentiation*. 1999; 6: 481-491.