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α-Lipoic Acid Increases Intracellular Glutathione in a Human T-lymphocyte Jurkat Cell Line

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Summary: The addition of exogenous α-lipoic acid to cellular medium causes a rapid increase of intracellular unbound thiols in Jurkat cells, a human T-lymphocyte cell line. The rise of cellular thiols is a result of the cellular uptake and reduction of lipoic acid to dihydrolipoic acid and a rise in intracellular glutathione. Although the level of dihydrolipoic acid is 100-fold lower than glutathione, the cellular concentration of dihydrolipoic acid might be responsible for the modulation of total cellular thiol levels. Rises in glutathione correlate with the levels of intracellular dihydrolipoic acid ($p<.01$). This increase in glutathione is not the result of expression of new proteins like γ-glutamylcysteine synthetase, since the rise in glutathione was not inhibited by cycloheximide, a protein synthesis inhibitor. Lipoic acid administration is therefore a potential therapeutic agent in an array of diseases with glutathione anomalies including HIV infection.

Glutathione (GSH) is an essential cellular compound which participates in a number of cell functions including amino acid transport, detoxification of exogenous compounds, protein synthesis and protection against oxidative damage(1). Glutathione has a high turnover rate and its synthesis is tightly regulated(1). Decreases in the level of intracellular glutathione have been implicated or associated with a wide range of diseases including cataracts(2), Parkinson's disease(3,4), diabetes(5), and AIDS(6). Replenishing glutathione levels in these cases has been suggested as possible therapeutic treatment(7,8). However, only a few compounds have been effectively shown to modulate glutathione levels. Certain compounds that enhance intracellular cysteine delivery such as N-acetyl -L-cysteine and 2-oxothiazolidine-4-carboxylic acid have been shown to be effective in increasing glutathione levels(1). Administration of glutathione itself has no effect on intracellular or tissue glutathione levels (1).

α-Lipoic acid, a precursor of the lipoamide prosthetic group in α-keto-acid dehyrogenase complexes, has been shown to have potent antioxidant abilities(9), and has been shown to inhibit HIV replication(10). Several preliminary reports indicate that lipoic acid can elevate total glutathione levels in some cell lines and tissues(11). The mechanism of action of lipoic acid in the modulation of glutathione levels is, however, still unknown. It is also not clear which lipoic

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acid derivative is responsible for the increase in glutathione levels, since lipoic acid has been shown to be intracellularly reduced to dihydrolipoic acid (DHLA)(12) and later metabolized by beta oxidation to short chain homologues (bisnorlipoic acid and tetranorlipoic acid) and to other substances(13). In the present study, we measured the oxidized and reduced forms of both glutathione and lipoic acid in an attempt to elucidate the mechanism of action of lipoic acid on intracellular thiol levels.

Materials and Method:

Cells: Jurkat cells (Clone E6-1) were obtained from American Type Culture Collection (ATCC TIB 152). Cells were incubated in RPMI 1640 with 10% foetal bovine serum as described (14). Racemic α-lipoic acid (ASTA Medica) was dissolved directly into the medium, and administered to Jurkat cells at 37 °C. For all experiments the cell suspensions were diluted two fold with either control medium or lipoic acid medium. At different time points, cells were removed from suspension and analyzed. In the cycloheximide experiments, cycloheximide was first dissolved in Dulbecco's Phosphate Buffered Saline and then given to cells one hour prior to measurement or lipoic acid addition. Cell number was determined using a hemocytometer counter (Sigma). Cell viability was determined using the trypan blue dye exclusion method.
HPLC: Glutathione levels, reduced and oxidized were determined using HPLC with a gold mercury electrode for detection as described in (15). For determination of lipoic acid and dihydrolipoic acid the same gold mercury HPLC system was used with some slight modifications(16). The gold mercury HPLC system allowed for the specific detection of thiols and disulfides in the picomole range.
Cellular analysis for glutathione and dihydrolipoic acid: For determination of both the reduced and oxidized forms of glutathione and lipoic acid, 10ml volume of cell suspension was taken at set time points. The cells were centrifuged and the medium carefully removed from the cells. Subsequently 400µl of 1% monochloroacetic acid was added to the cells and the solution was vortexed. This cell solution was split into two sets(200µl) and frozen in liquid nitrogen or in a -80 freezer for future analysis. At a later time the samples were thawed and given another 100µl of 1% monochloroacetic for glutathione analysis or 1% monochloroacetic in methanol for lipoic acid analysis. These samples were revortexed, centrifuged, and the supernant was analyzed by HPLC. This extraction and assay effectively analyzes unbound thiols and disulfides. Protein bound thiols and disulfides were not determined.

Previous work has shown, using trypan blue, that the medium constitutes 50% of the cell pellet after centrifugation(12). Because a large quantity of lipoic acid was dissolved in medium and because medium could not be completely separated from cells, intracellular lipoic acid content could not be accurately determined. Cells were not washed to remove lipoic acid contaminated medium because previous experiments have shown that washing leads to a partial release of intracellular DHLA. Therefore, lipoic acid could not quantifiable be measured inside cells. HPLC measurements have shown GSH, GSSG, and DHLA can be quantitatively determined because the medium contains none or very small amounts of these compounds after incubation of cells with lipoic acid.

Results:

When Jurkat cells were incubated with lipoic acid a time dependent and statistically significant rise in the concentration of unbound thiols occurred. This rise in thiols is primarily due to an increase in GSH, with DHLA making only a minor contribution (Table 1). In cells oxidized glutathione(GSSG) was below detectable limits using the HPLC system (detection limit ≥ 10 pmoles) and made no contribution to the glutathione increase. Since the cellular ratio of GSH/GSSG typically ranges from 30:1 to 100:1(17), it is not surprising that the increase of GSH was due only to an increase in the reduced form. No other unbound thiol species such as

Table 1: Intracellular changes in unbound thiols due to R,S -lipoic acid (100μM) addition to Jurkat cells

Time (hours)	GSH (Control)	GSH (LA)	GSH increase (%)	DHLA	Total thiols increase
LA	(-)	(+)		(+)	(+)
0	2.77 ± 0.40	2.82 ± 0.42	0	0	0
3	3.21 ± 0.39	3.51 ± 0.16	9.3	0.021 ± 0.012	0.321
6	3.49 ± 0.24	4.15 ± 0.25*	18.9	0.017 ± 0.008	0.677
12	3.36 ± 0.30	4.78 ± 0.06*	42.3	0.033 ± 0.020	1.453
24	3.43 ± 0.30	5.00 ± 0.27*	45.8	0.040 ± 0.003	1.610

Values are expressed in nanomoles/ 10^6 cells except GSH increase. No DHLA was detected in untreated cells. Data are shown as mean ± standard deviation. *$p < 0.01$ compared to control. (n =3-5). LA = lipoic acid.

bisnorlipoic acid or tetranorlipoic acid were observed in detectable quantities using the HPLC system.

The increase of cell glutathione levels was dependent on the concentration of lipoic acid added into the medium (Figure 1). Addition of fresh control medium or lipoic acid-treated medium always caused an initial rise in cellular GSH levels between 3 and 7 hours , which stabilized with time. This phenomenon could be the result of replenishment of certain nutrients

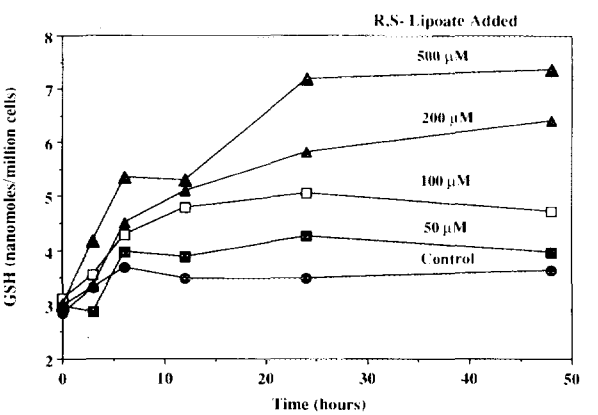

Figure 1. The concentration dependence of lipoic acid(lipoate) on cellular glutathione levels. Jurkat cells were incubated with several concentrations of lipoic acid in the medium to see the effect on glutathione levels. Since similar results were obtained from two separate experiments, the representative data are shown.

like cystine and needs to be further explored. However, it is clear that after 6 hours the higher concentrations of lipoic acid corresponded to greater levels of glutathione at all time points during incubation with lipoic acid. Along with this observation it was noticed that DHLA levels in cells were higher when cells were incubated with higher levels of lipoic acid (data not shown). Higher lipoic acid concentrations in the medium corresponded to greater levels of intracellular DHLA and a greater amount of intracellular glutathione.

To confirm a relationship between DHLA and GSH, we examined the relation between intracellular DHLA levels and GSH levels, after lipoic acid addition to cells (Figure 2). DHLA levels strongly correlated with increases in glutathione levels ($p<0.01$), suggesting that DHLA is contributing to the glutathione increase observed after α–lipoic acid administration. Intracellular lipoic acid could not be accurately quantified in cells due to contamination with culture medium. DHLA could not be directly given to cells to confirm the correlation with GSH because of its instability in cell medium (12).

γ-Glutamylcysteine synthetase, the enzyme catalyzing the rate-limiting step in glutathione synthesis, has been shown to be upregulated by methyl mercury hydroxide (18) and may be responsible for the glutathione increase induced by insulin or hydrocortisone (19). To determine if lipoic acid could be upregulating translation of enzymes in the glutathione biosynthetic pathway, cells were incubated with cycloheximide, a protein synthesis inhibitor. Administration of cycloheximide caused an intial 25% decrease in glutathione levels in both the control and lipoic acid incubated cells. However, cells incubated with lipoic acid even in the presence of cycloheximide experienced a significant increase in GSH levels compared to the control cycloheximide-treated samples. At 6 hours the (lipoic acid + cycloheximide)-treated cells

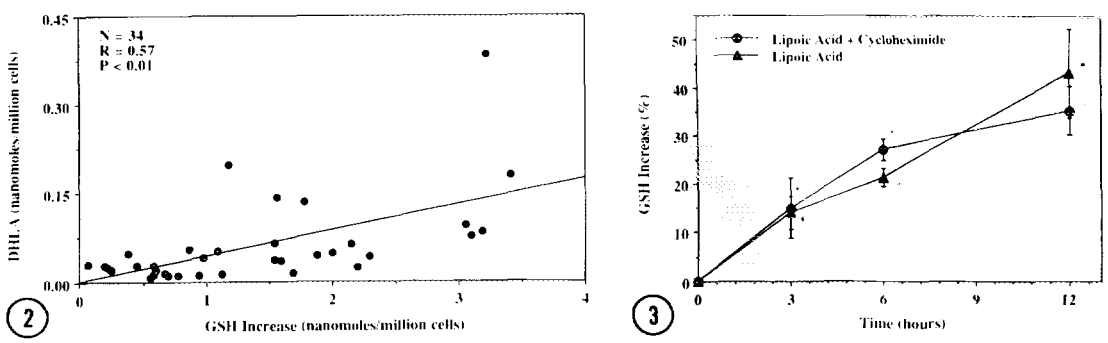

Figure 2. Correlation between DHLA and GSH increase after incubation with lipoic acid. Concentrations of DHLA and increased amounts of GSH in nanomoles/million cells were determined from the same cells and correlated. Nanomoles of GSH increase = (nanomoles GSH/million cells of lipoic acid treated cells - nanomoles GSH/million cells of control cells). The coefficient of correlation was 0.57. $p < 0.01$.

Figure 3. The effect of cycloheximide on cellular glutathione levels. The percent GSH increase by 100 μM of lipoic acid was determined in the presence and absence of cycloheximide(10μg/ml). (GSH increase = LA treated cells/ control cells -100) for both cycloheximide treated and untreated cells. *$p<0.05$ compared to control (no lipoic acid) samples (n=3).

exhibited a significant 26.9% increase in GSH levels relative to cycloheximide-treated samples(Figure 3). This value is similar to the 21.2% increase in glutathione by lipoic acid treated cells when compared to untreated cells. The conversion of lipoic acid to DHLA was also undisturbed by cycloheximide indicating that *de novo* enzyme synthesis is not needed for the reduction of lipoic acid to dihydrolipoic acid (data not shown).

Lipoic acid was also observed to have an adverse effect on the growth rate of Jurkat cells. Lipoic acid slowed down the mitotic activity of Jurkat cells in a concentration dependent manner (data not shown). At 100 μM lipoic acid, the growth rate decreased by 8.1% compared to control, and this decrease in growth rate was significant ($p < 0.05$). Lipoic acid treatment had no effect on cell viability, assessed by the trypan blue assay, over the time course of the experiment. A 26.8% decrease in cell number was experienced after 10 hours of cycloheximide treatment.

Discussion:

Lipoic acid treatment of Jurkat cells increases the intracellular level of total thiols in a time- and concentration-dependent manner. The increase of cellular thiols is due to a rise in reduced glutathione and a rise in cellular DHLA levels. The increase of DHLA can be explained by the cellular reduction of lipoic acid; however the mechanism of glutathione increase is unknown. Our work indicates that synthesis of new proteins are not involved in lipoic acid's modulation of GSH levels since, lipoic acid caused a rise in glutathione levels even in the presence of cycloheximide. The action of lipoic acid is not due an increase expression of γ-glutamylcysteine synthetase, as in the case of methyl mercury hydroxide(18).

These experiments indicate that DHLA may be the primary molecule responsible for the observed changes in glutathione levels. There is strong correlation between intracellular DHLA levels and increased GSH levels suggesting that DHLA is involved in the modulation of total thiol levels in cells. Intracellular lipoic acid itself cannot be ruled out in affecting GSH modulation but most research indicates that disulfides are rapidly converted to thiols once they enter cells(12,20). Thus a build up of intracellular lipoic acid is unlikely. The fact that DHLA may be responsible for GSH increase is not completely surprising since another thiol compound unrelated to cysteine, 2-mercaptoethanol, has been shown to increase GSH levels(21). There is probably a thiol-specific mechanism of glutathione modulation that requires further study.

The present studies were performed on the T-lymphocyte Jurkat line, because previous research with this cell line demonstrated lipoic acid's inhibition of HIV replication(10), and of NF-κB, a transcription factor needed for HIV activation(14). N-acetyl-L-cysteine is believed to inhibit NF-κB and HIV replication by replenishing cellular glutathione levels(22). We postulate that the action of lipoic acid inhibition of NF-κB and HIV replication involves lipoic acid's ability to increase total intracellular thiol levels. The intracellular build up of DHLA and increase GSH levels after administration of lipoic acid may shift the redox status of the cell, allowing it to inhibit reverse transcriptase and to counter the oxidative stress associated with HIV infection more effectively.

Lipoic acid is a commonly used therapeutic agents in wide array of disorders including diabetes(23) and diabetic polyneuropathy(24). Since lipoic acid has been shown to have similar

effects as N-acetyl-L-cysteine, lipoic acid should also be taken consideration in the treatment of diseases related to glutathione depletion. Because lipoic acid is effective in changing GSH levels in the micromolar range while N-acetyl-L-cysteine is effective only in the millimolar range, lipoic acid may have a considerable advantage over N-acetyl-L-cysteine. In addition lipoic acid treatment causes a build up of intracellular DHLA, a potent antioxidant in both the aqueous and the lipid phases(9).

The ability of lipoic acid to increase reduced GSH-levels presented here is further confirmed in experimental diabetic polyneuropathy (25). Lipoic acid has been shown in streptozotocin diabetic neuropathy to normalize decreased GSH-levels and reduce oxidative stress of diabetic nerves in a dose dependent manner (25). Therefore this mechanism of lipoic acid described here is of great importance for the therapeutic use of lipoic acid in diabetic polyneuropathy.

This ability of lipoic acid to increase total thiol levels in cells should, therefore, be considered in the potential treatment in a number of diseases including AIDS and neurodegenerative disorders.

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